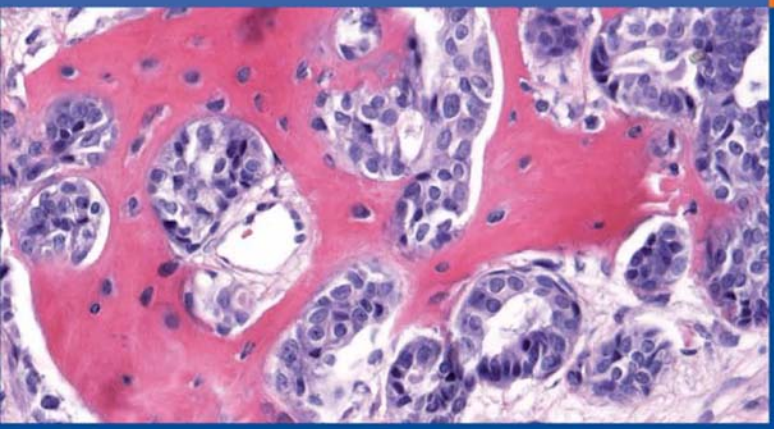
Methods of Cancer Diagnosis, Therapy, and Prognosis

Volume 1
Breast Carcinoma



M.A. Hayat
Editor

Springer

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Edited by

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Springer

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ISBN 978-1-4020-8368-6 e-ISBN 978-1-4020-8369-3

Library of Congress Control Number: 2008930172

Printed on acid-free paper

9 8 7 6 5 4 3 2 1

springer.com

New technology, for better or for worse, will be used, as that is our nature.

Lewis Thomas

You have been given the key that opens the gates of heaven; the same key opens the gates of hell.

Writing at the entrance to a Buddhist temple

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Preface

The enormity of the global healthcare costs as a result of cancer infliction cannot be overemphasized. There are more than 100 types of cancers; any part of the body can be affected. More than 11 million people are diagnosed with cancer every year, and it is estimated that there will be 16 million new cases per year by the year 2020. In 2005, 7.6 million people died of cancer, that is, 13% of the 58 million deaths worldwide. It is estimated that 9 million people will die from cancer worldwide in 2015 and 11.4 million will die in 2030. More than 70% of all cancer deaths occur in low and middle income countries.

Five major cancer causing overall mortalities per year worldwide are (WHO):

1. Lung: 1.3 million
2. Stomach: 1 million
3. Liver: 662,000
4. Colon: 655,000
5. Breast: 502,000

The five most common types of cancers that kill men worldwide (in order of frequency) are: lung, stomach, liver, colorectal, and esophagus. The five most common types of cancers that kill women worldwide (in order of frequency) are: breast, lung, stomach, colorectal, and cervical. One-fifth of all cancers worldwide are caused by a chronic infection, for example, human papilloma virus (HPV) causes cervical cancer and hepatitis B virus (HBV) causes liver cancer. Tobacco use is the most common preventable cause of cancer in the world. Approximately, 168,000 cancer deaths are expected to be caused by tobacco use. Approximately, 40% of cancer could be prevented, mainly by not using tobacco, having a healthy diet, being physically active, preventing infections that may cause cancer, reducing exposure to sunlight, and avoidance of excessive alcohol consumption and stress (anger). A third of cancers could be cured if detected early and treated adequately.

It is well established that scientific journals facilitate exchange of information, resulting in rapid progress. In this endeavor, the main role of scientific books is to present information in more detail after careful, additional evaluation of the investigational results, especially those of new or relatively new methods.

Although subjects of diagnosis, therapy, therapy assessment, and prognosis of various types of cancers, cancer recurrence, and resistance to chemotherapy are scattered in a vast number of journals and

books, there is need of combining these subjects in single volumes. An attempt has been made to accomplish this goal in these volumes. A constructive evaluation of commonly used methods for elucidating primary and secondary cancer initiation, progression, relapse, and metastasis is presented. Methods of cancer diagnosis discussed include various modalities of imaging, (e.g., ultrasound, computed tomography, and magnetic resonance imaging), histochemistry, and immunohistochemistry. In addition, detailed therapeutic protocols, both their benefits and side-effects, are discussed. Examples of cancer treatments are chemotherapy, radiation, surgery, chemoradiation, and immunotherapy.

In the era of cost-effectiveness, my opinion may be a minority prospective, but it needs to be recognized that the potential for false-positive or false-negative interpretation on the basis of a single laboratory test in clinical pathology does exist. Interobservor or intraobservor variability in the interpretation of results in pathology is not uncommon. Interpretive differences often are related to the relative importance of criteria being used.

Generally, no test performs perfectly. Although, there is no perfect remedy to this problem, standardized classifications with written definitions and guidelines will help. Standardization of methods to achieve objectivity is imperative in this effort. The validity of a test should be based on the objective interpretation of the photomicrographs or tomographic images. The interpretation of the results should be explicit rather than implicit. To achieve accurate diagnosis, and correct prognosis, the use of molecular criteria is important. Indeed, molecular medicine has arrived.

This volume discusses in detail all aspects of breast cancer, including diagnosis using molecular genetics, various imaging modalities, tumor markers (e.g., applying tissue microarrays), immunohistochemistry, biopsy specimens, blood, and serum, treatments such as chemotherapy, radiation, chemoradiation, hormonal therapy, immunotherapy, and surgery, and prognosis. The side effects of the treatments are also pointed out. Both primary and secondary cancers, and risk of cancer survivors developing other cancers are explained. An attempt is also made to translate molecular genetics into clinical practice. Evidence-based therapy is included. Other cancers will be discussed in subsequent volumes of this series.

Various anticancer drugs, such as tamoxifen, docetaxel, epirubicin, and anastrazol, are discussed, including their effectiveness and limitations, and their resistance by cancer cells. The role played by genes, including HER-2/*neu*, *BRCA1*, and *BRCA2*, in breast cancer initiation, progression, and metastasis is explained.

Each chapter is written by distinguished, practicing clinicians/surgeons/oncologists. Their practical experience highlights their writings, which should build and further the endeavors of the readers. This volume was written by 148 scientists representing 17 countries. It is my hope that these handbooks would assist in more complete understanding of at least some of the globally-encountered cancer problems. Successful cancer treatment, cure, and prevention are areas of immediate concern of and demand by the public.

I am grateful to the contributors for their promptness in accepting my suggestions, and appreciate their dedication and hard work in sharing their invaluable

knowledge with the readers. Each chapter provides unique individual, practical knowledge based on the expertise and practical experience of the authors. The chapters contain the most up-to-date practical as well as theoretical information. It is my hope that the book will be published expeditiously.

I am thankful to the Board of Trustees, Dr. Dawood Farahi, and Dr. Kristie Reilly for recognizing the importance of scholarship in an institution of higher education and providing the resources for completing this important project. I am thankful to Ayesha Muzaffar and Lina Builes for their expert help in preparing this volume.

M.A. Hayat
December 2007

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Breast Cancer: An Introduction

M.A. Hayat

Female breast cancer is the second leading cause of cancer-related deaths. It is estimated that 180,510 new cases of breast cancer would be reported and 40,460 patients will die in 2007 in the United States (Am. Cancer Soc. 2007), accounting for approximately one-third of all female cancers in the country. Approximately, 2,030 new cases of breast cancer are also expected in men in 2007, and 450 will die. Death rates from breast cancer have been steadily decreasing due to a combination of early detection and improved treatment. The use of adjuvant chemotherapy and hormone treatment have led to improvement in relapse-free and overall survival; these gains are impressive.

Nevertheless, ~ 30% of patients with node-negative breast cancer will present distant recurrence and will die as a result of their disseminated disease, while ~ 40% of the patients with involved axillary lymph nodes will survive for ~ 10 years without clinical recurrence. Considering these data, it is apparent that more effective strategies for the prevention and treatment of this malignancy are urgently needed.

The female hormone estrogen plays a critical role in breast cancer development by binding to the estrogen receptor and inducing the expression of peptide growth factors that are responsible for the proliferation of cancer cells. Approximately, 60% of premenopausal and 75% of postmenopausal patients have estrogen-dependent carcinomas. In fact, estrogen and progesterone are breast cell mitogens, and postmenopausal women with high circulating estrogen levels are at a greater risk of breast cancer. Therefore, it is expected that antiestrogen selective receptor modulators, such as tamoxifen and raloxifene inhibitors, will show reduced risk of breast cancer.

However, although these anti-hormonal therapies are effective for the presentation of estrogen receptor-positive breast cancer, they have no effect on the development of estrogen receptor-negative breast cancer. Thus, there remains a critical need to develop agents that will prevent estrogen receptor-negative breast cancer. Agents currently being investigated for the prevention of estrogen receptor-negative breast cancer include cyclooxygenase-2 inhibitors, tyrosine kinase inhibitors, and retinoids. However, their use in humans is limited because of their toxicity. We need to develop agents that will prevent cancer with reduced toxicity and increased efficacy.

Another related approach to reduce the risk of breast cancer is the use of aromatase inhibitors. Abnormal expression of aromatase (the enzyme responsible for estrogen synthesis) in breast cancer cells and/or surrounding adipose stromal cells may have a significant influence on tumor development and growth in breast cancer patients. These inhibitors are effective in the treatment of hormone-responsive breast cancer and significantly prevent contralateral cancers. Thus, suppression of estrogen formation in postmenopausal women by aromatose inhibitors may be a useful prevention/treatment strategy in these women.

The use of hormone therapy, especially the combination of estrogen and progesterone, increases breast cancer risk (van Duijnhoven, 2007). Such treatments may increase mammographic density of the breast; this density is determined by the relative amounts of the connective tissue, epithelial tissue, and fat in the breast; the proportion of fat increases as the density decreases. High mammographic density is associated with an increase in breast cancer risk. The degree of mammographic density is dependent on several factors including the age, the number of children, and body weight. High mammographic density is usually found in young women, whereas density decreases with age. This accelerated decrease in density does not occur in some women who use hormone therapy.

Breast cancer is a systemic disease in that cancer cells may start to be disseminated into blood and lymphatic systems even in early stages or when the tumor size is still small. Active angiogenesis may occur in breast tumor nodules as small as 2 mm in diameter. In metastatic state, each gram of tumor may shed ~ 10^6 cells per day into blood vessels. Detection of early breast cancer by studying disseminated tumor cells in the peripheral blood is explained in two chapters authored by Zehentner, and by Feng and Kiviat in this volume. This technique allows not only early detection of breast cancer but also the progression of the disease. That detection of peripheral cytokeratin-19 mRNA-positive cells is an independent predictive and prognostic factor for reduced disease-free interval and overall survival, respectively, in node-negative breast cancer patients is discussed by Xenidis, Perrakis, Kakolyris, Mavroudis, and Georgoulias in this volume.

Early detection is one of the most effective means to achieve better prognosis and lower rate of death. Randomized screening trials with mammography results in 15–20% reduction in the risk of breast cancer death. Early diagnosis of the cancer has the benefit of receiving a wider range of therapeutic options, leading to more successful treatment.

Although ^{18}F-fluorodeoxyglucose-positron emission tomography (FDG-PET) is commonly used to detect breast cancer, it has variable sensitivity (64–100%). This variation (uptake of FDG) is related to biologic factors such as tumor size, histological tumor grade and type, proliferation index, microscopic growth pattern, presence of BRAC1, BRAC2, HER-2/*neu*, and p53 status, hormonal receptor status, axillary lymph node status, and presence of inflammatory, necrotic, cystic, or fibrotic tissues. Tumor size is one of the more important factors, which determines the FDG-PET outcome. Increased tumor size results in increased accuracy of FDG-PET, while this imaging modality has limited sensitivity in detecting breast tumors of a small size (< 10 mm). Thus,

tumors of a small size invite false-negative results. On the other hand, as the tumor size increases, the standardized uptake value also increases; this subject is discussed in detail by Kumar in this volume. It should also be noted that tumor size and tumor grade are independent factors associated with false-negative results of FDG-PET.

Less common symptoms include persistent changes in the breast (e.g., thickening, swelling, distortion, tenderness, skin irritation, scaliness, or nipple abnormalities such as ulceration, retraction, or spontaneous discharge) (American Cancer Society, 2007). The earliest sign of breast cancer is usually an abnormality detected on a mammogram. However, although mammography is accurate, it is not always perfect.

REFERENCE

American Cancer Society 2007. Cancer facts and figures. American Cancer Society, Atlanta.

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Breast Cancer: Computer-Aided Detection

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INTRODUCTION

Breast cancer is one of the leading causes of death in women over the age of 50. Scientific evidence indicates that early diagnosis and treatment substantially reduces patient mortality and morbidity. Among the large number of imaging modalities and detection tools, mammography is considered the most cost-effective method for detecting breast cancers at an early stage. However, considering the large volume of screening mammograms, tissue overlap in the X-ray projection images, and the low yield of cancers (i.e., 3–5 cancers per 1,000 screening examinations), detecting breast abnormalities surrounded and probably overlapped by the complex normal anatomy background is a difficult and time-consuming task for radiologists. As a result, 10–30% of detectable breast cancers (visible in the retrospective review of mammograms) are either not initially detected or not interpreted as such due to the subtle nature (low conspicuity) of the lesion, relatively poor image quality, eye fatigue, or oversight by the radiologists. The specificity of mammography is also relatively low in that ~10% of cases are recalled for additional imaging procedures and only a small fraction (15–30%) of biopsies is positive. Although independent double reading may improve diagnostic accuracy of radiologists in interpreting mammograms, it is an inefficient and costly approach. Hence, for the last 2 decades there is growing interest in the development of computer-aided detection (CAD) schemes for mammography, which aims to provide radiologists with a valuable "second opinion" and help them detect more breast abnormalities (i.e., masses and micro-calcification clusters) at an early stage.

Since US Food and Drug Administration approved the CAD technology in 1998, commercialized CAD systems have been gradually installed and used in a large number of medical institutions and imaging facilities around the world. CAD of breast cancer is rapidly becoming a well accepted clinical practice to assist radiologists interpreting screening mammograms. Recently, a large number of studies have been conducted to investigate how CAD might affect radiologists' performance in detection of breast cancers and whether the potential utility of CAD schemes had been fully realized in the clinical practice. In this chapter, we review the latest development of CAD in assisting radi-

ologists interpret mammograms and detect breast cancers. The chapter is organized as follows. Section 2 briefly reviews the history of developing CAD schemes for automatic detection of micro-calcification clusters and masses depicted on digitized mammograms. Section 3 discusses the performance levels of current CAD schemes (including sensitivity, false-positive rate, and reproducibility). Section 4 describes how current CAD schemes actually affect radiologists' performance in clinical practice. Section 5 introduces several new approaches to improve CAD performance and clinical utility. Based on these descriptions and discussions, section 6 concludes the advantages and limitations of applying current CAD of mammography in the clinical environment.

DEVELOPMENT OF COMPUTER-AIDED DETECTION SCHEMES

Since 1980s a large number of CAD schemes have been developed and optimized by different research groups to detect one of the two common abnormalities (micro-calcification clusters and masses). Although different techniques or image processing algorithms have been reported for different CAD schemes, a typical scheme usually includes three distinct stages, (1) image filtering, (2) region growth (or segmentation) and feature computation, and (3) classification of segmented suspicious lesions. In the first stage, different image filtering methods have been tested to enhance the signal-to-noise ratio of the suspected areas depicting either micro-calcifications or masses. These filters include but not limited to bilateral image subtraction (Mendez *et al*., 1998), density-weighted contrast enhancement (DWCE) (Petrick *et al*., 1996), the difference of Gaussian (DOG) filter (Zheng *et al*., 2000), and wavelet transform (Mini *et al*., 2004). This stage aims to achieve the maximum detection sensitivity. As a result, it usually detects a large number of suspicious areas (or pixels).

The second stage of CAD scheme segments each suspicious region (lesion) and computes a set of image features to represent the region. After identifying a growth seed inside a detected suspicious area, a region growth or segmentation algorithm is applied to define the region boundary contour. Active (dynamic) contour modeling and adaptive region growth algorithm are two popular region growth methods to segment suspicious mass regions (Brake and Karssemeijer, 2001). Unfortunately, neither method has proven to be superior to the other, nor is optimal due to the overlap of dense fibro-glandular breast tissue on two-dimensional X-ray images which create fuzzy boundaries of suspicious mass regions. For example, the assumption that the edge of a mass region always has the strongest gradients as compared with the surrounding background is frequently violated when the boundary of the lesion is reasonably fuzzy and ill-defined. As a result, active contours can expand (penetrate) into the surrounding breast tissue. Finding an optimal initial contour (growth seed) and an optimal termination criterion (threshold) is the most important and difficult issue in lesion segmentation. Without accurate region segmentation, the CAD-computed image features cannot accurately describe or represent morphology of the suspicious abnormalities, which will severely limit the improvement of

CAD performance and robustness. Hence, improving the accuracy in segmentation of breast lesions is still an active research topic in CAD development to date.

Based on the image features computed from the segmented regions (stage two), the third stage of the CAD scheme optimizes and applies a feature-based machine learning classifier to distinguish between true-positive and false-positive regions. CAD scheme then only reports and prompts the identified suspicious abnormalities that have detection scores higher than the pre-optimized operating threshold level of the scheme. A large number of machine learning classifiers have been optimized for this purpose, which include artificial neural networks, Bayesian belief networks, decision trees, and rule-based expert systems. Although considerable effort has been made in the optimization of different classifiers, studies indicated that their performance levels actually converged to a very similar level. As a result, improving CAD performance is more likely to be dependent on feature selection, database diversity, and training sample size used in CAD schemes, rather than the specific classifier being implemented.

As an example, Figure 2.1 demonstrates a flow diagram of an integrated CAD scheme for the detection of both micro-calcification clusters and masses, which was developed at Imaging Research Center, Department of Radiology, University of Pittsburgh (Zheng *et al.*, 2000). Once a digitized mammogram is loaded, the scheme first crops the background (air) area of the image. Two independent processes are then sequentially applied to detect suspicious micro-calcification clusters and masses depicted on this image. To detect micro-calcification cluster, the first stage of the scheme uses a DOG filter (with two different kernel sizes of 0.3 mm and 0.5 mm) to enhance high frequency signals (pixels) that may correlate with micro-calcifications. The initial suspected pixels are further extracted using the local contrast-based threshold method. Because this process selects a large number of suspected pixels, in order to delete a high percentage of false-positive pixels while maintaining high detection sensitivity, the scheme applies a special local minimum search ring to analyze all selected pixels followed by "blob" labeling and clustering. In the second stage of the scheme, a multi-layer topographic region growth algorithm and rule-based feature analysis are used to identify each suspected micro-calcification and continuously delete false-positive pixels (or "blobs"). The remaining suspected micro-calcifications are clustered and a set of classification rules related to the clustering is applied to delete isolated regions. During this process the scheme computes 13 morphological and intensive distribution related features to represent each identified cluster. The third stage of the scheme uses an artificial neural network (ANN) to classify true-positive and false-positive clusters of micro-calcifications. The ANN involves 13 input neurons, 7 hidden neurons, and 1 output neuron. ANN generates a detection score (ranging from 0 to 1) for each suspected cluster, which indicates the likelihood of the cluster involving true-positive micro-calcifications.

For mass detection, the original digitized image (i.e., with pixel size of 100 × 100 μm) is first sub-sampled (pixel averaged) by a factor of four in both dimensions to reduce the size of each image to ~600 × 450 pixels (i.e., with pixel size of 400 ×

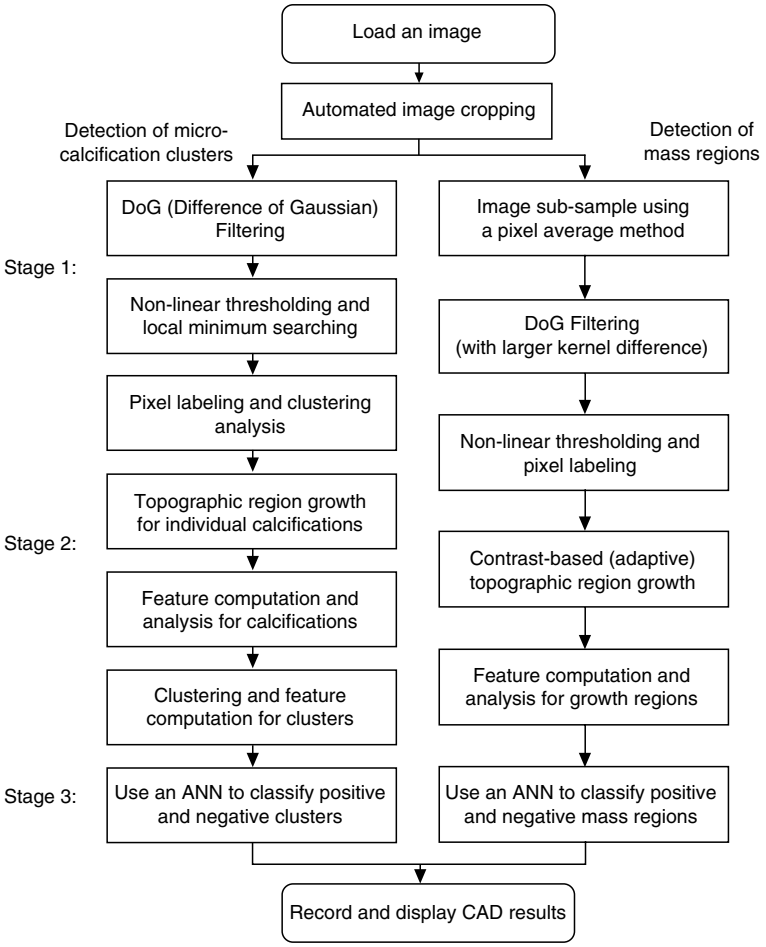


FIGURE 2.1. A flow diagram of an integrated CAD scheme for the detection of micro-calcification clusters and masses

400 μm). Then, three processing stages are applied to the sub-sampled images to identify suspected mass regions. In the first stage, another DOG filter with two different kernel sizes of Gaussian filters (e.g., 2.8 mm and 20.4 mm) is applied to the image to enhance the suspected mass regions (signals) and depress the background regions (noise) at different density levels. Threshold and labeling are followed to search for all initially suspected regions. Depending on the complexity of breast tissue structure, ~10–30 regions are likely to be identified at this stage in each image. Based on identification of a growth seed and corresponding local contrast measurement, the second stage of the scheme uses an adaptive multilayer topographic region growth algorithm to define three layers for each suspicious region. In each growth layer, a set of simple intra-layer boundary conditions on growth ratio and shape factor of the region are applied to eliminate a large portion of suspicious regions (~70–80%) while maintaining the higher detection sensitivity. For each of the remaining regions, the scheme computes a set of 14 morphological

and intensity distribution based features. In the third stage of the scheme, another ANN is applied to classify between true-positive and false-positive mass regions. Two optimal operating threshold values (one for micro-calcification cluster detection and one for mass detection) are then implemented in the integrated CAD scheme. CAD scheme only accepts and prompts the detected suspected regions with ANN-generated detection scores higher than the corresponding operating threshold. All other detected regions with detection scores lower than the operating threshold levels are discarded.

EVALUATION OF COMPUTER-AIDED DETECTION SCHEME PERFORMANCE

Currently several commercialized CAD systems (i.e., Image-Checker from Hologic Inc, Bedford, MA and Second-Look from CADX Systems, iCAD Inc., Nashua, NH) are available and have been installed in a large number of medical institutions and imaging facilities around the world to assist radiologists interpreting screening mammograms. CAD schemes have achieved very different performance levels on mass and micro-calcification cluster detection. Studies have reported that case-based CAD sensitivity for detecting micro-calcification clusters (in which a cluster is considered being detected as long as it is detected by CAD on at least one view) was as high as 98%, while CAD sensitivity on mass detection was generally reported in the range from 60% to 85% with relatively higher false-positive detection rate (Brem *et al*., 2005). While using CAD is rapidly becoming a well accepted clinical practice in interpreting screening mammograms, radiologists' attitude toward and acceptance of CAD-cued micro-calcification clusters and masses are substantially different. Several independent studies have been conducted and reported to assess performance and potential clinical utilities of the commercial CAD systems under different laboratory environments. Some of the studies are briefly described below.

Performance of Computer-Aided Detection Schemes on Several Specific Types of Micro-calcification Clusters

Although it is well accepted that current CAD schemes are performing at an excellent level in terms of detecting micro-calcification clusters and thereby truly aiding radiologists, CAD performance (e.g., in particular the sensitivity) on some specific type of micro-calcification clusters can be substantially low. Soo *et al*. (2005) applied a commercial CAD system to scan and process 85 mammography examinations in which the images depict histologically sampled groups of amorphous calcifications (including 21 malignant, 14 high risk, and 50 benign clusters). CAD detected amorphous calcifications in 43 of 85 cases (51% case-based sensitivity) and in 59 of 146 images (40% image-based sensitivity) with average 0.5 false-positive marks per image. The case-based CAD sensitivities by histologic outcome in this dataset were 57% for malignant, 29% for high-risk, and 54% for benign calcification clusters.

Because breast cancer is assumed to be a progressive disease, small tumors are generally considered to be an early disease with good prognosis. With increasing

compliance of the general population in undergoing mammography screening the search for optimal treatment plans becomes an extremely important issue. Aggressive treatment (e.g., mastectomy, chemotherapy) is often not recommended for the majority of patients diagnosed with small invasive cancers because in general there is a high survival rate in this group of patients. However, recent studies found that a small subset of patients whose mammograms depict casting-type calcifications has a significantly higher mortality rate. One study conducted by Tabar *et al*. (2004) reviewed survival of 714 women with small invasive cancers. Although only 7.3% of these cases depicted casting-type calcifications, this group contributed > 30% of the deaths during the period investigated, and in this study it accounted for 100% of deaths when any type of calcification was depicted on the mammograms. It is important to mention that unlike all other groups whose survival rates gradually decrease during the 20 year follow-up, all fatalities in this group occurred within the first 8 years. Although undocumented to date, current CAD schemes are likely to perform relatively poorly in detecting casting-type calcifications because these schemes are often designed to deliberately discard intra-vascular calcifications to reduce the false-positive detection rates, and casting-type calcifications frequently have an appearance quite similar to intra-vascular calcifications. Because radiologists heavily rely on CAD-prompted results to search for micro-calcification clusters, they should be aware of the lower sensitivity of CAD schemes in certain rare but more aggressive (lethal) types of micro-calcification clusters.

Performance of Computer-Aided Detection Schemes on False-Negative Cases and Prior Images

Because in the busy screening mammography environment radiologists can potentially miss a fraction of cancers (e.g., 10–30%) that are considered visible or actionable during the retrospective review, one of the most important advantages of using CAD is that CAD schemes can detect a high fraction of false-negative cancers. In one study, a panel of 14 radiologists selected from five medical facilities reviewed the most recent corresponding prior mammograms of 427 biopsy-proved cancer cases during a retrospective review. These cases were collected from 13 different medical facilities. These radiologists found that 67% of 427 cancers were "visible" on the prior images. An independent and blind review by panels of the radiologists indicated that 27% (115 of 427) cases were warranting recall. By processing images of these 115 cases using a commercial CAD system, the researchers found that CAD correctly marked 77% (or 89 of 115) of the cancers with an average one false-positive mark per image (Warren Burhenne *et al*., 2000).

In another multi-institutional trial, three radiologists retrospectively reviewed 377 screening mammograms interpreted as showing normal or benign findings 9–24 months before cancers were diagnosed and verified. The panel of radiologists determined that 123 of the 377 missed cancer cases warranted workup during the prior mammography examination. While applying another commercial CAD system to process this set of "prior" images, 65% (80 of 123) of cancers were correctly detected with average one false-positive mass region and 0.25 false-positive

micro-calcification clusters per image (Brem *et al.*, 2003). Because of these consistent findings with different commercialized CAD systems and diverse image databases, many researchers believe that CAD has potential to help radiologists detect a significant fraction of "missed" cancers in screening mammography.

Performance of Computer-Aided Detection Schemes on Spiculated Masses with Dense Background

Boundary margin spiculation of breast mass regions provides a direct radiographic manifestation of the local aggression of invasive breast cancer. It is a particularly valuable feature in detecting breast cancers. However, because the subtle masses have substantial variability in appearance and are often overlapped by heterogeneous or dense fibro-glandular breast tissue, a high fraction of cancers missed by radiologists during screening mammography are associated with spiculated masses and architectural distortion surrounded by dense breast tissue. Several studies evaluated CAD performance in detecting spiculated masses. In one study, 1,083 consecutive cases of biopsy-proved cancers detected at screening mammography were selected from 13 institutions. Among them, 677 cancers are associated with masses. Three radiologists examined these 677 cases and interpreted 375 (55%) masses as being spiculated on at least one view. Applying CAD to these 375 cases, 322 (86%) spiculated masses were correctly detected by the CAD scheme with an average 0.24 false-positive detections per image. The researchers also investigated the relationship of breast density ratings using four BI-RADS (Breast Imaging Reporting and Data System) categories versus consensus ratings of case subtlety and found that the relative proportion of subtle cases monotonously increased from the BI-RADS category 1 (fatty) to 4 (dense). The data analyses of the study indicated that CAD performance showed no significant dependence on breast density. Because radiologists' detection sensitivity is often lower in women with primarily dense breasts, the researchers predicted that CAD might help improve radiologists' performance in detecting spiculated masses depicted in dense breasts (Vyborny *et al.*, 2000).

However, several other studies reported that CAD performance depended on the case difficulty including the density of normal breast tissue. One group of researchers selected 264 sets of bilateral screening mammograms. Based on visual rating of a panel of radiologists, these images were divided into four density groups based on BI-RADS recommendation. A commercialized CAD system was used to process these images and detect suspected abnormalities. Researchers then analyzed CAD performance on each group of images and reported that the CAD sensitivity deteriorated significantly as the density of the breast increased while CAD specificity remained relatively constant (Ho *et al.*, 2003).

In another study, researchers collected four-view images acquired from 219 women underwent routine mammography examinations and divided the images into five groups. Group 1 included 58 cases depicting malignant masses that had been detected by radiologists during the screening interpretation and group 2 included all available "prior" examinations of the cases selected in group 1.

Group 3 included 22 false-negative examinations (in which the cancers were initially missed by radiologists in screening interpretation). All the cancers detected in this test image dataset are associated with malignant masses. Groups 4 and 5 included 50 negative cases each. The negative cases in group 4 were not recalled during the screening examinations, while the cases in group 5 were recalled and later proved to be negative by biopsy and follow-up. In general, group 5 included more dense images than group 4. Two leading commercialized CAD systems and one experimental CAD scheme were used to process these images to detect masses depicted in this dataset. CAD performance levels on each group were separately evaluated and compared. The results indicated that mass detection sensitivities in group 1 were 67–72% for three CAD schemes, which were not significantly different ($p \sim 0.05$). False-positive rates varied from 1.08 to 1.68 per examination (four images). In particular, the false-positive rates were significantly higher ($p < 0.01$) for recalled negative cases (group 5) than those not recalled (group 4). This work demonstrated that in general cases that were "difficult" to interpret by the radiologists during the clinical reading (partially due to the dominated dense breast tissue); hence, were recalled for additional procedures, were also difficult to assess by the CAD schemes, resulting in a higher false-positive prompted rates. In addition, between approximately 40% and 80% of masses (in different categories) were detected by CAD only on one view. The subtle masses are more likely to be detected by CAD only on one view (Gur *et al*., 2004b).

Performance of Computer-Aided Detection Schemes in Detecting Lesions with Architectural Distortion

Architectural distortion (i.e., a distortion of the parenchymal architecture without a concomitant mass) is the third most common mammographic appearance of breast cancers. In current CAD schemes, the architectural distortion is considered a type of special "mass" and the mass detection algorithm (or scheme) is also applied to detect the breast tissue with architectural distortion. Several studies have evaluated CAD performance of detecting architectural distortion. One study investigated CAD performance in detecting invasive lobular cancers. In 94 biopsy-proved invasive lobular carcinoma lesions, 56 manifested as masses of which 20 were interpreted by radiologists as architectural distortion. The commercialized CAD system correctly detected and marked 17 cancers with architectural distortion (85%). This sensitivity is similar or comparable to CAD sensitivity on other types of breast masses (Evans *et al*., 2002).

In another study, researchers compared the performance of two commercial CAD systems in detecting architectural distortion. In this study 45 cancer cases were retrospectively reviewed by a panel of radiologists. In each case architectural distortion was deemed present and considered actionable. One CAD system achieved 49% case-based sensitivity (detected 22 out of 45 architectural diction cases) and 38% image-based sensitivity. Another CAD identified 15 of 45 architectural distortion cases (33% case-based sensitivity as well as 21% image-based sensitivity). These researchers concluded that because fewer than one half of the architectural distortion cases were detected by two most

widely available CAD systems used in clinical practice for screening mammography, considerable improvement in the sensitivity of CAD schemes was needed for detecting architectural distortion type lesions. Meanwhile, radiologists who use CAD systems should remain vigilant for architectural distortion depicted on images (Baker *et al*., 2003).

Reproducibility of Results of Computer-Aided Detection Schemes

When applying CAD to screen-film based mammograms, a digitizer is used to convert the analog images (films) to digital images. Due to the digital noise generated during the digitization process and slight position shift of the film mammograms, the digitized images acquired at different times could have small variability in pixel values. Reproducibility is applied to measure how CAD is sensitive to the small variation of the pixel values in the digitized images. It is an important factor in determining the overall quality and performance of CAD schemes for digitized mammograms. Although great effort has been made to improve CAD performance by a large number of investigators, results are generally reported only in terms of case-based detection sensitivity and false-positive rates. The reproducibility of CAD-prompted results has never been reported by the companies that manufacture commercial CAD systems. During the last several years, a number of independent research groups investigated the reproducibility of CAD systems. For example, in one study researchers collected an image dataset involving 40 cancer cases. Among them 20 cancers that had been detected by radiologists during their original interpretation of screening mammograms, 10 interval cancers, and 10 false-negative cases (initially missed by radiologists) were detected. All film mammograms were scanned (processed) ten times using one commercial CAD system. Using case-based detection criterion, 15 cancers (37% sensitivity) were marked correctly in all ten scans, 10 were never detected (25%), and the remaining 15 cancers (37%) were prompted between two and nine times. In this study, the researchers also compared the reproducibility of two versions of the same commercial CAD software and reported the improvement of CAD reproducibility in the new version of CAD software. In addition, the researchers found that the least reproducible CAD marks were often subtle namely, visually difficult for radiologists to detect, as well (Taylor *et al*., 2003).

Another study reported that by scanning (digitizing) images of a set of 50 cancer cases 10 times, overall CAD performance (including both sensitivity and false-positive rate) remained relatively consistent in each digitization cycle. For example, CAD could consistently detect 40 to 43 of 50 cancers in each of 10 scans. The exactly detected cancers could be changed in different digitization cycle. Specifically, 14 cancers were non-reproducible (Baker *et al*., 2004). Because each radiologist is responsible to detect and diagnose suspicious findings on individual patients, generating reproducible CAD results for each examination may be more important than maintaining the consistent overall performance for a large and diverse database in the clinical application of CAD systems. However, whether and how the variation (lower reproducibility) of

CAD-generated results affects radiologists' confidence in CAD-cued results and their detection performance in the clinical practice has not been investigated to date.

Performance of Computer-Aided Detection Schemes on Full-Field Digital Mammograms

Regular screening mammograms are two-dimensional X-ray projection images and the presence of overlapping dense fibroglandular breast tissue reduces the conspicuity of breast lesions. This limits the sensitivity of mammography and constitutes another main cause of missing breast cancers by radiologists. The advent of full-field digital detectors with high-resolution and large sensor size offers great opportunities to develop advanced digital techniques to improve conspicuity of breast lesions overlapped by the dense breast tissue. As a result, full-field digital mammography (FFDM) systems have been developed and widely considered as a new promising imaging modality in improving breast cancer detection. Although CAD schemes were initially developed for digitized screen-film mammogram (SFM) images, CAD software is also available for FFDM images. Due to the substantial difference of image characteristics between the digitized SFM and FFDM images, CAD schemes optimized using digitized images cannot be directly applied to FFDM images. The parameters or threshold values used in the CAD schemes need to be adjusted and retested in order to achieve the optimal sensitivity in the detection of initial suspicious areas and improve accuracy of region segmentation. In region classification, different image features may also be used in CAD schemes for SFM and FFDM images. In one study to test the performance of a CAD scheme that was specially designed and optimized for FFDM images by the same company making the commercial CAD system for digitized SFM images, researchers selected an image dataset of 187 FFDM images involving 63 histologically verified carcinomas. When applying the scheme to this dataset, it achieved 89% and 81% sensitivities for micro-calcification cluster and mass detection, respectively. The overall average false-positive marks were 0.61 per image (including average 0.35 false-positive marks of micro-calcification clusters per image). As expected when applying CAD schemes to FFDM images, the overall detection performance on mass detection is very comparable to or better than the CAD schemes optimized for digitized images. However, in micro-calcification cluster detection, CAD schemes optimized for FFDM images generate relatively lower sensitivity and higher false-positive rate (Baum *et al.*, 2002). In addition, by avoiding possibly repeated digitization, one important advantage of applying CAD schemes to FFDM image is that CAD-generated prompts (marks) are always reproducible.

APPLICATION OF COMPUTER-AIDED DETECTION SCHEMES TO CLINICAL ENVIRONMENT

Because CAD systems have been applied in clinical practice to assist radiologists interpreting screening mammograms, a large number of research groups have conducted a variety of prospective and retrospective studies to assess how using

CAD systems may affect radiologists' performance. In these studies several CAD application issues have been investigated, which are briefly discussed and summarized below.

Rejection of Computer-Aided Detection Scheme prompted False-Negative Masses in Screening Environment

Although studies have reported that CAD could detect a high fraction of false-negative cancers and cancers depicted on prior examination (images) (Warren Burhenne *et al*., 2000; Brem *et al*., 2003), correctly accepting or discarding CAD-prompted suspected regions is not an easy task for radiologists in the screening environment due to low yield of cancers and a large number of CAD-generated false-positive marks. In a large prospective study 12 radiologists interpreted 6,111 screening mammography examinations. Images acquired from each examination were processed and analyzed by CAD. Mammograms were independently double read by two radiologists. During the interpretation of each examination (single-reading), one radiologist recorded the initial evaluation, viewed the CAD prompts, and recorded the final evaluation. A total of 62 cancers were detected in 61 women based on the double reading. This system correctly detected and prompted at least one true-positive region in 51 examinations diagnosed with cancers (84% of the case-based sensitivity) with false-positive rate of 1.59 per examination (four images). In single reading radiologists initially missed 12 cancers (19.7% false-negative rate). Of 12 missed cancers, nine were detected by CAD system. Among them, eight were malignant masses and one associated with micro-calcification cluster. Seven CAD-prompted mass regions were overruled (discarded) by the radiologists in their final evaluations. Action was taken in two CAD-prompted false-negative regions (one mass and one micro-calcification cluster) (Khoo *et al*., 2005).

The reasons for radiologists to discard CAD-prompted false-negative regions may not be well known. Some researchers believe that the CAD-generated high false-positive detection rate was shown to reduce radiologists' confidence in CAD-prompted suspected lesions, while others found that once a radiologist had made initial evaluation of the images, he/she usually would not make changes unless the CAD-prompted lesion was easy to verify (e.g., micro-calcification clusters) or it was substantially different from other false-positive prompts (e.g., a subtle mass that was prompted on both cranio-caudal (CC) and medio-lateral oblique (MLO) view). Because current CAD schemes are optimized to achieve the "optimal" case-based performance, a true-positive mass or micro-calcification cluster is counted as detected if it is correctly detected and marked by the CAD scheme on either one view or two views. As a result, similar to the majority of CAD-generated false-positive regions, most of the subtle cancers that are highly likely to be missed by radiologists in their initial evaluation are detected and prompted by CAD only on one view. In the busy screening environment, it is very difficult for radiologists to correctly distinguish between the subtle (false-negative) masses and false-positive regions if they all are prompted only on one view. In laboratory studies researchers have found that radiologists accepted more CAD-prompted false-negative lesions if

they were prompted on both CC and MLO views. In another prospective study, radiologists detected 43 of 48 cancers without using CAD. CAD detected three of five missed cancers (two were micro-calcifications and one was mass). Because the mass was detected only on one view by CAD, it was ultimately discarded by the radiologist and the two cancers associated with micro-calcification clusters were accepted (actually detected) by the radiologists. As a result, radiologists detected additional two cancers (4.7% sensitivity increase from 43 to 45) with 15% increase in recall rate when using CAD (Ko *et al.*, 2006).

Improvement of Detecting Cancers Associated with Micro-calcification Clusters

A large number of studies have been performed and reported to evaluate whether CAD can help radiologists detect more cancers. In one prospective study, two radiologists interpreted 12,860 screening mammography examinations at a community based breast imaging center with and without CAD. Before evaluating and diagnosing each examination, images were processed and analyzed by the CAD system. CAD generated average 2.8 marks (1.2 micro-calcification marks and 1.6 mass marks) per examination (four view images). Radiologist first evaluated images without viewing CAD-prompted result and recorded the initial interpretation results. Then, CAD-prompted results were provided and the radiologist had an option to change the initial evaluation results. Two sets of detection results for each examination were recorded and compared (even they were identical). In this study, two radiologists dismissed 97.4% of the CAD-prompted marks and considered the remaining 2.6% (368) marks actionable. Two radiologists initially detected 41 cancers (including 26 masses and 15 micro-calcification clusters) without viewing CAD-prompted results; while CAD independently detected 40 cancers (including 18 masses and 22 micro-calcification clusters). By viewing CAD-prompted results and making changes in their initial detections, the radiologists detected eight additional cancers from 41 to 49 (19.5% improvement in sensitivity) and recalled 156 more women (from 830 to 986) that represent an 18.8% increase in recall rate. Of the eight additionally detected cancers, seven were associated with micro-calcifications and one was mass (Freer and Ulissey, 2001).

At current CAD performance level, using CAD benefits radiologists in more efficiently searching for and identifying micro-calcification clusters depicted on images. Although CAD is intended to be used as a "second reader," due to the high confidence of radiologists in CAD performance of micro-calcification cluster detection and relatively easy to verify CAD-prompted results, more and more radiologists use CAD as a prescreening tool ("the first reader"). These radiologists rely heavily on CAD-prompted results and perform largely a search with magnifying glasses only in and around CAD-prompted regions to decide whether the CAD-prompted region depicts a true-positive micro-calcification cluster. As a result, using CAD in clinical practice may help radiologists (1) substantially improve efficiency in interpreting mammograms, (2) reduce inter-reader variability in interpretation of micro-calcification

clusters, and (3) detect more subtle cancers associated with micro-calcifications.

Impact of Computer-Aided Detection Schemes on Radiologists' Performance in Non-prompted Areas

Due to the relatively lower sensitivity and higher false-positive detection rate of current CAD schemes on mass detection, CAD could only detect false-positive regions and miss subtle masses in some true-positive examinations. While radiologists spend more time and effort to discard these false-positive prompts (i.e., discarding 97.4% of CAD-cued regions in a prospective study (Freer and Ulissey, 2001)), their attention to search for subtle lesions is reduced, which results in the degradation of their performance in detecting cancers in non-prompted areas. In a laboratory study, 20 radiologists read 60 cases twice (30 depicting cancers) with and without using CAD. CAD sensitivity was adjusted to 50% (only detecting 15 cancers). The average sensitivity of the readers when using CAD was significantly lower than the sensitivity without using CAD. The difference was most noted for cancers in the 15 non-cued positive cases (Alberdi *et al*., 2004). A recent large retrospective study involving 56,387 screening examinations reported that although using CAD did not significantly affect radiologists' overall sensitivity, the sensitivity was different in two groups. Radiologists' sensitivity increased in the true-positive examinations in which the cancers were correctly detected by CAD and decreased in cases in which CAD only prompted false-positive regions (Taplin *et al*., 2006). These studies clearly suggest the importance of reducing false-positive detection rate of the CAD scheme. Providing incorrect prompts not only reduce the radiologists' efficiency in interpreting mammograms, but also potentially reduce their detection accuracy. Radiologists should be aware of this issue and not ignore the suspicious findings in non-prompted areas to minimize this potentially negative impact of using CAD at current performance level.

Optimal Assessment of Computer-Aided Detection Effect on Radiologists' Performance

A number of studies have been reported in the recent years to compare detection performance between using independent double reading by two radiologists and a single reading of one radiologist with CAD assistance. While some studies reported that double reading could detect more cancers than a single reading with CAD, others reported that single-reading with CAD improved the overall sensitivity of detecting breast cancers. Although there is no agreement on which reading method results in the detection of more cancers by radiologists, one can find that in these studies when the sensitivity is higher using one reading method, the corresponding recall rate is also higher (or the specificity is lower). For example, in one study eight radiologists re-interpreted 10,267 cases using CAD. The researchers compared the reading results between this retrospective single reading with CAD and original double reading during the clinical practice. The results indicate that in this study single reading with CAD increases cancer detection and recall (related to false-positive) rates by 15% and 32%, respectively (Gilbert *et al*., 2006). It is also not clear how much of the observed difference was

due to a "laboratory effect" in the retrospective mode. Hence, using sensitivity as a sole criterion to report the potential effect or benefit of using CAD on radiologists' performance is often not sufficient.

In diagnosis of medical images, each radiologist operates at his/her own ROC (receive operating characteristic) curve. Two observers can operate at the similar ROC performance curves but choose different operating points. One observer has high detection sensitivity, thereby sacrificing specificity, while another observer is satisfied with lower sensitivity in order to achieve higher specificity. When comparing performance difference based on two pairs of sensitivity versus specificity or TPF (true-positive fraction) versus FPF (false-positive fraction) generated by two observers or one group of observers under two reading modes (e.g., with and without using CAD), it is often difficult to determine which pair represent higher performance. Although many researchers believed that using CAD could improve radiologists' sensitivity in detecting breast cancers, others argued that increasing false-positive (recall) rate due to the use of CAD might off-set the potential benefit of small sensitivity increase. The best approach to compensate the variability of observer preference in choosing different operating threshold of detection is to use ROC analysis. The performance difference of radiologists with *or* without the use of CAD assistance can be more reliably compared based on the difference of the areas under two ROC curves or the areas under the two partial ROC curves that are more clinically relevant. If the researchers want to compare observer performance at one specific operating point (threshold), they should compare the detection sensitivities at the same level of specificity (the false-positive rate).

Change of Cancer Detection and Recall Rates Before and After Introduction of Computer-Aided Detection Systems

To eliminate the potential bias of laboratory effect in observer performance studies, a number of research groups directly investigated changes in breast cancer detection and patient recall rates after the introduction of commercialized CAD systems in several medical centers. One study analyzed and compared cancer detection and patient recall rates for 24 radiologists before and after introducing CAD into the clinical practice of a University based medical center. In the data comparison, 56,432 screening cases were interpreted by radiologists without CAD and 59,139 cases were interpreted with CAD assistance. No statistically significant difference was found between two reading environments. The cancer detection rates were 3.49 versus 3.55 per 1,000 examinations (1.7% improvement, $p=0.68$); and the recall rates were 11.39% versus 11.40% ($p=0.96$) without and with using CAD, respectively. The 1.7% increase of cancer detection rate is mainly contributed by detecting more cancers associated with micro-calcification clusters (Gur *et al.*, 2004a).

Another research group investigated influence of CAD on performance of screening mammography at 43 medical facilities from 1998 to 2002. The study analyzed screening detection data of 222,135 women (or a total of 429,345 mammograms). The study reported that regular mammography screening without using CAD detected average 4.15 cancers

per 1,000 women screened, while interpreting mammograms using CAD detected 4.2 cancers per 1,000 examinations. The difference was not statistically significant ($p = 0.90$). However, the biopsy rate when using CAD was increased by 19.7%, which corresponds the reduction of positive prediction value from 4.1% to 3.2% ($p = 0.01$) and area under ROC curve from 0.919 to 0.871 ($p = 0.005$). The researchers concluded that the use of CAD was associated with reduced accuracy of interpretation of screening mammograms and the increased biopsy rate was not clearly associated with improved detection of invasive breast cancers (Fenton *et al*., 2007). These studies suggest that how to optimally use CAD in clinical practice and actually help improve radiologists' performance based on ROC evaluation criterion remains a challenge and unsolved issue.

NEW DEVELOPMENT IN COMPUTER-AIDED DETECTION RESEARCH

Studies have found that radiologists' attitude toward and acceptance of CAD-prompted micro-calcification clusters and masses were substantially different. Due to the high sensitivity, radiologists heavily rely on CAD-prompted results while searching for and identifying micro-calcification clusters. However, the lower CAD sensitivity for mass detection and the higher false-positive rates reduces the usefulness of CAD-prompted masses. It is also much more difficult for radiologists to verify and accept a CAD-prompted subtle mass than a CAD-prompted cluster of micro-calcifications. As a result, a number of studies found that radiologists either ignored CAD-prompted mass regions or substantially reduced the detection specificity. To improve CAD performance on mass detection and increase radiologists' confidence in CAD results, a number of new technologies and approaches have been recently investigated and tested. In this section, we discuss several new approaches in current CAD development and optimization.

Multi-View Based Computer-Aided Detection Schemes

During the interpretation of screening mammograms, radiologists rely heavily on the comparison of multiple images acquired from a single examination (including both bilateral and ipsilateral views) or sequential examinations ("prior" and "current" images). The multi-image based information plays one of the most important roles for radiologists to identify suspected lesions and discard false-positive detections. However, current commercialized CAD schemes are basically the single-image based detection schemes with the exception of possibly limiting the total number of CAD-prompted lesions in one examination. Multi-image (or multi-view) related information is not incorporated into the decision making process of these single-image based CAD schemes. Hence, CAD is somewhat "disadvantaged" as compared with radiologists and as a result the false-positive detection rate of current CAD schemes is substantially higher than that of the radiologists, in particular for mass detection. Another disadvantage of current CAD schemes is that since case-based sensitivity is commonly used to optimize CAD performance and a mass is considered "detected" if it is identified either on one or two views, a large fraction

of true-positive masses and the majority of false-positive regions are actually detected by CAD only on one view. Several studies from different groups suggested that radiologists were more likely to discard subtle masses detected only on one view as they routinely did in the case of false-positive detections.

To overcome these two disadvantages of current CAD schemes, researchers have attempted to develop multi-image based CAD schemes. A number of schemes has been developed and tested to identify specific landmarks (e.g., primarily nipple location and pectoral muscle) depicted on mammograms. Combining the global registration method and local area non-rigid adjustment (alignment), researchers have investigated different approaches in an attempt to match suspected mass regions detected on both ipsilateral (CC and MLO) views and thus to improve CAD performance. These approaches used two underlying assumptions: (1) a true-positive mass (TP) has a higher chance of being detected by the CAD scheme on both views than a false-positive region (FP), and (2) the same mass (TP) detected on two views (a TP-TP pair) has features that are measurably more similar than either TP-FP pairs or FP-FP pairs. For example, one study applied a geometrical model and a linear discriminant analysis (LDA) based on 15 texture and 31 morphological features to match (pair) regions detected on two views and identify TP-TP pairs. Using a dataset of 128 "current" and 41 "prior" image pairs as well as applying a threshold to initially select an average of 10 CAD-cued suspected regions (matching candidates) per image, the study found that by incorporating a two-view LDA based classifier, the overall case-based sensitivity was increased from 67% to 73% at one false-positive per image (Paquerault *et al.*, 2002). Because the correlations of image features between TP-TP pairs of subtle masses are often low, in order to maintain a relatively high sensitivity for the schemes, the matching performance was often assessed and reported at a relatively high false-positive detection rate (i.e., ≥ one false-positive detection per image). Due to the much higher false-positive detection rate than the current single-image based CAD schemes, no multi-view based CAD scheme has been used in clinical practice.

In order to increase the number of breast masses being detected on both views while maintaining a comparable case-based false-positive detection rate, a new multi-view CAD scheme was developed and tested (Zheng *et al.*, 2006). The scheme combines two independent modules. The first module is a single-image based scheme that detects initially suspected regions depicted on each image. For each identified region with detection score larger than the operating threshold, the second module is applied to match regions detected on both views. For this purpose, the module first identifies or estimates the locations of nipple and chest wall depicted on two images. To find matched areas of interest, the module computes the distance between the nipple and each CAD-prompted mass region projected onto the centerline (a line through the nipple that is perpendicular to the chest wall). Applying the same projected distance to the corresponding ipsilateral image, the module defines a "matching strip" of interest. The width of this strip is dependent on the effective size (e.g., the maximum length) of the originally CAD-prompted suspected mass

region. Then, the CAD scheme increases its detection sensitivity inside this matching strip to search for and identify a potentially matched region. For this purpose, the scheme gradually lowers its operating threshold on the detection scores until a suspected region is detected inside the strip. These two identified regions are considered to represent a mass depicted on two views. If no matched region can be identified inside the matching strip, the originally detected region is considered as an un-matched single region. Unless the single region meets one of either two exceptional rules, (1) the detection score is higher than a threshold (e.g., > 0.85) or (2) the matching strip of interest cannot be identified on the ipsilateral view because the region is located near the chest wall, the un-matched single region is discarded.

When applying both the single-image based CAD scheme and the multi-view based CAD scheme to detect suspected mass regions depicted on an image database involving 450 examinations (in which 250 involve malignant masses), the multi-view based scheme maintained a case-based detection sensitivity (74.4%) as the single-image based scheme at a reduced case-based false-positive detection rate by 23.7% (from 539 to 411). At the same time, the multi-view based scheme increased the number of true-positive masses being cued on both views from 91 to 169 (an 85.7% increase). Generally, attempts to increase the number of true-positive masses being cued by CAD on two views would substantially increase the number of false-positive detections as well. To overcome this difficulty, this scheme limits the number of possible matching pairs to be identified on two views from ≥ 5 to less than one per image, which is one of the primary reasons that the CAD scheme using a simple location based matching method has potential to substantially increase the number of masses being detected on two views while maintaining a comparable case-based false-positive rate to the CAD schemes used in the clinical environment. In addition, although the case-based sensitivity remains the same, a small fraction of the true-positive masses originally detected by the single-image based scheme is replaced by other masses with somewhat lower detection scores using the multi-view based CAD scheme, which may indicate that the multi-view based scheme actually detects and prompts more subtle masses. While this multi-view based CAD scheme increases the number of true-positive masses being detected on two views, it also prompted the majority of false-positive findings on both views. Although prompting true-positive masses on two views can help radiologists detect more subtle cancers, the issues of (1) whether radiologists can easily recognize the false-positive regions "matched" incorrectly on two views, and (2) whether these "paired" false-positive detections could significantly increase radiologists' recall rate need to be further investigated before this type of multi-view CAD scheme can be optimally applied in the clinical environment (Zheng *et al*., 2006).

Computer-Aided Detection Schemes with Interval Change Classifiers

In screening mammography, most women have sequential (yearly) examinations. When the radiologist interprets one examination, he/she often compare and analyze the interval changes of the suspected areas depicted on images acquired

from current and prior examinations. Comparison of sequential mammograms plays an important role in radiologists' decision making process. For example, if a suspected mass is visually identified in current image, the radiologist searches for the corresponding mass or dense tissue area depicts on the prior images (if available) to decide whether this is a new finding. If the corresponding mass is found in the prior image, the radiologist compares the change of size and density distribution between two masses to estimate the likelihood of malignancy of the finding.

To facilitate such comparison and analysis, a number of research groups have developed and tested the multi-image based CAD schemes that incorporate the information extracted from both the current and prior images into CAD classification process. For this purpose, the scheme needs a new computing module to detect and identify the corresponding mass regions depicted on current and prior images. A number of different algorithms using global image registration followed by a local area matching have been developed. To test the performance of CAD schemes to automatically link (or match) the temporal mass pairs depicted on current and prior images, one study reported that 87% of 124 mass pairs were overlapped at least 50% (Hadjiiski *et al*., 2001) and in another study the 82% of 389 temporal mass pairs were counted as correctly linked (Timp *et al*., 2005). After identifying the locations of matched temporal mass pairs, CAD schemes segment both regions and compute the features to represent two regions. Researchers then train and optimize the classifiers using features computed from both current and prior images. The testing results indicated that by incorporating corresponding feature information extracted from both current and prior images, CAD scheme achieved higher performance (e.g., increasing values of ROC curves from 0.82 to 0.88 (Hadjiiski *et al*., 2001)). A recent study developed and reported a dual CAD scheme, in which two single CAD schemes were first separately trained and optimized using current and prior images. The rule-based and linear discriminant analysis (LDA) based classifier was then used in each scheme to generate a detection score for each identified suspected mass region. The dual scheme was built by using an artificial neural network to merge LDA detection scores and differentiate true-positive masses from the normal breast tissue. The study reported that this dual CAD scheme could substantially reduce FP rate (i.e., from 1.6 to 0.9 per image at 85% sensitivity) while maintaining higher sensitivity (Wei *et al*., 2006).

To assess the potential benefit in the clinical practice when using a CAD scheme that involves an interval change classifier using the image features computed from current and prior images, a retrospective observer performance study was reported recently (Hadjiiski *et al*., 2006). In this study, 90 temporal pairs of two-view serial mammograms (depicting 47 malignant and 43-biopsy-proved masses) were selected. The CAD scheme was applied to process these images. Eight radiologists and two breast imaging fellows participated in this study to assess digitized two-view temporal pairs (in pre-selected regions of interest only) by estimating and reporting the likelihood of malignancy of each mass pair using BI-RADS categories. The study reported that the average area under ROC curve of 10 observers in classifying between the malignant and benign

masses improved from 0.83 without CAD to 0.87 with CAD ($p < 0.05$). In BI-RADS assessment, five observers improved both sensitivity and specificity. Five observers reduced specificity (by recommending more benign masses to biopsy) and among them one also reduced sensitivity (by incorrectly removing malignant masses from the biopsy recommendation list). This is a very limited study with restricted laboratory condition that may not be applicable in clinical screening environment. Hence, further investigations are needed to assess the benefit of using CAD schemes involving interval change classifiers.

Interactive Computer-Aided Detection and Visual Aid

Studies have demonstrated that unless CAD systems had extremely high performance levels and the results could be visually verified (e.g., CAD-prompted micro-calcification clusters), it was often difficult for radiologists to accept the CAD results without knowing the logic or reasoning for the detection. Current CAD systems prompted all detected suspected mass regions if their detection scores are higher than a predetermined operating threshold without explanation of why these regions are identified by the schemes as positive masses. This "black-box" type approach and the relatively low performance are the major factors that reduce radiologists' confidence in CAD-cued mass regions. To improve CAD performance and increase radiologists' confidence in CAD-prompted masses, several research groups have been developing interactive computer-aided diagnosis (ICAD) schemes to identify visually similar and clinically relevant mass regions. Once a suspected mass region is queried by the observer, ICAD scheme segments the queried region and computes the likelihood of this region being associated with cancer based on comparisons with sets of CAD-selected "similar" regions from a large reference library. These similar regions with known (verified) outcome are displayed on the ICAD workstation and used as a "visual aid" to assist radiologists in their decision making. Preliminary observer performance studies suggest that using an ICAD concept could improve radiologists' performance in classifying between malignant and benign masses as well as increase their confidence in the CAD-cued results (Giger *et al*., 2002).

Several techniques using multiple computer-extracted features, pixel value based mutual information, and content-based image retrieval network have been investigated and applied to compute "similarity" between the queried suspicious lesion and the selected reference lesions. One group developed an information-theoretic computer-assisted detection (IT-CAD) scheme to search for and evaluate image similarity measures (Tourassi *et al*., 2007). In this study the researchers first investigated eight IT similarity measures (including joint entropy, conditional entropy, mutual information, normalized mutual information, average Kullback-Leibler divergence, maximum Kullback-Leibler divergence, Jensen divergence, and arithmetic-geometric mean divergence) to search for similar mass regions stored in a reference library including 1,820 regions of interest. The study indicated that based on the top five retrievals, using normalized mutual information, the scheme achieved the highest retrieval precision, which is defined as number of relevant retrieval regions (e.g., if queried

region is malignant region, the retrieved regions are also malignant) divided by the total number of retrieved regions (including both malignant and benign or normal regions). The study also demonstrated that this IT-CAD scheme yielded a substantial reduction in false-positive detections while maintaining high detection sensitivity. The limitation of this study is that it assesses retrieval precision based on semantic, not visual content.

Although image content-based retrieval of the similar reference mass regions and providing radiologists "visual aid" is a new and attractive concept, it has only achieved limited success. One of the primary reasons for the limited enthusiasm is the substantial fraction of selected reference regions that are not considered to be "visually similar" by the observers due to the substantial difference between computer vision and human vision. In order to improve the clinical utility of ICAD system and increase radiologists' confidence in accepting and using ICAD results in their decision making, improving the clinical relevance and visual similarity of CAD-selected similar reference regions are the most two important issues. Recently, one research group developed a new ICAD scheme that aims to improve visual similarity between the queried region and ICAD-selected reference regions (Zheng *et al.*, 2007). First, the scheme uses a hybrid region growth algorithm (including a multi-layer topographic region growth and an active contour algorithm) to segment queried suspected mass region. Second, the scheme detects region boundary spiculated pixels and computes an index to classify the suspected regions into one of the three groups of spiculation levels ("none/minimal," "moderate," and "severe/significant"). Third, the scheme computes a set of image features for each queried region and applies a distance-weighted k-nearest neighbor (KNN) algorithm with three restricted conditions that guarantee the comparable size, circularity, and boundary margin spiculation level among the selected similar regions. Using the feature-based KNN algorithm, the scheme searches through the reference library and selects a small set of similar candidate regions by quickly discarding the majority regions as irrelevant regions. Fourth, the scheme computes mutual information (MI) between the queried mass region and each of the similar regions selected by KNN algorithm and sorts the regions starting from the highest MI score. The scheme finally selects a set of similar regions with the higher MI scores. Using these approaches, the ICAD system maintains the capability of real-time interaction between the observer and the computer scheme. Specifically, the response time for ICAD system to segment the queried region, select the similar reference regions, and display the results is less than one second. A two-alternative forced choice observer preference study was conducted to compare the "visual similarity" between a queried mass region and two sets of similar regions selected from a library of 3,000 reference regions. Nine observers participated in the study to select more visually similar region sets for each of 85 queried regions. The results demonstrate that the new CAD scheme can select reference sets that are significantly more "visually similar" ($p < 0.05$) than when using traditional CAD schemes in which the mass boundary spiculation levels are not accurately detected and quantified.

In conclusion, CAD of mammography has been routinely used in the clinical

practice to assist radiologists in interpreting screening mammograms. Evaluation of the impact of using CAD on the actual improvement of radiologists' performance in cancer detection is not a simple task and the results heavily depend on CAD performance, the size and diversity of selected dataset, the experience of the observers who participated in the studies, and the reporting methods (or evaluation criteria). Despite the different study results and disagreement among researchers whether current CAD is (or is not) an effective aid for screening mammography, CAD clearly increases the efficiency and the confidence level of radiologists while searching for micro-calcification clusters due to the high sensitivity of CAD schemes. As a result, radiologists typically feel less fatigued at the end of a CAD supported reading session. This is an important benefit in the clinical practice to help radiologists make correct diagnostic decisions. However, the lower CAD sensitivity for mass detection and the higher false-positive rates reduces the usefulness of CAD-prompted masses. Although CAD can detect a high fraction of subtle cancers missed by radiologists, most of the subtle malignant masses are detected only on one view (similar to the most of false-positive detections). As a result, due to the lower confidence level of radiologists in the CAD results of mass detection, majority of CAD-prompted false-negative masses are discarded by radiologists in busy screening environment. As researchers better understand how CAD is currently used in the clinical practice and how CAD impacts on radiologists' performance, a number of new approaches has been proposed and investigated to develop and optimize CAD schemes for mass detection. To realize the full potential of CAD in the clinical practice, the issues of improving CAD performance in mass detection as well as increasing radiologists' confidence in and reliance on CAD-prompted masses during the interpretation of screening mammograms need to be further investigated.

REFERENCES

Alberdi, E., Povyakalo, A., Strigini, L., and Ayton, P., (2004). Effects of incorrect computer-aided detection (CAD) output on human decision-making in mammography. *Acad. Radiol. 11*: 909–918.

Baker, J.A., Rosen, E.L., Lo, J.Y., Gimenez, E.I., Walsh, R., and Soo, M.S. (2003). Computer-aided detection (CAD) in screening mammography: sensitivity of commercial CAD systems for detecting architectural distortion. *Am. J. Roentgenol. 181*: 1083–1088.

Baker, J.A., Lo, J.Y., Delong, D.M., and Floyd, C.E. (2004). Computer-aided detection in screening mammography: variability in cues. *Radiology 233*: 411–417.

Baum, F., Fisher, U., Obenauer, S., and Grabbe, E. (2002). Computer-aided detection in direct digital full-field mammography: initial results. *Eur. Radiol. 12*: 3015–3017.

Brake, G.M., and Karssemeijer, N. (2001). Segmentation of suspicious densities in digital mammograms. *Med. Phys. 28*: 259–266.

Brem, R.F., Baum, J., Lechner, M., Kaplan, S., Souders, S., Naul, L.G., and Hoffmeister, J. (2003). Improvement in sensitivity of screening mammography with computer-aided detection: a multi-institutional trial. *Am. J. Roentgenol. 181*: 687–693.

Brem, R.F., Hoffmeister, J.W., Zisman, G., DeSimio, M.P., and Rogers, S.K. (2005). A computer-aided detection system for evaluation of breast cancer by mammographic appearance and lesion size. *Am. J. Roentgenol. 184*: 893–896.

Evans, W.P., Warren Burhenne, L.J., Laurie, L., O'Shaughnessy, K.F., and Castellino, R.A. (2002). Invasive lobular carcinoma of the breast: mammographic characteristics and computer-aided detection. *Radiology 225*: 182–189.

Fenton, J.J., Taplin, S.H., Carney, P.A., Abraham, L., Sickles, E.A., D'Orsi, C., Berns, E.A., Cutter, G.,

Hendrick, R.E., Barlow, W.E., and Elmore, J.G. (2007). Influence of computer-aided detection on performance of screening mammography. *New Engl. J. Med. 356*: 1399–1409.

Freer, T.M., and Ulissey, M.J. (2001). Screening mammography with computer-aided detection: prospective study of 12,860 patients in a community breast center. *Radiology 220*: 781–786.

Giger, M.L., Huo, A., Vyborny, C.J., Lan, L., Bonta, I., Horsch, K., Nishikawa, R.M., and Rosenbourgh, I. (2002). Intelligent CAD workstation for breast imaging using similarity to known lesions and multiple visual prompt aids. *Proc. SPIE 4684*: 768–773.

Gilbert, F.J., Astley, S.M., McGee, M.A., Gillan, M.G., Boggis, C.R., Griffiths, P.M., and Duffy, S.W. (2006) Single reading with computer-aided detection and double reading of screening mammograms in the United Kingdom national breast screening program. *Radiology 241*: 47–53.

Gur, D., Sumkin, J.H., Rockette, H.E., Ganott, M., Hakim, C., Hardesty, L., Poller, W.R., Shah, R., and Wallace, L. (2004a). Changes in breast cancer detection and mammography recall rates after the introduction of a computer-aided detection system. *J. Nat. Cancer Inst. 96*: 185–190.

Gur, D., Stalder, J.S., Hardesty, L.A., Zheng, B., and Sumkin, J.H. (2004b). Computer-aided detection performance in mammographic examination of masses: assessment. *Radiology 223*: 418–423.

Hadjiiski, L., Sahiner, B., Chan, H.P., Petrick, N., and Helvie, M. (2001). Analysis of temporal changes of mammographic features: computer-aided classification of malignant and benign breast masses. *Med. Phys. 28*: 2309–2317.

Hadjiiski, L., Sahiner, B., Helvie, M.A., Chan, H.P., Roubidoux, M.A., and Shen, J. (2006). Breast masses: computer-aided diagnosis with serial mammograms. *Radiology 240*: 343–356.

Ho, W.T., and Lam, P.W. (2003). Clinical performance of computer-assisted detection (CAD) system in detecting carcinoma in breast of different densities. *Clin. Radiol. 58*: 133–136.

Khoo, L.A., Taylor, P., and Given-Wilson, R.M. (2005). Computer-aided detection in the United Kingdom National Breast Screening Programme: prospective study. *Radiology 237*: 444–449.

Ko, J.M., Nicholas, M.J., Mendel, J.B., and Slanetz, P.J., (2006). Prospective assessment of computer-aided detection in interpretation of screening mammograms. *Am. J. Roentgenol. 187*: 1483–1491.

Mendez, A.J., Tahoces, P.G., Lado, M.J., Souto, M., and Vidal, J.J. (1998). Computer-aided diagnosis: automatic detection of malignant masses in digitized mammograms. *Med. Phys. 25*: 957–964.

Mini, M.G., Tech, M., Devassia, V.P., and Thomas, T. (2004). Multiplexed wavelet transform technique for detection of microcalcification in digitized mammograms. *J. Digital Imag. 17*: 285–291.

Paquerault, S., Petrick, N., Chan, H.P., Sahiner, B., and Helvie, M.A., (2002). Improvement of computerized mass detection on mammograms: fusion of two-view information. *Med. Phys. 29*: 238–247.

Petrick, N., Chan, H.P., Wei, D., Sahiner, B., Helvie, M.A., and Adler, D.D. (1996). Automated detection of breast masses on mammograms using adaptive contrast enhancement and texture classification. *Med. Phys. 23*: 1685–1696.

Soo, M.S., Rosen, E.L., Xia, J.Q., Ghate, S., and Baker, J.A. (2005). Computer-aided detection of amorphous calcifications. *Am. J. Roentgenol. 184*: 887–892.

Tabar, L., Chen, H.H., Yen, M.F., and Tot, T. (2004). Mammographic tumor features can predict long-term outcomes reliably in women with 1-14-mm invasive breast carcinoma. *Cancer 101*: 1745–1759.

Taplin, S.H., Rutter, C.M., and Lehman, C. (2006). Testing the effect of computer-assisted detection on interpretive performance in screening mammography. *Am. J. Roentgenol. 187*: 1475–1482.

Taylor, C.G., Champness, J., Reddy, M., Taylor, P., Potts, H.W., and Given-Wilson, R. (2003). Reproducibility of prompts in computer-aided detection (CAD) of breast cancers. *Clin. Radiol. 58*: 733–738.

Timp, S., van Engeland, S., and Karssemeijer, N. (2005). A regional registration method to find corresponding mass lesions in temporal mammogram pairs. *Med. Phys. 32*: 2629–2638.

Tourassi, G.D., Harrawood, B., Singh, S., Lo, J.Y., and Floyd, C.E. (2007). Evaluation of information-theoretic similarity measures for content-based retrieval and detection of masses in mammograms. *Med. Phys. 34*: 140–150.

Vyborny, C.J., Doi, T., O'Shaughnessy, K.F., Romsdahl, H.M., Schneider, A.C., and Stein, A.A. (2000). Breast cancer: importance of

spiculation in computer-aided detection. *Radiology 215*: 703–707.

Warren Burhenne, L.J., Wood, S.A., D'Orsi, C.J., Feig, S.A., Kopans, D.B., O'Shaughnessy, K.F., Sickles, E.A., Tabao, L., Vyborny, C.J., and Castellino, R.A., (2000) Potential contribution of computer-aided detection to the sensitivity of screening mammography. *Radiology 215*: 554–562.

Wei, J., Chan, H.P., Sahiner, B., Hadjiiski, L.M., Helvie, M.A., Roubidoux, M.A., Zhou, C., and Ge, J. (2006). Dual system approach to computer-aided detection of breast masses on mammograms. *Med. Phys. 33*: 4157–4168.

Zheng, B., Sumkin, J.H., Good, W.F., Maitz, G.S., Chang, Y.H., and Gur, D. (2000). Applying computer-assisted detection schemes to digitized mammograms after JPEG data compression: An assessment. *Acad. Radiol. 7*: 595–602.

Zheng, B., Leader, J.K., Abrams, G.S., Lu, A., Wallace, L., Maitz, G.S., and Gur, D. (2006). Multiview-based computer-aided detection scheme for breast masses. *Med. Phys. 33*: 3135–3143.

Zheng, B., Mello-Thoms, C., Wang, X.H., Abrams, G.S., Sumkin, J.H., Chough, D.M., Ganott, M.A., Lu, A., and Gur, D. (2007). Interactive computer-aided diagnosis of breast masses: computerized selection of visually similar image sets from a reference library. *Acad. Radiol. 14*: 917–927.

3
Sebaceous Carcinoma of the Breast: Clinicopathologic Features

Masanori Hisaoka and Tetsuo Hamada

INTRODUCTION

Sebaceous carcinoma is a distinctive breast carcinoma characterized by unequivocal morphologic differentiation towards sebaceous epithelium or sebocytes, whereas its cutaneous counterpart is better described most commonly in ocular and only occasionally in extraocular regions. Despite the possible derivation from a putative common origin of the mammary glands and skin appendages, it is notable that primary sebaceous carcinoma of the breast is exceedingly rare.

The sebaceous feature in mammary carcinomas was initially recognized by van Bogaert and Maldague (1977), who described such lesions in three cases as a variant of lipid-secreting carcinoma of the breast. Subsequently, the sebaceous differentiation was identified in mammary adenoid cystic carcinoma by Tavassoli and Norris (1986) and in peculiar mammary carcinoma with ductal, squamous, and myoepithelial elements by Prescott *et al.* (1992). In 1999, Tavassoli first described the mammary carcinoma with well-differentiated sebaceous morphology under the rubric of sebaceous carcinoma in her monograph.

Although cutaneous sebaceous carcinomas may also occur in the breast or involve the mammary gland, such lesions should not be included in the category of mammary sebaceous carcinoma.

METHODS

We reviewed clinicopathologic findings of the cases with sebaceous carcinoma of the breast or mammary carcinoma with sebaceous differentiation. In addition, the formalin-fixed, paraffin-embedded tumor specimens studied by us were examined microscopically, histochemically (periodic acid-Schiff (PAS), mucicarmine), and immunohistochemically using the following primary antibodies and a labeled polymeric secondary antibody (EnVision system, DAKO Cytomation, Kyoto, Japan) with or without appropriate antigen retrieval; anticytokeratin (AE1/AE3, 1:50, DAKO Cytomation), anti-α-smooth muscle actin (1A4, 1:150, DAKO Cytomation), anti-S-100 protein (polyclonal, 1:200, DAKO Cytomation), antisynaptophysin (SY38, 1:100, DAKO Cytomation), antineurofilament (DA2/FNP7/RMdO20. 11, 1:50, Zymed, South San Francisco, CA),

antiPGP9.5 (13C31A3, 1:400, UltraClone, Isle of Wight, UK), anticarcinoembryonic antigen (II-7, 1:200, DAKO Cytomation), antiGCDFP-15 (23A3, 1:50, Novocastra, Newcastle-upon-Tyne, UK), antiestrogen receptor (1D5, 1:50, DAKO Cytomation), antiprogesterone receptor (1A6, 1:50, Immunotech, Marseille, France), and anti-HER-2/neu (polyclonal, prediluted, DAKO Cytomation). Frozen sections were prepared from the formalin-fixed wet tumor tissue and stained with oil-red-O.

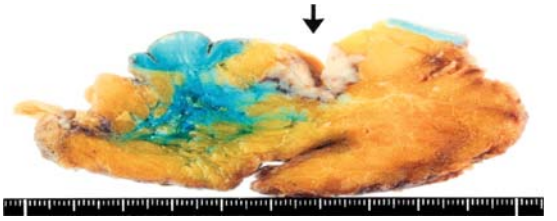


FIGURE 3.1. Macroscopic view of sebaceous carcinoma (arrow), showing a well delineated grayish nodule

CLINICAL FEATURES

According to the previously described but still limited cases including the most recent one by Hisaoka *et al*. (2006), the carcinomas with sebaceous differentiation primarily affected middle-aged or elderly women. A single case of a male patient was documented by Mazzella *et al*. (1995). The patients usually presented with palpable nodules or masses lacking any other clinical signs or symptoms. The right breast was more frequently affected by the tumor than the left one.

PATHOLOGIC FINDINGS

Macroscopically, the tumors are commonly well-circumscribed but occasionally ill-defined nodules or masses with size ranging from 2.5 to 7.5 cm and a firm consistency (Figure 3.1). The lesion has a solid, white or gray cut surface. Any connection between the tumor and the overlying skin or its adnexal structures is not found.

Microscopically, the lesion is composed of a nested or lobular and solid or sheet-like proliferation of large polygonal epithelial cells with abundant multivacuolated cytoplasm and often scalloped nuclei, resembling epithelial cells in sebaceous glands of the skin, which are variably admixed with smaller non-vacuolated cells having eosinophilic cytoplasm (Figures 3.2, 3.3). Tumor cells usually show mild or moderate nuclear atypia with low mitotic activity, but frequent mitotic figures may be present. Intracytoplasmic lipids are demonstrated in a majority of the tumor cells with fat staining with oil-red-O or ultrastructurally. PAS-positive intracytoplasmic glycogen granules are few, and no intra- or extracellular mucin production is evident histochemically. The lesion with predominant tumor cells showing such sebaceous features fits well with the designation of sebaceous carcinoma in the narrow sense, although non-sebaceous elements of the lesion may display other distinct morphologic appearances such as those of mammary ductal carcinoma and adenoid cystic carcinoma. The lesion is usually recognized as an invasive carcinoma with or without minor *in situ* components. Foci of squamous differentiation (or squamous morules) or myoepthelial cells may be also present.

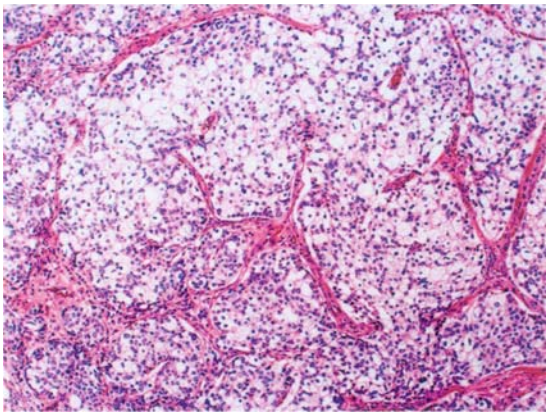


FIGURE 3.2. Microscopically, the tumor cells are arranged in a nested or solid lobular growth fashion (Hematoxylin & Eosin, ×100)

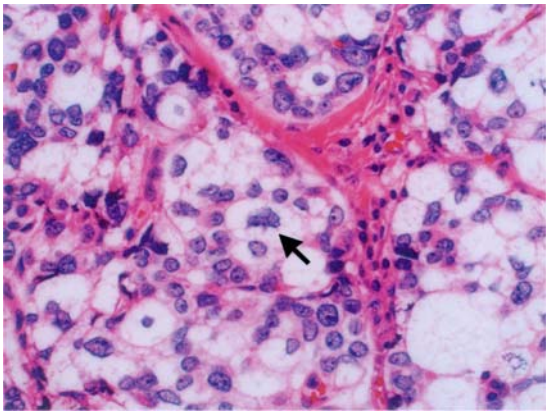


FIGURE 3.3. Ample multivacuolated cytoplasm with often scalloped nuclei (arrow) suggests sebaceous differentiation of the tumor cells (Hematoxylin & Eosin, ×400)

Immunohistochemically, the tumor cells are invariably positive for epithelial markers such as cytokeratin and epithelial membrane antigen. S-100 protein, smooth muscle actin, gross cystic disease fluid protein-15 (GCDFP-15), and carcinoembryonic antigen were consistently negative in the previously analyzed cases and ours. Focal expression of some neural or neuroendocrine markers such as synaptophysin, neurofilament, and PGP9.5 have been demonstrated in a single study by Hisaoka *et al*. (2006). Receptors of estrogen and progesterone were expressed in most of the cases examined. No immunohistochemical overexpression of HER-2/neu has so far been described.

DIFFERENTIAL DIAGNOSIS

Because of the potential intracytoplasmic production or accumulation of lipids, lipid-rich carcinoma and apocrine carcinoma are main differential diagnoses of sebaceous carcinoma. Apocrine carcinoma is characterized by cytologic features of apocrine differentiation such as abundant eosinophilic or oncocytic cytoplasm. Although the tumor cells of apocrine carcinoma usually lack overt sebaceous differentiation, they may display a histiocytoid appearance with vacuolated or foamy cytoplasm (so-called sebocrine cells). The histologic architecture of apocrine carcinoma often exhibits an intraductal solid or papillotubular or cribriform pattern as also seen in nonapocrine type mammary carcinomas, and essentially differs from the nested or lobular structures of sebaceous carcinoma. Furthermore, sebaceous carcinoma is immunohistochemically negative for GCDFP-15, a specific marker of apocrine differentiation.

Lipid-rich carcinoma is a rare variant of invasive ductal carcinoma of the breast, in which a majority of the tumor cells also contain abundant cytoplasmic lipids. In lipid-rich carcinoma, the tumor cells have clear or vacuolated cytoplasm, and are often arranged in infiltrating nests or cords or alveolar structures rather than

in a lobular or solid growth pattern as seen in sebaceous carcinoma. Moreover, cytoplasmic lipid vacuoles may be much more subtle in lipid-rich carcinoma. Because of the extreme rarity of both lipid-rich carcinoma and sebaceous carcinoma, they may, however, represent a single disease spectrum with some cytoarchitectural variations as initially reported by van Bogaert and Maldague (1977) rather than two distinct tumor entities. Although other primary mammary carcinomas, such as adenoid cystic carcinoma and ductal carcinoma, may show focal but unequivocal sebaceous differentiation, they would be better labeled as such carcinomas with sebaceous features (or differentiation).

PROGNOSIS AND TREATMENT

Because of the very limited number of patients with available follow-up information, the biological behavior of sebaceous carcinoma remains largely unknown. Tavassoli (1999) suggested that it is a low-grade tumor because her study showed well-differentiated histology and no evidence of lymph node metastasis despite the relatively large size of the lesion. However, Varga *et al*. (2000) described a case that developed distant metastases involving the skin and bones 8 years after the first operation. Therefore, sebaceous carcinoma is potentially a more aggressive phenotype of mammary carcinoma than considered previously. Besides, the case reported by Hisaoka *et al*. (2006) had a metastatic deposit in one of the nine regional lymph nodes excised at a muscle-preserving radical mastectomy.

Practically, any lesion should be surgically removed with an adequate lymph node dissection, followed by necessary adjuvant chemotherapy and/or radiotherapy essentially according to the risk assessment and therapeutic recommendations suggested in the recent expert consensus meeting, which has been reviewed and summarized by Goldhirsch *et al*. (2005). An effectiveness of Herceptin (an antibody raised against HER-2/neu), however, has not been guaranteed because of lack of any reported examples over-expressing the oncogenic receptor protein.

REFERENCES

Goldhirsch, A., Glick, J.H., Gelber, R.D., Coates, A.S., Thürlimann, B., Senn, H.-J., and Panel Members. 2005. Meeting highlights: international expert consensus on the primary therapy of early breast cancer. *Ann. Oncol. 16*: 1569–1583.

Hisaoka, M., Takamatsu, Y., Hirano, Y., Maeda, H., and Hamada, T. 2006. Sebaceous carcinoma of the breast: case report and review of the literature. *Virch. Arch. 449*: 484–488.

Mazzella, F.M., Sieber, S.C., and Braza, F. 1995. Ductal carcinoma of male breast with prominent lipid-rich component. *Pathology 27*: 280–283.

Prescott, R.J., Eyden, B.P., and Reeve, N.L. 1992. Sebaceous differentiation in a breast carcinoma with ductal, myoepithelial and squamous elements. *Histopatholgy 21*: 181–184.

Tavassoli, F.A. 1999. Sebaceous carcinoma. In: Tavassoli, F.A., ed. *Pathology of the Breast*, 2nd edn. Appleton and Lange, Stamford, pp 555–558.

Tavassoli, F.A., and Norris, H.J. 1986. Mammary adenoid cystic carcinoma with sebaceous differentiation. A morphologic study of the cell types. *Arch. Pathol. Lab. Med. 110*: 1045–1053.

van Bogaert, L.-J., and Maldague, P. 1977. Histologic variants of lipid-secreting carcinoma of the breast. *Virch. Arch. Pathol. Anat. 375*: 345–353.

Varga, Z., Kolb, S.A., Flury, R., Burkhard, R., and Caduff, R. 2000. Sebaceous carcinoma of the breast. *Pathol. Int. 50*: 63–66.

4
Breast Cancer: Detection by In-Vivo Imaging of Angiogenesis

Tore Bach-Gansmo and Derek Tobin

INTRODUCTION

The theory that tumor growth is dependent upon angiogenesis started with the works of Folkman in the 1970s (Folkman, 1971). Since then, research activities in this field have led to the discovery of cellular markers and mediators associated with angiogenesis. One such family of markers is the integrin proteins (Tamkun *et al*., 1986), the structure and functions of which have been the subject of numerous publications, totaling in excess of 35,000 hits in PubMed.

Integrins are essential for the formation of new blood vessels, and this dependency is mediated by the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, which are specifically expressed and activated during the angiogenic process. These molecules thereby provide a potential key to two medical needs, firstly a therapeutic target to turn off angiogenesis and provide a strangle-hold on growing tumors and secondly, a specifically expressed target for molecular imaging agents.

Understanding angiogenesis for research or medical needs requires tools for monitoring angiogenic activity. Of particular interest is the development of non-invasive *in vivo* monitoring of angiogenesis, preferably by visualizing particular molecular events. Such a tool has the potential to monitor the presence of an angiogenic process, its relative activity, the efficacy of anti-angiogenic agents, and possibly modify the prognosis for cancer. This chapter relates the initial clinical experience with one such new agent, Technetium 99 m-NC100692. This agent binds to the $\alpha_v\beta_3$ and the $\alpha_v\beta_5$ integrins and has provided exciting initial images that suggest a positive role for this agent in *in vivo* imaging of angiogenic activity.

ANGIOGENESIS

Angiogenesis is the creation of new blood vessels and is considered an essential requirement for the development of a solid tumor beyond the size of 1–2 mm (Hanahan and Folkman, 1996). Mammalian cells require a continuous supply of oxygen (and nutrients) for their survival, and, therefore, must be located within the diffusion limit for oxygen which is generally considered to be $\leq 200\,\mu m$ from the nearest blood vessel. As tissue mass increases, cells located furthest from the nearest blood vessel become hypoxic and release

a host of growth factors such as hypoxic inducible factors; these, in turn, stimulate the release of proangiogenic factors such as vascular epithelial growth factor (VEGF) and fibroblastic growth factors. At present there are > 20 angiogenic factors and 30 anti-angiogenic factors known. Release of proangiogenic factors causes a variety of reactions including endothelial cell proliferation, release of metaloproteases for the breakdown of the extracellular matrix (ECM), and migration of the new endothelial cells such that the *de novo* blood vessels grow towards the hypoxic area. The growing needs of the ever hungry tumor are, therefore, met by a responsive vascular support. Without angiogenesis, cell proliferation is hampered such that the rate of growth is matched by the rate of apoptosis and the tumor remains dormant.

Newly formed blood vessels have characteristics that differentiate them from normal blood vessels, with important functional and structural abnormalities. Angiogenic vessels are highly disorganized, they are unevenly dilated and show excessive branching and shunting, as a result, with chaotic and irregular blood flow. The vessel walls show endothelial fenestration and transcellular holes, widened interendothelial junctions, and interruptions or a total lack of basement membrane. The poor integrity of tumor related angiogenic blood vessels may have an important role in the ability of the tumor to metastasize by providing an easily accessible escape route from the tumor into the body's vasculature. Another particularity interesting feature of these vessels is the expression of the $\alpha_v\beta_3$ integrin. This integrin is expressed specifically on endothelial cells associated with this process.

INTEGRINS

Integrins are transmembrane receptor proteins with both intracellular and extracellular components, composed of two variable subunits named α and β. As many as 18 α and 8 β variants have been described, which undergo heterodimerization to form 24 different combinations of functional integrins. A common feature for many of these, including the $\alpha_v\beta_3$ integrin, is the recognition of a configuration of a tripeptide sequence arginine-glycine-aspartic acid (single-letter codes RGD). This RGD recognizing structure is a central component of the extracellular binding site. The binding specificity of any individual RGD peptide for a given integrin depends on the conformation immediately surrounding the RGD motif. The substrate specificity shown by integrin receptors varies from one integrin to the other. The $\alpha_v\beta_3$ integrin is a promiscuous partner and is able to bind a variety of ECM proteins (Alghisi and Rüegg, 2006).

Integrins bind to ECM proteins, and thereby offer a means of communication from the extracellular milieu into the cell. Cell to cell contact is also mediated by integrin receptors facilitating cell-cell communication. This generic form of communication means that integrins are involved in a huge variety of processes, including lymphocytes polarization (following β_3 integrin activation by certain antibodies), oocytes fertilization, organization of muscle and nervous tissues during development, cardiac growth, repair and contractility, pathological conditions such as wound healing and cancer cell biology (Mahabeleshwar *et al.*, 2006). With a multitude of activators, it is unsurprising that there are a variety of downstream

intracellular pathways known to mediate the effects of integrin activation including inositol lipid synthesis, tyrosine phosphorylation of a wide range of nonreceptor tyrosine kinases, and activation of Ras/mitogen-activated protein (MAP) kinase pathway (Eliceiri and Cheresh, 1999).

THE $\alpha_V\beta_3$ INTEGRIN

In the normal human adult, the $\alpha_v\beta_3$ integrin has a limited tissue distribution, it is not normally expressed on epithelial cells to any extent, and appears only at minimal levels on intestinal, vascular, and uterine smooth muscle cells and on a small percentage of activated leukocytes, macrophages, and osteoclasts, where it appears to contribute to immune function and bone resorption. Certain invasive tumors such as malignant melanoma and certain sarcomas also express $\alpha_v\beta_3$, where it contributes to the malignant phenotype of the tumor (Eliceiri and Cheresh, 1999). Endothelial cells exposed to growth factors, or those undergoing angiogenesis in tumors, wounds, or inflammatory tissue, express high levels of $\alpha_v\beta_3$. As might be expected the spatial and temporal expression of $\alpha_v\beta_3$ integrin during angiogenesis is reflected in the functional importance the molecule plays in this process. Disruption of $\alpha_v\beta_3$ integrin function by specific antibodies (Vitaxin, and LM609) or antisense RNA has shown some efficacy in reducing tumor growth and angiogenesis *in vitro*; however, β_3 knock-out mice have normal vasculature, and the in the rare disease Glanzmann thrombaesthenia, in which patients lack β_3 subunits, there are no apparent vascular aberrations. This contradiction in data has led to the developing idea that unligated $\alpha_v\beta_3$ plays a role in activating apoptosis such that endothelial cells die when in contact with an inappropriate extracellular matrix (Stupack *et al*., 2001; Kim *et al*., 2002).

The particular expression pattern of $\alpha_v\beta_3$ integrin in vascular cells has made this molecule not only a target for therapeutic agents but also for imaging agents. Sipkins (Sipkins *et al*., 1998) demonstrated that a monoclonal antibody that specifically binds to the $\alpha_v\beta_3$ integrin accumulates in areas of tumor-related angiogenesis. Bound to the paramagnetic metal gadolinium, the antibody-gadolinium complex allows detection of angiogenesis *in vivo* using magnetic resonance imaging. This approach provides enhanced and detailed imaging of the neovasculature associated with the growth of a carcinoma model in rabbits.

Recent efforts in angiogenesis imaging have led to the development of an $\alpha_v\beta_3$ integrin marker for use with positron emission tomography (PET). Fluorinated (^{18}F) agents are expected to provide information not only concerning the expression of $\alpha_v\beta_3$ but also the functional status of this integrin (Haubner *et al*., 2005). This hypothesis first materialized the same year as the publication of an initial clinical investigation (Beer *et al*., 2005) of pharmacokinetics and biodistribution of such an agent, which was chosen by The Journal of Nuclear Medicine as the most outstanding contribution from the literature published by the journal during 2005.

SCINTIMAMMOGRAPHY

Scintimammography (SMM) with sestamibi or tetrofosmin has been performed for many years (Khalkhali *et al*., 1994; Cwikla

et al., 2001). The procedure has, however, remained a third line investigation in specialized centers. This may change with the publication by Boyd *et al*. (2002) showing evidence that dense breast may be a particular risk factor for breast cancer. Women with extensive dense breast tissue visible on a mammogram have a risk of breast cancer that is 1.8–6.0 times that of women of the same age with little or no density. In addition, dense breast tissue is a confounding factor in identifying tumors from a mammogram. The number of missed cancers estimated in women with extremely dense breasts is 3.0 compared to 0.4 for fatty breasts in women from 60–69 years (Kerlikowske *et al*., 2007).

As breast tissue density does not influence the results of SMM, there is a window of opportunity for this procedure. However, there are additional requirements that must be settled before nuclear medicine represents a valid supplement to X-ray mammography (XMM). Particularly, the sensitivity of the method has been under question, and until recently, published data suggested that the sensitivity of ^{99m}Tc-sestamibi SMM had limited diagnostic value in lesions below 1 cm, although lesions of 1 cm and above could be detected with a sensitivity as high as 92%. However, the sensitivity of SMM is only as good as the tracer used. The development of a new generation of molecular markers, with a different physiological mechanism than conventional sestamibi or tetrofosmin may increase the utility of SMM in patients with difficult-to-interpret mammographic images. Finally, new technical improvements in semi conductor gamma camera development may be the turning point in a more widespread use of SMM.

TECHNETIUM LABELED NC100692

The radiopharmaceutical ^{99m}Tc-NC100692 is a cyclic, chelate-petide conjugate containing an RGD motif, under development by GE Healthcare. In the mother compound NC100692, the C-terminal amino acid carries an ethylene glycol chain, and a chelator capable of binding ^{99m}Tc linked to the N-terminal amino acid (Figure 4.1). The agent demonstrates high affinity for, and a relatively specific binding to the $\alpha_v\beta_3$ integrin, with a K_i in the order of 1 nmol/L (Oulie *et al*., 2007). Some affinity for another integrin associated with angiogenesis, the $\alpha_v\beta_5$ integrin, has also been demonstrated for NC100692.

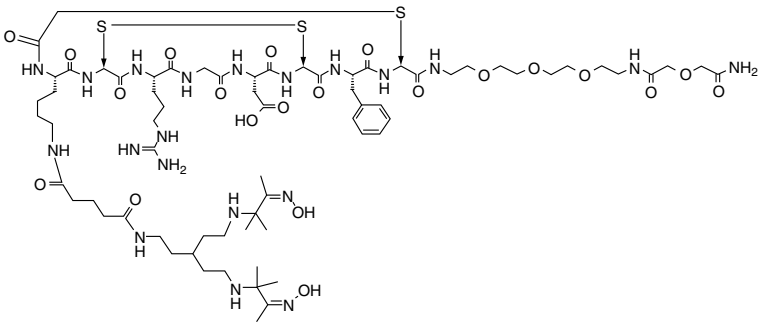


FIGURE 4.1. Structure of NC100692. (From Oulie *et al*., 2007 with permission.)

Labeling of NC100692 with ^{99m}Tc provides a potential tool for imaging the $\alpha_v\beta_3$ integrin and hence indirectly visualizing angiogenesis, providing the possibility to demonstrate the presence of malignant lesions using conventional gamma camera technology.

In a study in a murine model of hind leg ischemia, ^{99m}Tc-labeled NC100692 was shown to target the $\alpha_v\beta_3$ integrin, enabling the detection of angiogenesis associated with peripheral limb ischemia. The imaging was performed with a high-resolution gamma camera, the results were validated with gamma well counting of tissue, and the endothelial cell specificity was confirmed by immunohistochemistry. The conclusion of this study was that this agent demonstrated acceptable pharmacokinetics and *in vivo* efficacy for imaging angiogenesis (Hua *et al*., 2005). The Tc-labelled peptide formulated for use as a radiopharmaceutical *in vivo* marker has been named NC100962 Injection.

INITIAL CLINICAL EXPERIENCE

Breast cancer is a common disease, often detected at an early stage. The cancer is situated in a region which is readily accessible for surgery and complete anatomic-pathological correlation. Breast cancer was, therefore, chosen as a primary model for the clinical evaluation of NC100692 in the detection of angiogenesis associated with a malignant process. An important hypothesis, however, is that it is not the region examined, but rather the process which is being imaged. Hence, it may be postulated that the results from this limited indication could be valid for a variety of malignant diseases.

This first clinical trial, a phase IIa study, was thus performed to provide an initial indication of the efficacy of ^{99m}Tc-NC100692 Injection as a marker of angiogenesis in malignant breast tumors.

^{99M}TC-NC100692 in Breast Cancer

In a two-center clinical study, 16 female patients aged 40–76 years with a high suspicion of malignant breast disease, were included. An additional four patients with benign disease were included as controls after a protocol amendment. The trial was conducted according to the Helsinki Declaration, and was approved by the local independent ethics committees and national review boards. Initial subject inclusion was based upon a screening XMM, demonstrating a presumptive malignant breast tumor. This finding was subject to a confirmatory ultrasound examination. Most subjects were scheduled to undergo breast biopsy immediately after the SMM, and surgery within 3 weeks of inclusion. For most of the benign lesions, histopathology after aspiration cytology or biopsy was a part of the final clinical diagnosis (Bach-Gansmo *et al*., 2006).

Investigational Imaging agent

A vial containing 75 μg of ^{99m}Tc-NC100692 Injection reconstituted in 3.5 ml of sterile saline was administered as a single bolus injection followed by a 10 ml saline flush. The mean activity injected was 694 MBq.

Gamma cameras and imaging protocols

A static frontal view image was acquired (10 min acquisition time) at 45 min and 1 h 45 min post-injection. At 1 h and 2 h

post-injection a static lateral image was acquired. SPECT images were acquired at 1 h 15 min post-injection using a 2 headed camera. During the acquisition of the static lateral images the subject was prone using a specially designed mattress with the breast pending freely. Imaging was performed using a dual headed gamma camera (ADAC Genesys and Siemens E-CAM). SPECT acquisitions were performed in the same manner using a 360° circular orbit, 128 steps (64 steps, two heads), each of 30 s. The images were evaluated by two experienced on-site nuclear medicine physicians and confirmed from histopathology.

Surgery

All except two subjects with malignant lesions underwent surgery within 3 weeks. There was a histopathological verification of all malignant tumors. Biopsy, but no further follow-up was performed in the patients with benign lesions. Histological investigation of the axillary lymph nodes was performed in all patients with malignant lesions from sentinel node surgery or axillary lymph node dissection.

Results and efficacy

There were no significant safety issues from the study. One subject experienced a metallic taste for a short period of time during the night following the injection. A total of 22 malignant lesions were detected in 16 patients, mostly invasive ductal carcinoma (IDC) (n = 8) (Figure 4.2), invasive ductal carcinoma with *in situ* components (n = 7), and invasive lobular carcinoma (n = 5) and isolated ductal carcinoma *in situ* (DCIS) (n = 2), The size of the lesions, as found by histopathological examination, varied from 6–40 mm. Of these 22 lesions, 19 (86%) were detected with the prescribed ^{99m}Tc-NC100692 scintigraphy imaging protocol. Three lesions were not identified: two tumors measuring 2.5 and 6 mm and a case of multifocal invasive lobular carcinoma, where one of three lesions was missed. Although a 10 mm large area of DCIS in one subject was clearly defined, components of DCIS in patients with well defined IDC were generally not identified.

As axillary regions were included in the gamma camera field of view, an evaluation of uptake of the study drug in lymph nodes was made. For 9 of the 16 subjects with malignant lesions, no lymph node metastases were detected by histopathology and SMM showed no uptake in the axillary area. Seven subjects had lymph node metastases detected by histopathology. Four of these subjects had lymph nodes ≥ 20 mm and focal uptake in the axillary region associated with lymph node metastasis was clearly identified in three of these patients. The fourth subject also had focal uptake in the axilla but this could not be confirmed as lesion-specific, as the subject also had inflammatory skin lesions which showed moderate to high uptake of NC100962 both in the axilla, and in other

FIGURE 4.2. Lateral view (in specially designed mattress) and anterior view of a 2.5 cm large invasive ductal carcinoma using a conventional gammacamera.

regions. Three subjects had small, non-palpable lymph node metastases (≤ 5 mm) which were not detected by scintigraphy.

Not surprisingly, only large axillary (and potentially internal mammary) lymph nodes could be imaged. There is no evidence or reason to believe that submillimeter lesions associated with lymphatic spread of breast cancer could be imaged. The sentinel node procedure will remain as important a procedure if and when scintimammography with NC100692 becomes a clinical reality, only obviating the procedure in the very few patients with large lymph nodes that the pathologist might erroneously call benign after a non-diagnostic ultrasound guided biopsy.

Five benign changes were detected on SMM. These benign lesions were 4 fibrocystic changes and one infected cyst. All the fibrocystic changes detected by scintigraphy were seen as heterogeneous diffuse areas of uptake and had a different appearance from the distinct focal uptake seen with malignant lesions. One subject had intense uptake in an infected cyst. Interestingly this lesion showed a similar level of uptake as malignant lesions which may be due to invading leucocytes which are known to express $\alpha_v\beta_3$ during inflammation.

In summary, the findings suggest that fibroadenomas are not visualized on SMM and fibrocystic changes can lead to diffuse uptake. The imaging results from this study demonstrated that ^{99m}Tc-NC100692 Injection scintigraphy can detect malignant lesions. However, it should be noted that no cases classified as difficult or inconclusive by XMM/US were included in this study and that no lesions < 6 mm were evaluated.

All lesions ≥ 10 mm were clearly detected by ^{99m}Tc-NC100692 Injection scintigraphy. Similar results have been achieved and documented for other scintigraphic agents (e.g., ^{99m}Tc-sestamibi and ^{99m}Tc-tetrofosmin). ^{99m}Tc-NC100692 Injection scintigraphy using the prescribed imaging protocol detected lesions as small as 7 mm, but failed to visualize a 6 mm lesion located close to the chest wall. Because it is generally accepted that growth of solid cancers beyond a diameter of 2–3 mm requires new growth of blood vessels (angiogenesis), it is unlikely that scintigraphy with ^{99m}Tc-NC100692 Injection will be able to detect very small malignant lesions in which there is not a significant angiogenic process.

IMPROVEMENTS IN GAMMA CAMERA TECHNOLOGY

During the last 5 years technical improvements in gamma camera technology has led to the construction of dedicated cameras for breast imaging. Imaging with one of these new generation gamma cameras was performed as an investor driven, independent clinical trial, to assess the full potential of SMM. The gamma camera used was a prototype LumaGem camera. The LumaGem (Gamma Medica – Idea, Northridge, CA) is a small digital semiconductor camera in which the scintillation crystals have been replaced by 2.5 × 2.5 mm large elements of the semiconductor Cadmium-Zinc-Telluride (CZT) (Mueller *et al.*, 2003; O'Connor *et al.*, 2007). The size of the detector is 30 × 40 cm with only a narrow (8 mm) dead space between the edge of the camera and the detector field of view. Images

are acquired in an 80 × 80 matrix, with a pixel size of 2.5 × 2.5 mm. The system is equipped with a general purpose collimator with 2.3 × 2.3 mm square holes and a depth of 35 mm. The size of the gamma camera allows positioning between the breasts. It is thus possible to image structures close to the thoracic wall and in the medial quadrants, regions where the utility of ordinary SMM has been limited.

Supplementary Clinical Study

Classical scintimammography with a conventional gamma camera presents two orders of limitations, the first as discussed, being the sensitivity of the radiopharmaceutical. The selectivity of such an agent for tumor tissue remains a key issue to any development in this field. However, the inherent limitations of the Anger gamma camera presents another order of limitations to the development of scintimammography. A large dead space imposes the use of prone lateral imaging with the patient's breast pending in specially designed mattresses. The area close to they chest wall and medial quadrants of the breast are regions where the prone scintimammography has severe limitations.

Technical developments have led to the introduction of a new generation of small gamma cameras based on semi-conductors, such as the cadmium zinc telluride system With an improved spatial resolution and high energy discrimination, with the same order of magnitude as conventional gamma cameras, these new cameras are expected to provide a reliable diagnosis of tumors down to 5–7 mm in diameter (Mueller *et al*., 2003).

Eight of the nine patients included in the primary center for the phase II trial were included in this additional imaging study, being performed in parallel to the principal study. Two acquisitions using a craniocaudal and a lateral view were made, and moderate compression of the breast was always applied. Each acquisition lasted 10 min. The procedure was performed between 40 min and 2¼ h after injection of the agent. The prototype camera used was LumaGem, as described in the previous paragraph.

LumaGEM scintigraphy revealed all malignant lesions identified with conventional ultrasound or X-ray mammography, except in one patient with large hypertrophic breasts. In this patient the procedure was not conclusive because the breasts exceeded the size of the camera. In a patient with multifocal lobar carcinoma, only two of three foci were identified prior to surgery, by any of the diagnostic methods. Also a 2.5 mm large lesion was only found at histopathology. The remaining nine tumors in the seven patients were all diagnosed with LumaGEM (Figure 4.3). The combined use of the new angiogenesis based receptor imaging agent NC100629 and a dedicated digital gamma camera (LumaGem) for breast imaging was highly effective in detecting breast cancers. In this limited number of patients, LumaGEM identified all lesions visualized by the conventional gamma camera, including SPECT, and identified an additional 6 mm lesion not found using the gamma camera (Bach-Gansmo *et al*., 2007).

Future of Integrin Scintigraphy

This initial clinical trial demonstrates promising breast cancer detection using integrin scintigraphy. With the introduction

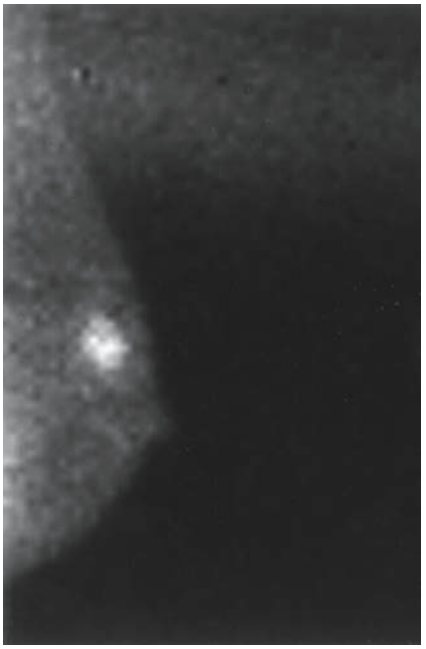


FIGURE 4.3. Lateral view of a 1 cm large invasive ductal carcinoma using LumaGEM.

of dedicated breast imaging gamma cameras, nuclear medicine may have found an alternative to X-ray mammography in subgroups of patients where this examination is known to have limitations. The agent distinguishes itself from contrast agent agents used in routine practice by providing physiological information regarding the lesion of interest. The hypothesis is that ^{99m}Tc-NC100692 provides information on the angiogenic status of malignant lesions. Similar markers of angiogenesis have shown a good correlation between signal intensity obtained on nuclear imaging, integrin expression, and microvessel density. Growth rate and prognosis of a lesion is associated with the microvessel density of the lesion, and therefore visualizing angiogenesis may provide additional clinical information of a given lesion.

One area of potential interest is the use of the agent for the monitoring of response to cancer treatment with the aim of distinguishing between responders and non-responders. Response to standard chemotherapy regimens may be monitored by assessing angiogenicity as a surrogate marker of lesion growth versus lesion death. In addition, it may also be possible to monitor lesion response to the newer biological anti-cancer agents, many of which have anti-angiogenic activities (e.g., bevacizumab). Continued work is required to validate the findings presented here and to investigate the clinical potential of one of the first candidates in the promising field of *in vivo* physiological markers.

REFERENCES

Alghisi, G.C., and Rüegg, C. 2006. Vascular integrins in tumor angiogenesis: mediators and therapeutic targets. *Endothelium 13*: 113–135.

Bach-Gansmo, T., Danielsson, R., Saracco, A., Wilczek, B., Bogsrud, T.V., Fangberget, A., Tangerud, Å., and Tobin, D. 2006. Integrin receptor imaging of breast cancer: a proof of concept study to evaluate ^{99m}Tc-NC100692. *J. Nucl. Med. 47*: 1434–1439.

Bach-Gansmo, T., Skretting, A., and Bogsrud, T.V. 2007. Integrin scintimammography using a dedicated breast imaging, solid state γ-camera and Tc-labelled NC100692. Submitted for publication.

Beer, A.J., Haubner, R., Goebel, M., Luderschmidt, S., Spilker, M.E., Wester, H.-J., Weber, W.A., and Schwaiger, M. 2005. Biodistribution and pharmacokinetics of the αvβ3-selective tracer ^{18}F-galacto-RGD in cancer patients. *J. Nucl. Med. 46*: 1333–1341.

Boyd, N.F., Dite, G.S., Stone, J., Gunasekara, A., Dallas, R., English, D.R., McCredie, M.R.E., Giles, G.G., Tritchler, D., Chiarelli, A., Yaffe, M.J., and Hopper, J.L. 2002. Heritability of mammographic density, a risk factor for breast cancer. *N. Engl. J. Med. 347*: 886–894.

Cwikla, J.B., Buscombe, J.R., Holloway, B., Parbhoo, S.-P., Davidson, T., McDermott, N., and Hilson, A.J. 2001. Can scintimammography with (99 m)Tc-MIBI identify multifocal and

multcentric primary breast cancer? *Nucl. Med. Commun. 22*: 1287–1293.

Eliceiri, B.P., and Cheresh, D.A. 1999. The role of α_v integrins during angiogenesis: insights into potential mechanisms of action and clinical development. *J. Clin*. Invest. *103*: 1227–1230.

Folkman, J. 1971. Tumor angiogenesis: therapeutic implications. *N. Eng. J. Med. 285*: 1182–1186.

Hanahan, D., and Folkman, J. 1996. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell 86*: 353–364.

Haubner, R., Weber, W.A., Ambros, J., Beer, A., Vabuliene, E., Reim, D., Sarbia, M., Becker, K.-F., Goebel, M., Hein, R., Wester, H.J., Kessler, H., and Schwaiger, M. 2005. Noninvasive visualization of the activated αvβ3 integrin in cancer patients by positron emission tomography and [^{18}F]Galacto-RGD. *PLoS Med 2*(3): e70 doi:10.1371/journal.pmed.0020070.

Hua, J., Dobrucki, L.W., Sadeghi, M.M., Zhang, J., Bourke B.B., Cavaliere, P., Song, J., Chow, C., Jahanshad, N., van Royen, N., Buschmann, I., Madri, J.A., Mendizabal, M., and Sinusas, A.J. 2005. Noninvasive imaging of angiogenesis with a ^{99m}Tc-labeled peptide targeted at $\alpha_v\beta_3$ integrin after murine hindlimb ischemia. *Circulation 111*: 3255–3260.

Kerlikowske, K., Ichikawa, L., Miglioretti, D.L., Buist, D.S.M., Vacek, P.M., Smith-Bindman, R., Yankaskas, B., Carney, P.A., and Ballard-Barbash, R. 2007. Longitudinal measurement of clinical mammographic breast density to improve estimation of breast cancer risk. *J. Natl. Cancer Inst. 99*: 385–395.

Khalkhali, I., Mena, I., Jouanne, E., Diggles, L., Venegas, R., Block, J., Alle, K., and Klein, S. 1994. Prone scintimammography in patients with suspicion of carcinoma of the breast. *J. Am. Coll. Surg. 178*: 491–497.

Kim, S., Bakre, M., Yin, H., and Varner, J.A. 2002. Inhibition of endothelial cell survival and angiogenesis by protein kinase A. *J. Clin. Invest. 110*: 933–941.

Mahabeleshwar, G.H., Feng, W., Phillips, D.R., and Byzova, T.V. 2006. Integrin signalling is critical for pathological angiogenesis. *J. Exp. Med. 203*: 2495–2507.

Mueller, B., O'Connor M.K., Blevis I., Rhodes D.J., Smith R., Collins D.A., Phillips S.W. 2003. Evaluation of a small Cadmium Zinc Telluride detector for scintimammography. *J. Nucl. Med. 44*: 602–609.

O'Connor, M.K., Phillips, S.W., Hruska, C.B., Rhodes, D.J., and Collins, D.A. 2007. Molecular breast imaging: advantages and limitations of a scintimammographic technique in patients with small breast tumors. *Breast J. 13*: 3–11.

Oulie, I., Roed, L., Toft, K.G., and Skotland, T. 2007. Quantification of NC100692, a new tracer for (99 m)Tc-imaging of angiogenesis, in human plasma using reversed-phase liquid chromatography coupled with electrospray ionization ion-trap mass spectrometry. *J. Chromatogr. B Analyt. Technol. Biomed Life Sci. 852:* 605–610.

Sipkins, D.A., Cheresh, D.A., Kazemi, M.R., Nevin, L.M., Bednarski, M.D., and Li, K.C. 1998. Detection of tumor angiogenesis in vivo by alphaVbeta3-targeted magnetic resonance imaging. *Nat. Med. 4*: 623–626.

Stupack, D.G., Puente, X.S., Boutsaboualoy, S., Storgard, C.M., and Cheresh, D.A. 2001. Apoptosis of adherent cells by recruitment of caspase-8 to unligated integrins. *J. Cell Biol. 155*: 459–470.

Tamkun, J.W., DeSimione, D.W., Fonda, D., Patel, R.S., Buck, C., Horwitz, A.F., and Haynes, R.O. 1986. Structure of integrin, a glycoprotein involved in the transmembrane linkage between fibronectin and actin. *Cell 46*: 271–282.

5
Breast and Prostate Biopsies: Use of Optimized High-Throughput MicroRNA Expression for Diagnosis (Methodology)

Michael D. Mattie and Robert C. Getts

INTRODUCTION

Recent technological advancements and methodologies have been developed to perform global analysis of tissue-specific 'signatures' of microRNAs in humans. The emerging view from such studies is that altered miRNA expression commonly occurs in a variety of cancers, which underscores the potential utility of miRNA profiling for diagnostic and prognostic applications. We present here a high-throughput methodology for miRNA profiling that is sufficiently sensitive for routine use on clinical specimens with limited starting material.

MicroRNA Biogenesis

MicroRNAs (miRNA) are a class of small non-coding RNAs encoded in the genomes of animals and plants (reviewed in Bartel, 2004) that play a role in targeting messages of protein-coding genes for cleavage or translational repression (Ambros, 2003; Bartel and Bartel, 2003). In mammalian cells, the active miRNA products are formed from a larger primary transcript (pri-miRNA) generated in the form of long, polycistronic messages by a type II RNA polymerase (Cai *et al*., 2004; Lee *et al*., 2004). The pri-miRNAs form specific hairpin-shaped stem–loop secondary structures that are processed in the nucleus by the RNase III Drosha to release a 60–110 bp miRNA precursor (pre-miRNA) (Gregory *et al*., 2005). Pre-miRNAs may be further post-translationally edited to modulate or modify targeting (reviewed in Landgraf *et al*., 2007). The pre-miRNAs are then transported to the cytoplasm by Exportin-5 (Bohnsack *et al*., 2004), where the RNase III endonuclease Dicer processes the molecule into double-stranded, 18–24-bp mature miRNAs (Ketting *et al*., 2001; Hutvagner and Zamore, 2002), one strand of which is incorporated into the ribonucleoprotein complex RISC (RNA-induced silencing complex) for subsequent targeting to mRNAs (Lin *et al*., 2005, Gregory *et al*., 2005). Target messenger RNA (mRNA) sequences are inactivated by cleavage in a fashion similar to RNAi, while pairing with partially complementary sequences in the 3′ UTR of target mRNAs can either repress translational efficiency or induce transcript decay (Olsen and Ambros, 1999). Binding through imperfect complementarity allows a single miRNA to potentially target multiple transcripts, as predicted by

multiple algorithms (Lewis *et al*., 2003; John *et al*., 2004). Present estimates suggest that nearly a third of all cellular transcripts may be regulated by the few hundred human miRNAs currently known to exist (Bartel, 2004). There are > 450 miRNAs in the human genome annotated in the miRNA registry to date (Griffiths-Jones *et al*., 2006), with some estimates suggesting there might be as many as 1,000 in the human genome. The majority of human miRNAs are located within the introns of protein-coding or noncoding mRNA transcripts (Rodriguez *et al*., 2004), with the remaining miRNAs being located within the exons of noncoding mRNA genes and the 3' UTRs of mRNA genes, or in clusters such as one found on chromosome 19 that is comprised of 54 novel miRNAs (Cai *et al*., 2004; Bentwich *et al*., 2005; Baskerville and Bartel, 2005).

Biological Roles of MicroRNAs

To date, functional roles have been characterized for only a relatively small fraction of the hundreds of currently known miRNAs. Of the ones that have been characterized, several play key roles in a variety of processes, including early development, cell proliferation and cell death, fat metabolism, cell differentiation, insulin secretion, and neuronal development (reviewed in Cao *et al*., 2006; Plasterk, 2006; Shivdasani, 2006). The first evidence linking miRNAs to cancer first appeared in 2002, which reported deletion or downregulation of specific miRNAs in cancer cells (Calin *et al*., 2002). Since then, a number of studies have reported altered expression of a variety of miRNAs in various cancer types. Takamizawa *et al*. (2004) reported that reduced expression of the *let-7* miRNA in human lung cancer was significantly associated with shortened postoperative survival of patients after potentially curative resection. This evidence, in conjunction with functional studies, suggests that *let-7* could be used diagnostically for patients with non-small-cell lung carcinomas to determine their prognosis for postoperative survival (Johnson *et al*., 2005; Yanaihara *et al*., 2006). Lu *et al*. (2005) used a novel bead-based flow cytometric method to profile 217 mammalian miRNAs across 334 primary tumors, normal tissues, and tumor-derived cell lines representing a diverse panel of human tissues and tumors. The researchers found that the expression profiles of only a relatively small panel of miRNAs (~ 200 genes) were required to accurately classify human cancers. The expression profiles reflected the developmental lineage and differentiation state of the tumors, with tumors clustering according to their embryonic lineage. An examination of miRNA profiles of various human malignancies, including breast and prostate cancer (Iorio *et al*., 2005), revealed a small subset of deregulated miRNAs (including mir-125b, mir-145, mir-21, and mir-155) that unequivocally distinguish normal from malignant breast tissue. Other differentially expressed miRNAs appear to correlate with breast cancer histopathologic features such as tumor size, nodal involvement, proliferative capacity and vascular invasiveness (Iorio *et al*., 2005). The emerging view from these studies is that altered miRNA expression commonly occurs in a variety of cancers, which underscores the potential utility of miRNA profiling for diagnostic and prognostic applications (Takamizawa *et al*., 2004; Iorio *et al*., 2005; Calin *et al*., 2005; Lu *et al*., 2005). However, while there appears to be a significant population of ubiquitously expressed microRNAs, most of the differences between tissues and cell types are accounted for by only a small subset of these molecules (Landgraf *et al*., 2007).

Focus of this Chapter

Adapting high-throughput methods for mRNA profiling to study miRNAs involves a number of technical challenges. Cloning and Northern blot analyses typically require large amounts of starting material, more than is typically available from clinical biopsy samples, prohibiting their use in high-throughput miRNA profiling. The small size of miRNAs provides very little sequence for labeling or designing probes. A number of high-throughput approaches have recently been developed, including microarray (reviewed in Einat, 2006), quantitative RT-PCR (Chen *et al*., 2005; Schmittgen *et al*., 2004) and bead-based flow cytometry (Lu *et al*., 2005), which have been useful in performing global analysis of tissue-specific 'signatures' of miRNAs in humans. However, several of these methods can require initial inputs up to several micrograms of total RNA for global miRNA analysis. A number of new methods have been developed for miRNA isolation, labeling, probe design, data analysis, and array production, many of which are now commercially available. We adapted a previously described method (Goff *et al*., 2004) for the amplification of mRNAs that allows for sensitive high throughput microarray analysis of miRNA expression. We have been able to successfully amplify miRNA with as little as 250 pg of enriched miRNA (~ 2 ng total RNA), an amount which is easily obtainable from needle core biopsies or other clinical samples with very limited cellular material. The amplification method demonstrates good replication fidelity in comparison to unamplified miRNA, suggesting that all miRNAs are being amplified efficiently and with little bias. We present here a high-throughput methodology for miRNA profiling that is sufficiently sensitive for routine use on clinical specimens with limited starting material.

METHODOLOGY FOR HIGH-THROUGHPUT MIRNA PROFILING

Purification of miRNA

The quality of gene expression analysis by microarray is heavily dependent on RNA quality and requires robust and reproducible methods for the quantitative isolation of miRNA. Many RNA isolation methods, such as chemical extraction or solid-phase extraction on glass filters, were originally designed to capture longer mRNA species, but do not quantitatively retain the small RNA fraction. However, a number of isolation methods have been developed that are available in convenient kit formats that retain the small RNAs during total RNA isolation or enrich for the small RNA population. In our experience with the methodologies presented in this chapter, further enrichment for the small RNA fraction improves the amplification of miRNAs, as well as improves microarray analysis by reducing nonspecific hybridization to longer miRNA precursors, homologous regions of target mRNAs, and other unrelated RNA species.

Isolation methods

Trizol: Chemical extraction method that uses highly concentrated chaotropic salts in conjunction with guanidinium isothiocyanate and acidic phenol or phenol-chloroform solutions to inactivate RNases and purify RNA results in highly pure recovery of total RNA, but the RNA must typically be desalted and concentrated with an alcohol precipitation step, which may not quantitatively recover the small RNA fraction.

Solid-phase extraction (column-based RNA isolation) relies on high salt or salt and alcohol to decrease the affinity of RNA for water and increase its affinity for the

solid support (usually a silica filter). The conditions used do not effectively retain the small RNA fraction. MicroRNAs can potentially be isolated from flow-through fractions, but this is not as ideal as other options below.

Denaturing polyacrylamide gel electrophoresis (PAGE) has been used as an alternative means for isolating miRNAs from total RNA, but is a time-consuming procedure that results in variable yield. The flashPAGE fractionator system from Ambion can rapidly and reproducibly fractionate total RNA samples with greater efficiency than traditional PAGE; however, the yield of small RNAs is typically < 1 ng per 10 µg of total RNA from mammalian tissue, which may not be suitable for samples of limited size.

Microfiltration requires isolation of total RNA by one of the other methods listed, usually Trizol-extracted. Microcon columns (Millipore) can be used to enrich for the small RNA fraction by size exclusion. These are centrifugal columns with a low-binding regenerated cellulose membrane that allows for concentration, desalting, and purification of nucleic acid samples.

Column-based miRNA isolation kits. Our preferred method for miRNA enrichment with prostate and breast tissue biopsies involves isolation with the mirVana miRNA isolation kit (Ambion) or the PureLink miRNA isolation kit (Invitrogen). These methods utilize columns with glass fiber filters. An ethanol concentration is used that is sufficient enough to immobilize the large RNA fraction in an initial column, but not the small RNA fraction. The flow-through fraction is then bound to a second column with an increased ethanol concentration, washed and eluted. The small RNA fraction contains RNA species 200 nt.

Procedure for miRNA isolation from prostate and breast tumor biopsies

1. RNA was isolated from fresh frozen tissue biopsies.
2. Tissue sections were cut in 14 µM sections with a microtome onto glass slides.
3. Dehydrate sections on slides for 1 min each in 70%, 95%, and 100% ethanol.
4. Dehydrate in xylene for 5 min and let slides dry.
5. Representative slides stained with hematoxylin and eosin were reviewed by a pathologist.
6. Areas of interest for each tissue section were manually microdissected with a scalpel. If available, laser capture microdissection offers better precision. Scrape and put each sample into lysis buffer from mirVana or PureLink miRNA isolation kit.
7. Homogenize samples with a motorized rotor-stator homogenizer (Polytron).
8. Proceed with miRNA enrichment procedure according to manufacturer instructions.

Formalin-fixed paraffin-embedded (FFPE samples). Due to their small size, miRNAs are relatively resistant to RNase degradation and can be successfully isolated from routinely processed FFPE tissue. In our experience, the RecoverAll Total Nucleic Acid Isolation kit for FFPE from Ambion is suitable for isolation of RNA and enrichment of the miRNA fraction for microarray analysis. In a recent study, Lawrie *et al.* (2007) isolated miRNAs from FFPE sections using the RecoverAll kit and performed microarray analysis using the same labeling procedure described in this

chapter. They found that the FFPE samples gave similar results to matched frozen tissue by qRT-PCR, even when > 8-years old. FFPE-isolated miRNA also gave comparable data to frozen material in microarray analysis.

AMPLIFICATION OF LOW MOLECULAR WEIGHT RNA

An adaptation of the SenseAMP™ Plus RNA amplification protocol (Genisphere, Inc.) was utilized for amplification of low molecular weight (LMW) RNA for miRNA microarray analysis of prostate and breast cancer biopsies as described by Mattie *et al*. (2006). This method is based on the use of T7 RNA polymerase enzyme to generate amplified RNA for further analysis by microarrays, quantitative RT-PCR, ELOSA, or other relevant RNA-based applications. Up to 30 ng of enriched LMW RNA sample can be used in this amplification procedure. The procedure described below has recently been made available in a commercially available kit from Genisphere (SenseAmp™ Plus Low Molecular Weight RNA Amplification kit); this procedure is also available as a similar version from Invitrogen (NCode™ miRNA amplification kit, cat. no. MIRAS-20).

Depending on the amount of available starting material from a given sample, amplification of miRNA may be necessary prior to the labeling procedure to ensure reliable detection of miRNAs expressed at low levels. The following guidelines on various means of miRNA enrichment and resulting yields that are suitable for the labeling procedure can be used to determine whether sufficient material is available for the labeling procedure or if amplification may be necessary prior to labeling of samples:

RNA sample	Input needed for labeling
Total RNA containing LMW RNA	0.5–3 μg
LMW RNA enriched by Microcon YM-100 Filter (Millipore cat. no. 42413)	10–80 ng
LMW RNA enriched by PureLink™ miRNA Isolation Kit (Invitrogen cat. no. K1570-01)	50–300 ng
LMW RNA enriched by mirVana™ miRNA Isolation Kit (Ambion cat. no. 1560)	50–300 ng
LMW RNA enriched by flashPAGE™ Fractionator (Ambion cat. no AM13100)	0.1–1 ng
LMW RNA enriched by Qiagen RNA/DNA Midi Kit (Qiagen cat. no. 14142)	50–300 ng
senseRNA* amplified by Genisphere's SenseAMP Plus LMW RNA Amplification Kit (cat. no. RAMP110MIR or RAMP120MIR)	125–750 ng

Note: The amplification procedure described here is not compatible with the TaqMan® miRNA qRT-PCR assays from Applied Biosystems because the addition of the polyA tail to the 3′ end of miRNAs inhibits binding of the hairpin primer to correct position. This procedure is compatible with other qRT-PCR approaches (Shi and Chiang, 2005; NCode™ qRT-PCR from Invitrogen). One should also ensure that the senseRNAs produced with this amplification method are compatible with the probe design of the particular detection platform selected.

Enrichment of LMW RNA

This amplification protocol will result in amplification of all RNA species. As a result, it is optimal to enrich the sample for low molecular weight RNA species to allow better amplification of miRNAs. Samples can be enriched as described previously for the LMW RNA fraction using commercially available columns such as the mirVana™ miRNA Isolation kit (Ambion), the PureLink™ miRNA isolation system (Invitrogen), or the FlashPage fractionator according to manufacturer instructions. For total RNA samples that were isolated by a method that retains the LMW RNA fraction, such as Trizol extractions, the procedure below can be used to enrich for the LMW RNA fraction.

1. Dilute the total RNA sample to 100 µL with 10 mM Tris (pH 8).
2. Heat to 80°C for 3 min, then cool on ice for 3 min.
3. While the sample is cooling on ice, add 50 µl of 10 mM Tris (pH 8) to a Microcon YM-100 column (Millipore, cat. no. 42413), and spin for 3 min at top speed.
4. Discard the flow-through and the collection tube. Place the column into a new collection tube.
5. Add the 100 µl of RNA to the Microcon column, and centrifuge for 7 min at top speed.
6. Save the eluate in the collection tube. This is the enriched LMW RNA. Yields may be quantitated with the RiboGreen RNA Quantitation Kit (Molecular Probes).

Poly(A) Tailing

Using Poly(A) polymerase enzyme (PAP), a short tail of ~ 70 adenine residues is added to the 3' end of all LMW RNAs. This is a modification of the Poly(A) tailing kit protocol from Ambion.

1. In this protocol, up to 30 ng of low molecular weight RNA can be used. Adjust the volume to 18 µl with nuclease-free water.
2. For LMW RNA samples between 1 and 30 ng: dilute the ATP(10 mM) in 1 mM Tris (pH 8.0) according to the following formula:

ATP dilution factor = 5,000/ng of LMW RNA

For example, if starting with 2 ng RNA, the ATP dilution factor = 5000 ÷ 2 ng = 2500.

Dilute the ATP 1:2500 by adding 1 µl of ATP to 2499 µl of 1 mM Tris (pH 8.0).

For LMW RNA samples > 1 ng: dilute the ATP 1:5000 in 1 mM Tris (pH 8.0).

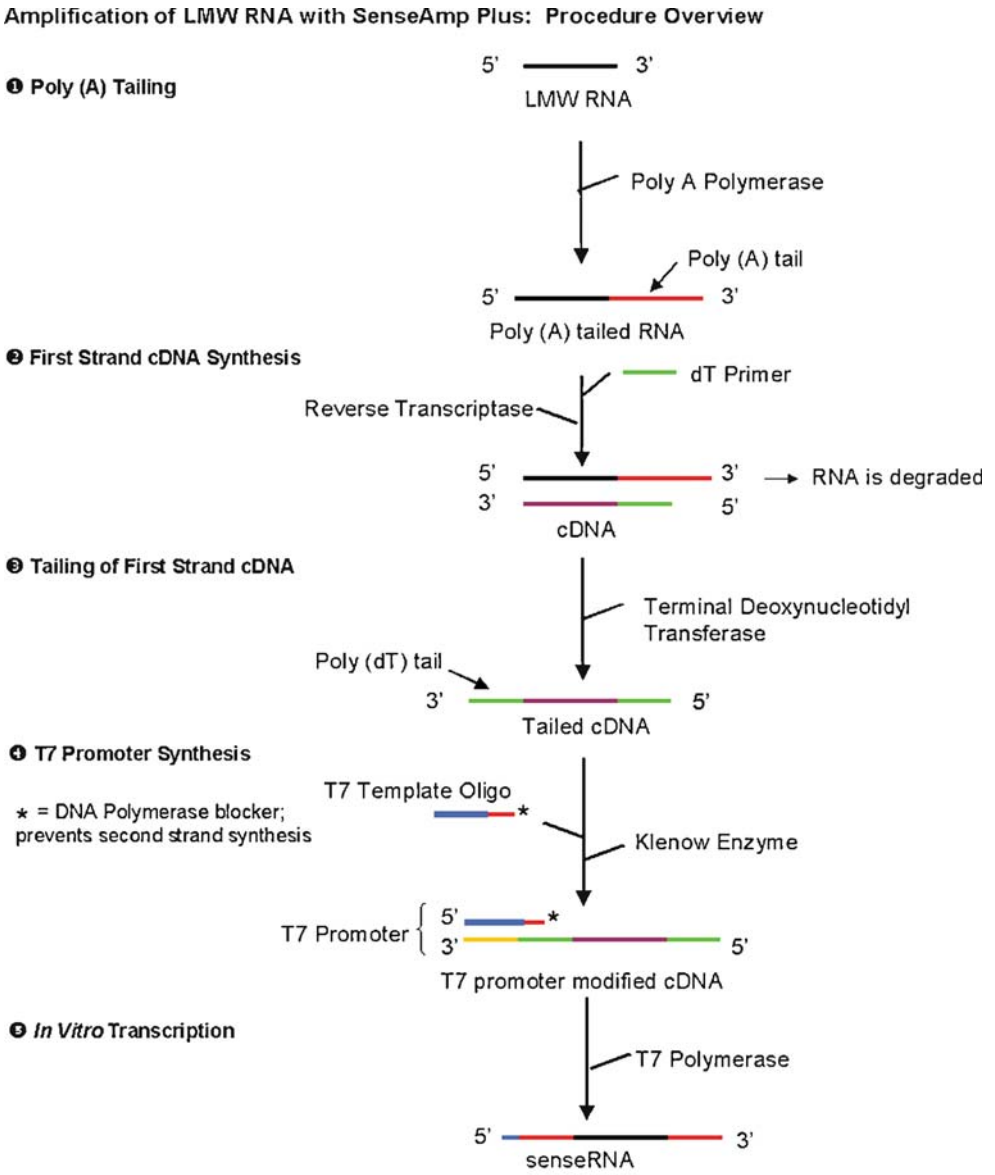
3. Add the following components to the 18 µl LMW RNA, for a volume of 25 µl:

   2.5 µl 10X Reaction Buffer (PAP Buffer)
   2.5 µl 25 mM $MnCl_2$
   1 µl diluted ATP
   1 µl PAP Enzyme

4. Mix gently and microfuge.
5. Incubate in a 37°C heat block for 15 min.

First Strand cDNA Synthesis

1. Briefly microfuge the 25 µl of tailed RNA and place on ice.
2. Prepare a 1:10 dilution of SenseAmp dT primer by adding 1 µl SenseAmp dT primer to 9 µl 0.1X TE. Vortex and briefly microfuge. The SenseAmp dT primer is an oligo dT24 primer (50 ng/µl).
3. On ice, add 2 µl of 1:10 diluted SenseAmp dT primer.



❶ **Poly (A) Tailing**: Poly (A) tails are generated on all LMW RNA molecules.

❷ **First Strand cDNA Synthesis**: RNA is primed using an Oligo (dT) and/or random primer to produce single-stranded cDNA.

❸ **Tailing of First Strand cDNA**: First strand cDNA is purified then tailed with dTTP using Terminal Deoxynucleotidyl Transferase.

❹ **T7 Promoter Synthesis**: The T7 Template is annealed to the 3' end of the cDNA. Klenow enzyme fills in the 3' end of first strand cDNA to produce a double-stranded T7 promoter. The T7 Template contains a blocker to prevent second strand synthesis.

❺ **In Vitro Transcription**: senseRNA copies of the original RNA molecules are generated.

4. Mix gently and microfuge. Incubate at 65°C for 10 min and immediately transfer to ice for 2 min.
5. Add the following components on ice, for a volume of 50 µl:

   10 µl 5X First Strand Buffer (or equivalent buffer supplied with the RT)
   5 µl 0.1 M DTT (If supplied with the RT; otherwise use nuclease free water)
   2.5 µl dNTP mix (10 mM each concentration)
   1 µl Superase-in™ RNase inhibitor
   2 µl SuperScript II reverse transcriptase, 200 units
   2.5 µl Nuclease-Free Water

6. Gently mix (do not vortex) and incubate at 42°C for 1 h.
7. Stop the reaction by adding 8.75 µl of 0.5 M NaOH/50 mM EDTA. Briefly vortex and microfuge. Note: the reaction may turn to a brown color; this is normal.
8. Incubate at 65°C for 30 min to degrade the RNA. Note: the reaction may turn from brown to clear; this is normal.
9. Neutralize the reaction by adding 12.5 µl of 1 M Tris (pH 8.0). Bring the sample to 100 µl by adding 28.75 µl 1X TE Buffer. Briefly vortex and microfuge. This is the cDNA.

Purification and Concentration of cDNA

Purify the cDNA using a Microcon YM-100 column and a conventional tabletop microfuge.

1. Add the 100 µl cDNA to the sample reservoir. Do not touch the membrane with the pipette tip. Secure the tube cap and centrifuge for 6 min at 13,000 × g.
2. Add 200 µl of 1X TE buffer to the sample reservoir without touching the membrane. Gently mix by pipetting up and down 5 times. Secure the tube cap and centrifuge for 6 min at 13,000 × g.
3. Carefully separate the sample reservoir from the collection tube. Discard the flow-through. Place the YM-100 column into the same collection tube.
4. Add 200 µl of 1X TE buffer to the sample reservoir without touching the membrane. Gently mix by pipetting up and down 5 times. Secure the tube cap and centrifuge for 6 min at 13,000 × g.
5. Carefully separate the sample reservoir from the collection tube. Discard the collection tube.
6. Add 5 µl 1 mM Tris (pH 8.0) to the sample reservoir without touching the membrane. Gently tap the side of the reservoir to mix.
7. Carefully place the sample reservoir upside down in a **new collection tube**. Centrifuge for 3 min at 13,000 × g.
8. Note the volume of cDNA collected in the bottom of the tube (5–10 µl). Bring the volume of cDNA to 10 µl with nuclease free water.

Tailing of First Strand cDNA

1. Heat purified cDNA (10 µl) to 80°C for 10 min. Ice immediately for 1–2 min. Briefly microfuge and return to ice.
2. For each reaction, prepare a Master Mix in a separate tube on ice:

   2 µl 10X Reaction Buffer (supplied with TdT enzyme)
   2 µl Nuclease Free Water
   4 µl 10 mM dTTP
   2 µl TdT Enzyme (Terminal Deoxynucleotidyl Transferase)
   10 µl

3. Combine the Master Mix and the cDNA for a volume of 20 µl. Mix gently and microfuge.

4. Incubate in a 37°C heat block for 3 min. **Do not exceed 3 min.**
5. Stop the reaction by heating to 80°C for 10 min. Briefly microfuge and cool to room temperature for 1–2 min.

T7 Promoter Synthesis

1. Add 2 µl of SenseAmp T7 Template Oligo to the tailed cDNA for a volume of 22 µl. Briefly vortex and microfuge. The T7 Template Oligo consists of a T7 promoter sequence on the 5' end of a 3' end blocked poly dA oligo sequence.
2. Incubate at 37°C for 10 min to anneal the strands.
3. To each reaction, add the following components for a volume of 25 µl:

   1 µl 10X Reaction Buffer
   1 µl dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP)
   1 µl Klenow Enzyme (5 U/µl)

4. Mix gently and microfuge. Incubate at room temperature for 30 min.
5. Stop the reaction by heating to 65°C for 10 min. Place on ice.
6. Proceed to the *In Vitro Transcription* reaction using half (12.5 µl) of the promoter-modified cDNA. Save the remaining modified cDNA at −20°C for future use or for use in a parallel amplification reaction.

In Vitro Transcription

1. Incubate the 12.5 µl of cDNA at 37°C for 10 min to re-anneal the strands.
2. Thaw the T7 Nucleotide Mix *(ATP, CTP, GTP, UTP, 75 mM each)* and 10X T7 Reaction Buffer at room temperature, and **keep at room temperature until use**. Thoroughly vortex the 10X T7 Reaction Buffer to avoid precipitation of certain buffer components.
3. For each reaction, add the following components to the 12.5 µl of cDNA **at room temperature**, for a final volume of 25 µl:

   8.0 µl T7 Nucleotide Mix
   2.5 µl 10X T7 Reaction Buffer
   2.0 µl T7 Enzyme Mix

4. Mix gently and microfuge. Incubate in a thermalcycler (with heated lid) at 37°C for 4–16 h. Or, place the reaction in a 37°C heat block for 5 min and then transfer to a 37°C air hybridization oven for 4–16 h. **It is essential to avoid evaporation and condensation of the reaction during this step.**

Purification of senseRNA

Purify the senseRNA using the RNeasy MinElute Kit (Qiagen cat. no. 74204) following Qiagen's protocol for RNA Cleanup. To elute, add 14 µl Nuclease Free Water, incubate for 2 min, and then spin. The recovered volume should be ~ 12 µl.

Quantitation of senseRNA

Determine the concentration of the senseRNA using the RiboGreen RNA Quantitation Kit (Molecular Probes cat. no. R-11490). Use the Ribosomal RNA standard provided with the kit to prepare a standard curve. Use 1 µl of the purified senseRNA to quantitate. Concentrations can also be determined using spectrophotometers that utilize low volumes (1–2 µl) such as a NanoDrop ND-1000 (NanoDrop Technologies).

LABELING OF miRNA

The described procedure (FlashTag™, Genisphere) will label any RNA sample, including total RNA, senseRNA, severely

degraded RNA, and low molecular weight RNA. This protocol describes labeling low molecular weight RNA (snRNA, hnRNA, piRNA, miRNA, etc.) for microarray analysis. Starting with ~ 1μg of total RNA (or LMW RNA enriched from 1 μg of total), the process begins with a brief tailing reaction followed by ligation of the signal molecule to the target RNA sample. Samples can be labeled with either Oyster®-550 (equivalent to Cy™3) or Oyster®-650 (equivalent to Cy™5). This procedure has been tested and is compatible with several array platforms (see below). The procedure described here utilizes Genisphere's proprietary 3DNA dendrimer signal amplification technology. The 3DNA dendrimer is a branched structure of single and double stranded DNA conjugated with numerous fluorescent dyes (Nilsen *et al*., 1997; Stears, *et al*., 2000). Whereas other labeling strategies typically target a single fluor to the sample, a 3DNA molecule delivers ~ 15 fluors to the sample. The FlashTag™ protocol presented here is similar to the microRNA 3′ tagging portion of the NCode™ miRNA labeling procedure from Invitrogen. In the case of FlashTag™, a small DNA dendrimer is directly attached to the 3′ end of a poly A tail enzymatically synthesized on each miRNA molecule in the sample. As a result, one hybridization step is required to develop the signal and establish an expression pattern.

Microarrays

FlashTag™ has been tested to be compatible with the following commercially available miRNA microarrays:

- NCode Multispecies miRNA Array (Invitrogen)
- miRCURY LNA Array (Exiqon)
- CustomArray 4X2 MicroRNA Array (CombiMatrix)
- Non-commercially printed arrays*

***See notes in microarray platform section regarding compatibility with array platforms**

RNA Samples

FlashTag labeling can accommodate any RNA sample, including intact or degraded total RNA, enriched LMW RNA, or sense RNA that has been amplified from LMW RNA using the SenseAMP™ Plus LMW RNA Amplification Kit. While it is not required to enrich the RNA sample, some RNA samples may require enrichment in order to achieve accurate results. For example, to distinguish mature and precursor miRNAs, enrichment may be necessary.

For microarray analysis: Flashtag™ RNA Labeling Kit (Genisphere)

*Note: This protocol can accommodate enriched RNA or senseRNA that has been amplified from LMW RNA using the SenseAMP™ Plus LMW RNA Amplification Kit. If labeling senseRNA, the "Poly (A) Tailing" reaction is not required. Proceed directly to *FlashTag Ligation* step, using 125–750 ng adjusted to a volume of 15 μl with Nuclease-Free water.

Enrichment and Concentration of LMW RNA Using YM-100 and YM-3 Columns

For total RNA samples that have not been enriched for the low molecular weight RNA fraction using the column-based procedures previously mentioned, enrich the total RNA sample using a Microcon YM-100 column (Millipore cat. no. 42413) and a conventional tabletop microfuge.

1. Dilute the total RNA sample to 100 µl with 10 mM Tris (pH 8.0).
2. Heat to 80°C for 3 min, then immediately cool on ice for 3 min.
3. While the sample is cooling on ice, add 50 µl of 10 mM Tris (pH 8.0) to the Microcon column, and spin for 3 min at 13,000 × g.
4. Discard the flow-through and the collection tube. Place the column into a new collection tube.
5. Add the 100 µl of RNA to the Microcon column, and centrifuge for 7 min at 13,000 × g.
6. Save the eluate (~ 95 µl) in the collection tube. This is the enriched LMW RNA. The LMW RNA can be quantitated with the Quant-iT RiboGreen RNA Assay Kit or the NanoDrop ND-1000 Spectrophotometer. Proceed to Concentration or "FlashTag RNA Labeling Procedure".

Note: To collect the high molecular weight RNA, add 5 µl of 10 mM Tris (pH 8.0) to the Microcon column and gently mix by tapping the side. Carefully place the sample reservoir **upside down** in a **new collection tube** and centrifuge for 3 min at 13,000 × g.

Concentration of LMW RNA with Microcon YM-3 Column (Millipore cat. no. 42404)

1. Add the LMW RNA from step 6 above (~ 95 µl flow-through of YM-100) to the YM-3 sample reservoir. Do not touch the membrane with the pipette tip. Secure the tube cap and centrifuge for 30 min at 13,000 × g.
2. Check the volume of the flow-through and continue the centrifugation if necessary. For adequate concentration, the flow-through volume should be equal to the loaded volume minus 5 µl. For example, if 95 µl was loaded, then the flow-through volume should measure 90 µl.
3. Add 5 µl of 10 mM Tris-HCl (pH 8.0) to the sample reservoir and gently mix by tapping the side.
4. Carefully place the sample reservoir **upside down** in a **new collection tube**. Centrifuge for 3 min at 13,000 × g to collect the concentrated LMW RNA (~ 5–10 µl). The LMW RNA can be quantitated with the Quant-iT RiboGreen RNA Assay Kit or the NanoDrop ND-1000 Spectrophotometer. Proceed to "FlashTag RNA Labeling Procedure".

FlashTag RNA Labeling Procedure

Procedure Overview:

Step 1: Poly A tail the 3' end of the RNA (15 min)
Step 2: Ligate the FlashTag™ dendrimer to the 3' end of the tailed miRNA (30 min).
Step 3: Prepare Hybridization Mix and apply to microarray (Overnight)
Step 4: Wash microarray (30 min)
Step 5: Scan and analyze results.

Note: If labeling **senseRNA**, the "Poly (A) Tailing" reaction is not required. Adjust the volume of senseRNA to 15 µl with Nuclease-Free water and proceed directly to "FlashTag Ligation" below.

Poly (A) Tailing

1. Adjust the volume of RNA, including any spike-in controls, to 10 µl with nuclease-free water.
2. Dilute the ATP mix (**10 mM**) in 1 mM Tris (pH 8.0) as follows:

   For **total RNA samples,** dilute the ATP Mix 1:500.

For **enriched samples**, calculate the dilution factor according to the following formula:

5000 ÷ ng input LMW RNA

Example: If using 100 ng of enriched LMW RNA, the dilution factor is 5000 ÷ 100 = 50. Dilute the ATP Mix 1:50.

3. Add the following components to the 10 µl RNA, for a volume of 15 µl:

   1.5 µl 10X Reaction Buffer (PAP Buffer)
   1.5 µl 25 mM MnCl2
   1 µl diluted ATP Mix
   1 µl PAP Enzyme

4. Mix gently (do not vortex) and microfuge.
5. Incubate in a 37°C heat block for 15 min. Discard any unused, diluted ATP Mix.

FlashTag Ligation

Perform the following steps in the dark to avoid degradation and fading of the fluorescent dyes.

1. Briefly microfuge the 15 µl of tailed RNA and place on ice. If labeling **senseRNA**, adjust the volume to 15 µl with Nuclease-Free water and proceed to step 2.
2. Add 4 µl of the appropriate 5X FlashTag Ligation Mix (**Oyster-550 or Oyster-650**). Oyster-550 and Oyster-650 have similar spetral characteristics to Cy3™ and Cy5™, respectively.
3. Add 2 µl of T4 DNA Ligase (2 U/µl).
4. Mix gently and microfuge.
5. Incubate at room temperature for 30 min. Protect the reactions from light.
6. Stop the reaction by adding 1.5 µl of 0.5 M EDTA, pH 8.0. Mix and microfuge the 22.5 µl of ligated sample. The samples are now ready for hybridization to microarrays.

Hybridization Procedures (Spotted Arrays)

*Hybridization procedures presented below are general procedures for arrays spotted with oligonucleotides having ~ 22 bases of Tm optimized sequence complementary to microRNAs. For commercially available arrays (Invitrogen, Exiqon or CombiMatrix) refer to recommended conditions from manufacturer.

Additional required materials:

- Glass coverslips (Corning, VWR or other manufacturer)
- Heat blocks set to 65°C and 70–80°C
- Wash buffers: 2X SSC/0.2% SDS, 2X SSC and 0.2X SSC
- Hybridization oven set to 52°C

Array Hybridization

1. Resuspend the 2X Hybridization Buffer or 2X Enhanced Hybridization Buffer (recommended). Other hybridization buffers, including 2X SDS-based Hybridization Buffer, can be used. Heat the hybridization buffer to 70–80°C for 10 min, vortex, and repeat as necessary to resuspend the buffer. Microfuge for 1 min.
2. For dual-color assays: combine the two ligation reactions and add 2 µl of FlashTag Hyb. Blocker and 5 µl of 10% BSA. Then add 52 µl of hybridization buffer. The Hybridization Mix is 104 µl.
   For single-color assays: add 1 µl of FlashTag Hyb. Blocker, 5 µl of 10% BSA and 28.5 µl of hybridization buffer. The Hybridization Mix is 57 µl. For larger volumes, add equal parts 2X hybridization buffer and water.
3. Heat the Hybridization Mix to 65°C for 10 min. Gently vortex and briefly microfuge.

4. Apply some or all of the Hybridization Mix to a microarray, and cover with a glass coverslip. Hybridization chambers can also be used. **Note:** 2X Enhanced Hybridization Buffer requires higher hybridization volumes due to increased viscosity.

Recommended **Hybridization Mix** volumes for glass coverslips:

5. Incubate the array 6 h to overnight (6–20 h) in a dark, humidified chamber at 52°C.

Array Washing

1. Prewarm the 2X SSC/0.2%SDS wash buffer to 52°C.
2. Remove the coverslip by washing the array in 52°C 2X SSC/0.2% SDS for 2 min or until the coverslip floats off.
3. Wash for 15 min in 52°C 2X SSC/0.2%SDS.
4. Wash for 15 min in 2X SSC at room temperature.
5. Wash for 15 min in 0.2X SSC at room temperature.

	24×40 mm coverslip	24×50 mm coverslip	24×60 mm coverslip
2X Enhanced buffer	43 µl	53 µl	63 µl
2X SDS buffer	37 µl	45 µl	53 µl

6. Transfer the array to a dry 50 mL centrifuge tube or slide rack. Immediately centrifuge for 2 min at 800–1,000 RPM to dry the slide (any delay in this step may result in high background). Avoid contact with the array surface.

Signal Detection

Scan the array according to the manufacturer's recommendations, or first apply DyeSaver 2 coating (Genisphere cat. no. Q500500) to preserve fluorescent signal.

Detection Platforms for MicroRNA Profiling

Microarray platforms can be classified on the basis of whether they support one-color or two-color analysis. Fabricated (printed) microarrays are typically produced by robotic spotting of synthetic oligonucleotides probes onto the array and bound to it, usually at the 5' end through an amino group. These types of arrays can generally support two-color experiments, requiring that a test and reference sample are each labeled with a fluorescent dye and cohybridized on the same array. Alternatively, printing and fabrication technologies can be used to produce in situ synthesized microarrays. In this approach the probes are synthesized on the array and are bound to the surface at the 3' end since the oligonucleotide synthesis is carried in a 3' to 5' direction. When choosing a detection platform, compatibility between miRNA labeling/amplification methods and detection platforms should be confirmed, which means ensuring that the design of the probes are compatible (see below) and, in the case of microarrays, knowing the surface chemistry of the arrays and how they are fabricated. Composition of the hybridization buffer will also determine compatibility. For example, the custom spotted arrays described in the experiments by Mattie *et al*. (2006) were made by spotting oligonucleotides with an arrayer onto glass slides coated with poly-L-lysine. During evaluation of various labeling approaches with these arrays, it was discovered that hybridization buffers containing formamide will strip the poly-L-lysine coating, along with the probes, from the surface of the array. Likewise, the precursor to the FlashTag™ labeling procedure, the Array900 miRNA labeling

kit (Genisphere), was not compatible with CombiMatrix arrays. Subsequent changes to the dendrimer design for the FlashTag™ kit have resolved this issue.

In designing a microarray for miRNA analysis, optimal oligonucleotide probe design is essential. Prior studies have demonstrated that the closer the miRNA complementary sequence is to the surface, the lower the signal obtained. Binding may be physically hindered because of the close proximity and rigidity of the molecules at the surface-binding position. A recent study that evaluated probe design examined the effect of probes containing two or three copies of the target miRNA (Goff *et al*., 2005). The results of the study demonstrated that the copy found at the probe end that is farthest away from the surface is the most relevant, not the number of copies. As a result, various groups have used either tandem copies of the mature miRNA sequence or non-human genome sequences as a "stuffer" or linker to ensure the complementary miRNA sequence has enough distance from the surface of the array. Orientation of the probes should also be determined to ensure that the hybridized-labeled material is in the right orientation for complementarity. Inclusion of control probes, mismatch probes, or spike-in control probes can be used to assess aspects of the methodology, including specificity, labeling efficiency, hybridization efficiency, and accuracy in measuring differential expression. Both the probe design and the efficiency of the labeling method can contribute to the overall sensitivity of the platform.

Choice of a detection platform should weigh a number of factors including sensitivity, specificity, amount of input RNA required and available, cost, versatility or compatibility of sample processing with other detection platforms, and the number of miRNAs that need to be measured. The methods presented in this chapter allow for highly sensitive miRNA expression profiling for samples with limited input. These methods were especially ideal for tissue sections of biopsy samples. The low inputs required for the amplification and/or labeling procedure leaves enough material for follow-up validation with other detection platforms that do not require high amounts of sample input to survey a smaller panel of miRNAs, such as qRT-PCR or bead-based methods.

Analysis of MicroRNA Expression Data

A number of considerations should be taken into account when analyzing miRNA expression data. For a more thorough review on this subject see Davison *et al*. (2006). In summary, several of the fundamental assumptions that underlie conventional mRNA array analysis do not apply to the current state of miRNA analysis. First, the amount of RNA added to mRNA arrays is held constant, whereas the amount of miRNA loaded is unknown and can fluctuate widely between different tissues and samples. Second, the typical mRNA array experiment assays tens of thousands of genes that represent nearly the entire genome of transcripts. Consequently, the assumption for mRNA microarrays is that the expression levels of the vast majority of genes will have little or no variation across a set of experiments, and most mRNA normalization methods rely on this stabilizing, central trend in the data. In contrast, miRNA expression patterns and the number of miRNAs with detectable expression levels can vary widely between tissues. As a result, mean centering the data can yield false results. Finally, no reliable

set of constitutive or housekeeping miRNA genes has been reported, so it is not yet appropriate to normalize to an internal standard such as housekeeping miRNAs.

As a relatively new field, consensus on normalization and analysis of miRNA array data has not been achieved and the appropriate selection of methods continues to be debated. In terms of array data we have found that a scale factor normalization approach may be more appropriate for miRNA array analysis, though other valid approaches have been described as well (see Davison *et al*., 2006 review). TaqMan analysis has also had its own issues in terms of analysis. Early on, certain miRNAs like mir-16 and let-7a were used to normalize the data against because these miRNAs were found to be highly abundant within many tissues. However, these same miRNAs have also been found to be differentially expressed in several cancers. Normalization to any particular miRNA is really only appropriate if it is found to remain constant across your sample set. Other genes that have been suggested for the purposes of miRNA TaqMan normalization include 18 S rRNA or snoRNAs, but again this assumes that these remain constant across your data set and that your RNA isolation method gives consistent quantitative retention of these RNA species. As with mRNA microarray analysis, microRNAs found to have significantly differential expression between groups should be confirmed by other measurement options that have been mentioned in this chapter (e.g., qRT-PCR or bead-based profiling).

MicroRNAs in Cancer Diagnosis and Prognosis

Recent evidence from translational studies suggests that miRNA signatures may be useful in categorizing, detecting and predicting the course of an increasing number of human cancers. Translating miRNA signatures into clinical biomarkers is subject to the lessons learned from previous biomarker validation studies. Such validation studies should include a well-defined clinical question, a statistically valid experimental design, selection of highly characterized cases appropriate to the question and representative of the population, consideration of tumor heterogeneity, identification of normal controls, a robust platform, and robust statistical and computational analysis of diagnostics and predictors that are validated on independent data sets. The clinical usefulness of a biomarker should be assessed in randomized clinical trials and subsequently validated in follow-up trials (Wilson, 2006). Such studies will be critical for translating miRNA expression into clinical application as biomarkers for the diagnostic, prognostic, and predictive management of cancer.

REFERENCES

Ambros, V. 2003. MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing. *Cell 113*: 673–676.

Bartel, D. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell 116*: 281–297.

Bartel, B., and Bartel, D.P. 2003. MicroRNAs: at the root of plant development? *Plant Physiol. 132*: 709–717.

Baskerville, S., and Bartel, D.P. 2005. Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes. *RNA 11*: 241–247.

Bentwich, I., Avniel, A., Karov, Y., Aharonov, R., Gilad, S., Barad, O., Barzilai, A., Einat, P., Einav, U., Meiri, E., Sharon, E., Spector, Y., and Bentwich, Z. 2005. Identification of hundreds of conserved and nonconserved human microRNAs. *Nat. Genet. 37*: 766–770.

Bohnsack, M.T., Czaplinsk, K., and Gorlich, D. 2004. Exportin 5 is a RanGTP-dependent

dsRNA-binding protein that mediates nuclear export of pre-miRNAs. *RNA 10*: 185–191.

Cai, X., Hagedorn, C.H., and Cullen, B.R. 2004. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. *RNA 12*: 1957–1966.

Calin, G.A., Dumitru, C.D., Shimizu, M., Bichi, R., Zupo, S., Noch, E., Aldler, H., Rattan, S., Keating, M., Rai, K., Rassenti, L., Kipps, T., Negrini, M., Bullrich, F., and Croce, C.M. 2002. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc. Natl. Acad. Sci. 99*: 15524–15529.

Calin, G.A., Ferracin, M., Cimmino, A., Di Leva, G., Shimizu, M., Wojcik, S.E., Iorio, M.V., Visone, R., Sever, N.I., Fabbri, M., Iuliano, R., Palumbo, T., Pichiorri, F., Roldo, C., Garzon, R., Sevignani, C., Rassenti, L., Alder, H., Volinia, S., Liu, C.G., Kipps, T.J., Negrini, M., and Croce, C.M. 2005. A microRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *N. Engl. J. Med. 353*: 1793–1801.

Cao, X., Yeo, G., Muotri, A.R., Kuwabara, T., and Gage, F.H. 2006. Noncoding RNAs in mammalian central nervous system. *Annul. Rev. Neurosci. 29*: 77–103.

Chen, C., Ridzon, D.A., Broomer, A.J., Zhou, Z., Lee, D.H., Nguyen, J.T., Barbisin, M., Xu, N.L., Mahuvakar, V.R., Andersen, M.R., Lao, K.Q., Livak, K.J., and Guegler, K.J. 2005. Real-time quantification of microRNAs by stemloop RT-PCR. *Nucl. Acids Res. 33*: e179.

Davison, T.S., Johnson, C.D., and Andruss, B.F. 2006. Analyzing microRNA expression using microarrays. *Meth. Enzymol. 411*: 14–34.

Einat, P. 2006. Methodologies for high-throughput expression profiling of microRNAs. *Meth. Mol. Biol. 342*: 139–157.

Goff, L.A., Bowers, J., Schwalm, J., Howerton, K., Getts, R.C., and Hart, R.P. 2004. Evaluation of sense-strand mRNA amplification by comparative quantitative PCR. *BMC Genomics 5*: 76–80.

Goff, L.A., Yang, M., Bowers, J., Getts, R.C., Padgett, R.W., and Hart, R.P. 2005. Rational probe optimization and enhanced detection strategy for microRNAs using microarrays. *RNA Biol. 2*: E9–E16.

Gregory, R.I., Chendrimada, T.P., Cooch, N., and Shiekhattar, R. 2005. Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. *Cell 123*: 631–640.

Griffiths-Jones, S., Grocock, R.J., van Dongen, S., Bateman, A., and Enright, A.J. 2006. miRBase: microRNA sequences, targets and gene nomenclature. *Nucl. Acids Res. 34*: D140–D144.

Hutvagner, G., and Zamore, P.D. 2002. A microRNA in a multiple turnover RNAi enzyme complex. *Science 297*: 2056–2060.

Iorio, M.V., Ferracin, M., Liu, C.G., Veronese, A., Spizzo, R., Sabbioni, S., Magri, E., Pedriali, M., Fabbri, M., Campiglio, M., Menard, S., Palazzo, J.P., Rosenberg, A., Musiani, P., Volinia, S., Nenci, I., Calin, G.A., Querzoli, P., Negrini, M., and Croce, C.M. 2005. MicroRNA gene expression deregulation in human breast cancer. *Cancer Res. 65*: 7065–7070.

John, B., Enright, A.J., Aravin, A., Tuschl, T., Sander, C., and Marks, D.S. 2004. Human microRNA targets. *PLoS 2*: e363.

Johnson, S.M., Grosshans, H., Shingara, J., Byrom, M., Jarvis, R., Cheng, A., Labourier, E., Reinert, K.L., Brown, D., and Slack, F.J. 2005. RAS is regulated by the *let-7* microRNA family. *Cell 120*: 635–647.

Ketting, R.F., Fischer, S.E., Bernstein, E., Sijen, T., Hannon, G.J., and Plasterk, R.H. 2001. Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. *Genes Dev. 15*: 2654–2659.

Landgraf, P., Rusu, M., Sheridan, R., Sewer, A., Iovino, N., Aravin, A., Pfeffer, S., Rice, A., Kamphorst, A.O., Landthaler, M., Lin, C., Socci, N., Hermida, L., Fulci, V., Chiaretti, S., Foa, R., Schliwks, J., Fuchs, U., Novosel, A., Muller, R.-U., Schermer, B., Bissels, U., Inman, J., Phan, Q., Chien, M., Weir, D.B., Choksi, R., De Vita, G., Frezzetti, D., Trompeter, H.-I., Hornung, V., Teng, G., Hartmann, G., Palkovits, M., Di Lauro, R., Wernet, P., Macino, G., Rogler, C.E., Nagle, J.W., Ju, J., Papavasilliou, F.N., Benzing, T., Lichter, P., Tam, W., Brownstein, M.J., Bosio, A., Borkhardt, A., Russo, J.J., Sander, C., Zavolan, M., and Tuschl, T. 2007. A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell 129*: 1401–1414.

Lawrie, C.H., Soneji, S., Marafioti, T., Cooper, C.D., Palazzo, S., Paterson, J.C., Cattan, H., Enver, T., Mager, R., Boultwood, J., Wainscoat, J.S., and Hatton, C.S. 2007. MicroRNA expression

distinguishes between germinal center B cell-like and activated B cell-like subtypes of diffuse large B cell lymphoma. *Intl. J. Cancer 121*: 1156–1161.

Lee, Y., Kim, M., Han, J., Yeom, K.H., Lee, S., Baek, S.H., and Kim, V.N. 2004. MicroRNA genes are transcribed by RNA polymerase II. *EMBO J. 23*: 4051–4060.

Lewis, B.P., Shih, I.H., Jones-Rhoades, M.W., Bartel, D.P., and Burge, C.B. 2003. Prediction of mammalian microRNA targets. *Cell 115*: 787–798.

Lin, S.L., Chang, D., and Ying, S.Y. 2005. Asymmetry of intronic pre-miRNA structures in functional RISC assembly. *Gene 356*: 32–38.

Lu, J., Getz, G., Miska, E.A., Alvarez-Saavedra, E., Lamb, J., Peck, D., Sweet-Cordero, A., Ebert, B.L., Mak, R.H., Ferrando, A.A., Downing, J.R., Jacks, T., Horvitz, H.R., and Golub, T.R. 2005. MicroRNA expression profiles classify human cancers. *Nature 435*: 834–838.

Mattie, M.D., Benz, C.C., Bowers, J., Sensinger, K., Wong, L., Scott, G.K., Fedele, V., Ginzinger, D., Getts, R., and Haqq, C. 2006. Optimized high-throughput microRNA expression profiling provides novel biomarker assessment of clinical prostate and breast cancer biopsies. *Mol. Cancer 5*: 24.

Nilsen, T.W., Grazel, J., and Prensky, W. 1997. Dendritic Nucleic Acid Structures. *J. Theor. Biol. 187*: 273–284.

Olsen, P.H., and Ambros, V. 1999. The *lin-4* regulatory RNA controls developmental timing in *Caenorhabditis elegans* by blocking LIN-14 protein synthesis after the initiation of translation. *Dev. Biol. 216*: 671–680.

Plasterk, R.H. 2006. MicroRNAs in animal development. *Cell 124*: 877–881.

Rodriguez, A., Griffiths-Jones, S., Ashurst, J.L., and Bradley, A. 2004. Identification of mammalian microRNA host genes and transcription units. *Genome Res. 14*: 1902–1910.

Schmittgen, T.D., Jiang, J., Liu, Q., and Yang, L. 2004. A high-throughput method to monitor the expression of microRNA precursors. *Nucl. Acids Res. 32*: e43.

Shi, R., and Chiang, V.L. 2005. Facile means for quantifying microRNA expression by real-time PCR. *Biotechniques 39*: 519–525.

Shivdasani, R.A. 2006. MicroRNAs: regulators of gene expression and cell differentiation. *Blood 108*: 3646–3653.

Stears, R.L., Getts, R.C., and Gullans, S.R. 2000. A novel, sensitive detection system for high-density microarrays using dendrimer technology. *Physiol. Genomics 3*: 93–99.

Takamizawa, J., Konishi, H., Yanagisawa, K., Tomida, S., Osada, H., Endoh, H., Harano, T., Yatabe, Y., Nagino, M., Nimura, Y., Mitsudomi, T., and Takahashi, T. 2004. Reduced expression of the *let-7* microRNAs in human lung cancers in association with shortened postoperative survival. *Cancer Res. 64*: 3753–3756.

Wilson, J.F. 2006. The rocky road to useful cancer biomarkers. *Ann. Intern. Med. 144*: 945–948.

Yanaihara, N., Caplen, N., Bowman, E., Seike, M., Kumanoto, K., Yi, M., Stephens, R., Okamoto, A., Yokota, J., Tanaka, T., Calin, G., Liu, C., Croce, C., and Harris, C. 2006. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer Cell 9*: 189–198.

6
Familial Breast Cancer: Detection of Prevalent High-Risk Epithelial Lesions

Peter Bult and Nicoline Hoogerbrugge

INTRODUCTION

BRCA1 and *BRCA2* mutation carriership is associated with a highly increased risk of developing breast carcinoma. For women carrying a *BRCA* mutation, the risk of breast cancer begins to increase before the age of 25, with a steep increase after the age of 40 years. The cumulative risk of developing breast cancer before the age of 40 years is ~ 15%, while the cumulative risk of developing breast cancer before the age of 50 is 40–50% (Ford *et al*., 1998). The life time risk of invasive breast cancer is 60–80%. Very little is known regarding the early stages of breast cancer development in the inherited forms of the disease. Women with hereditary predisposition to breast cancer are prone to develop epithelial lesions that indicate a high risk of subsequent invasive breast cancer (Dupont and Page, 1985; Hoogerbrugge *et al*., 2003). These high-risk lesions include atypical lobular hyperplasia (ALH), atypical ductal hyperplasia (ADH), lobular carcinoma in situ (LCIS), and ductal carcinoma in situ (DCIS) (Kauff *et al*., 2003). These epithelial lesions may predict the occurrence of subsequent invasive breast cancer (Singletary, 1994).

Women with a clear autosomal dominant family history for breast cancer that is not caused by a *BRCA1* or *BRCA2* mutation also have a prevalence of epithelial high-risk lesions, which is at least as high as that of mutation carriers. The presence of high-risk lesions is associated with age; especially at the age over 40 years a large number of women with or without a *BRCA* mutation have high-risk epithelial lesions. Previous oophorectomy should not be taken to indicate low-risk of epithelial lesions in women at high risk of hereditary cancer (Hoogerbrugge *et al*., 2006).

The options for women with a deleterious germ-line mutation in *BRCA1* or *BRCA2* to handle their high risk are either regular surveillance or prophylactic mastectomy. Bilateral prophylactic mastectomy in healthy women with a *BRCA* mutation is associated with a 90% reduction in breast cancer incidence (Meijers-Heijboer *et al*., 2001; Rebbeck *et al*., 2004). When applied at young age, at or before the age of 40 years, this may lead to a significant survival advantage (van Roosmalen *et al*., 2002). This procedure is much less accepted for women who appeared to be negative for a *BRCA* mutation even though they have an apparent autosomal

dominant family history for breast cancer (Lostumbo *et al*., 2004). Especially women with breast cancer at young age and a strong family history but without a *BRCA* mutation may want to opt for contralateral prophylactic mastectomy.

After initial diagnosis of breast cancer in a *BRCA1* or *BRCA*2 mutation carrier, the risk of developing cancer in the opposite breast is ~ 30% in 10 years (Metcalfe *et al*., 2004), increasing to 40% in 10 years when breast cancer occurred before the age of 40 (Verhoog *et al*., 2000). Data concerning the risk of cancer in the opposite breast of women with an autosomal dominant family history for breast cancer without a *BRCA* mutation are lacking. Most likely the risk of a second primary breast cancer in these women greatly depends on the age of the breast cancer patient, as well as on family history. The optimal management of patients with breast cancer and an autosomal dominant family history for breast cancer is still controversial. Simple mastectomy is an effective way to prevent breast cancer (Meijers-Heijboer *et al*., 2001). However, it is unknown whether contralateral prophylactic mastectomy leads to survival advantage (Lostumbo *et al*., 2004). The stage and thereby the prognosis of the treated breast cancer may greatly influence survival of patients with contralateral prophylactic mastectomy. Especially young women who had breast cancer with characteristics indicating a good prognosis and a high genetic risk of a second primary breast cancer may benefit from contralateral prophylactic mastectomy. Most likely these patients have a heterogeneous genetic origin, consisting of women carrying a yet unknown *BRCA* mutation, or a combination of several less potent susceptibility genes (Easton *et al*., 2007). Although prophylactic mastectomy can be an option for these women, it is not the only option. Improved surveillance with advanced technologies such as magnetic resonance imaging (MRI) may be able to detect cancers at an early stage (Kriege *et al*., 2004). In theory chemoprevention, for example with tamoxifen, may be an option (Bramley *et al*., 2006). It is important to realize that for women with an autosomal dominant family history but without a personal history of breast cancer and no detectable *BRCA* mutation, alternative options instead of preventive mastectomy should be considered, because risk status cannot adequately be measured and the ability to modify this risk by mastectomy is not sufficiently known.

Careful examination of prophylactically removed breasts by the pathologist is important to exclude occult invasive carcinoma. Additionally, identification of premalignant lesions in prophylactically removed breasts supports the decision of prophylactic mastectomy in women at high hereditary risk of breast cancer.

In general practice, sampling of the mastectomy specimen is performed by macroscopical examination and palpation, before and after manual slicing of the specimen. Excisions are taken at least from the nipple and the four quadrants of the breast, and from suspicious areas. Special attention should be paid to changes in color and to palpable lesions not seen on the radiograms or not apparent with inspection. For example, ochreous colored fat tissue may indicate an invasive carcinoma, and point-shaped necrosis is suspicious for comedo type DCIS. Specimen radiography can guide this sampling of the mastectomy specimen (Egan, 1982; Holland *et al*., 1985; Hoogerbrugge *et al*., 2003, 2006). Resolution of the specimen radiograms is much higher than that of mammography.

Therefore, small architectural distortions, small densities (Figures 6.1 and 6.2), and tiny microcalcifications (Figures 6.3 and 6.4) in the breast tissue, skin, or fascia can be detected and subsequently sampled for histopathological examination.

MATERIALS

1. Measuring staff.
2. Scale.
3. Indian ink.
4. Waterproof felt pen.
5. Sutures.
6. Surgical needles.
7. Refrigerator which cools down to 32 degrees Fahrenheit (0 degrees Celsius).
8. Meat slicing machine.
9. Old mammograms or hard plastic pads.
10. Digital camera.

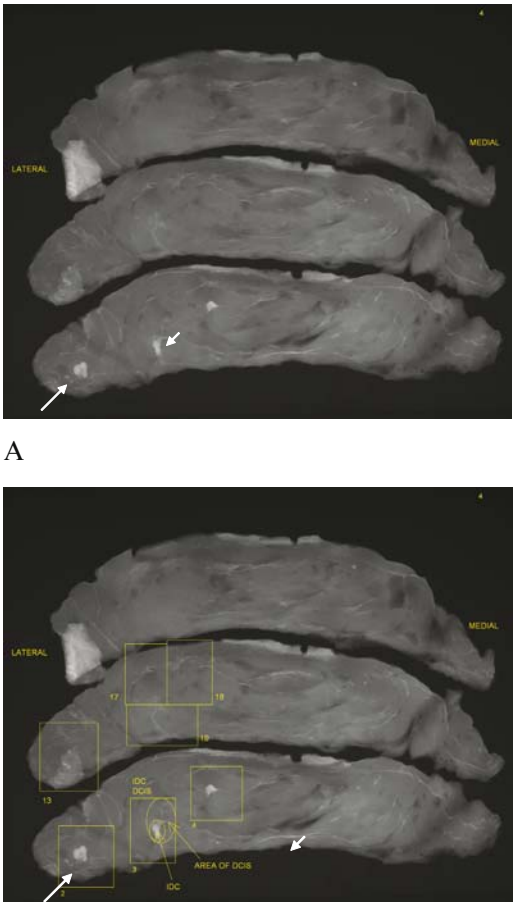


FIGURE 6.1. Specimen radiograms of a right breast without (A) and with (B) pathological findings. A small partly speculated density is seen (short arrow), besides an intramammary lymph node (long arrow)

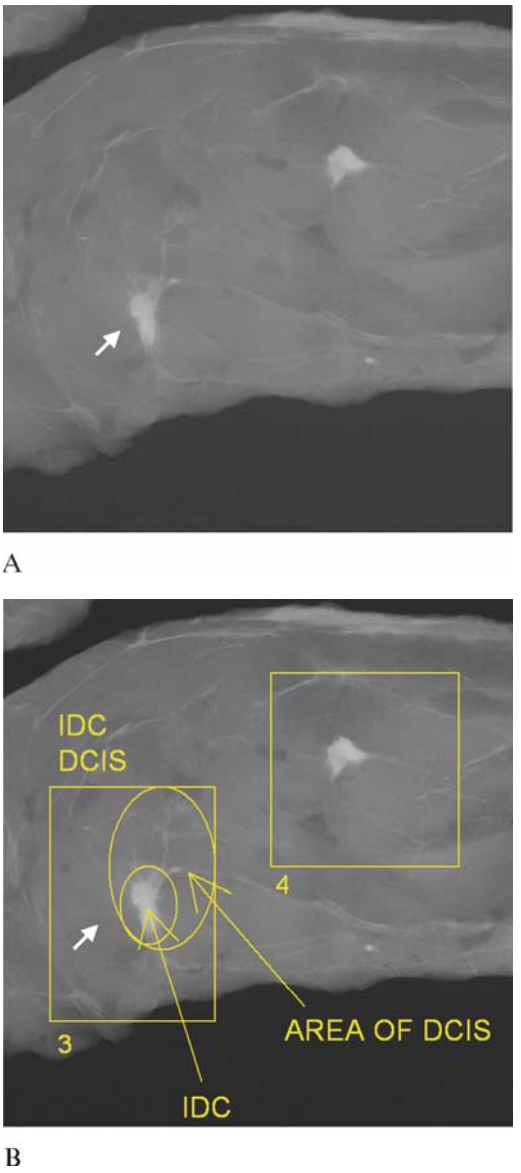
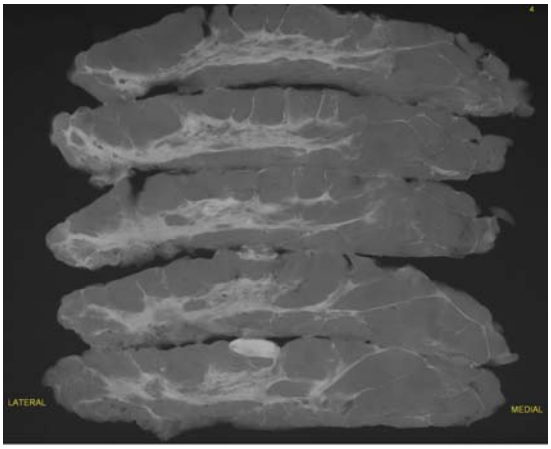
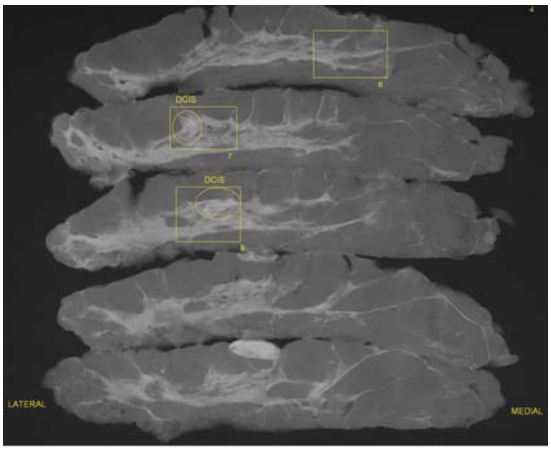


FIGURE 6.2. Magnified radiograms of Figure 6.1 with a small partly speculated density (arrow), representing invasive ductal carcinoma (IDC), with occult surrounding ductal carcinoma in situ (DCIS)



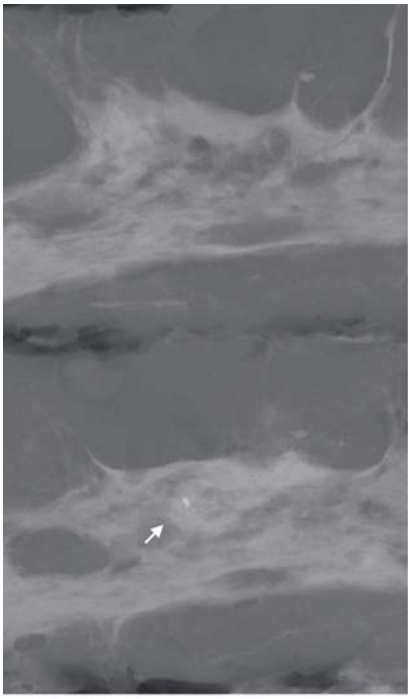
A



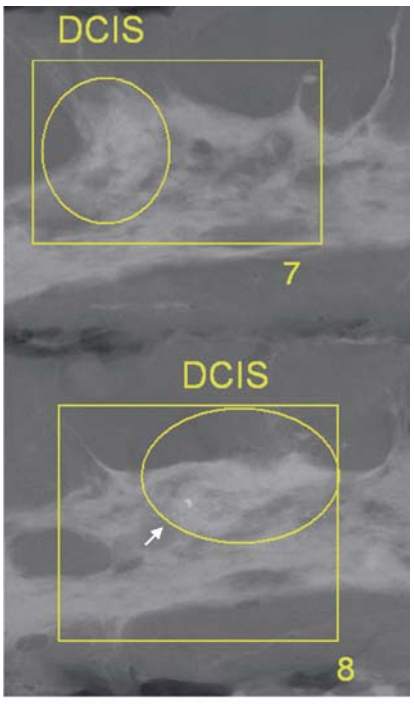
B

FIGURE 6.3. Specimen radiograms of a right breast without (A) and with (B) pathological findings

11. Digital radiography/mammography machine.
12. Software to retrieve the digital radiograms from the radiology/hospital database.
13. Software to annotate the digital radiograms; to add pathology data to the digital images.
14. Software to return the annotated digital radiograms to the radiology/hospital database.



A



B

FIGURE 6.4. Magnified radiograms of Figure 6.3 with tiny microcalcifications (arrow), pointing towards a part of an area of ductal carcinoma in situ (DCIS)

METHODS

The following procedure of mastectomy handling is partly based on the description of Egan (1982).

Handling of the mastectomy specimen

1. The excised simple mastectomy specimen should arrive at the pathology department within 1 h after removal to prevent autolysis of the tissue.
2. At the pathology department the whole specimen is weighed and measured immediately.
3. The excised skin is measured.
4. The fascia is inked with Indian ink.
5. The specimen is inspected and palpated for lesions. If a palpable lesion is found, this lesion is cut from the fascia side in medial-lateral direction. If a solid lesion is found the lesion is measured and the aspect of the lesion is described. A tissue slice is taken for direct formalin fixation and one or more small tissue fragments of this lesion are frozen in liquid nitrogen. If the resection margins other than the fascia are involved or suspected for involvement these are also inked with Indian ink.
6. The artificial fascia defect is closed with sutures.
7. The lateral and medial parts of the skin are marked with a waterproof felt pen with a “L” and “M”, respectively.
8. The mastectomy specimen is placed in the refrigerator for overnight cooling to stiffen the breast and fat tissue.
9. The next morning the specimen is palpated. If the tissue is stiff, the specimen can be processed. If the tissue is still soft, the specimen is put in a freezer and turned up side down every 15 min and every time the issue is inspected. If the tissue is stiff it can be processed further. Special attention should be paid to prevent the tissue to be frozen.
10. The breast specimen is sliced on a meat slicing machine from cranial to caudal in 5 mm thick slices.
11. The slices are placed on old mammograms or hard plastic pads in sequence from cranial to caudal, as if looking at the tissue from feet to head.
12. Digital radiograms of the slices are made on a digital radiography/mammography machine in sequence from cranial to caudal and stored in the hospital database.
13. The radiograms are retrieved from the hospital database.
14. All radiograms are numbered in sequence from cranial to caudal.
15. The radiograms are marked; cranial and caudal are added to the first and the last radiograms, respectively. Lateral and medial is added to all radiograms.
16. The tissue (breast, fascia, and skin) is inspected and palpated and the findings are correlated with the radiograms and vice versa.
17. Tissue sampling is performed from the nipple and the four quadrants and areas which are aberrant in the breast tissue, fascia, and skin (palpable lesions, discolorations e.g. ochreous colored fat tissue, point shaped necrosis), architectural tissue distortion, densities, microcalcifications, retraction of the skin or fascia, thickened skin or fascia), and (intramammary) lymph nodes. The number of tissue samples can vary considerably from specimen to specimen depending on the size and weight of the specimen,

the amount of fibrose (which might hamper the judgment of the radiograms leading to more tissue samples), the amount and distribution of microcalcifications, etc.

18. The total number of samples should be ≈18 (range 7–39).
19. The tissue excisions are marked on the digital radiograms.
20. The tissue excisions are processed to paraffin blocks, and from the paraffin blocks 4 micron thick slices are cut for hematoxylin and eosin staining.
21. In the pathology report all aberrant findings are described, with special attention to the premalignant lesions (ALH, ADH, LCIS, and DCIS) and eventual invasive carcinoma. If an invasive carcinoma is present, all relevant aspects of the tumor are reported (e.g., tumor type, tumor size, histological grade, vessel invasion, skin invasion, invasion of the fascia, estrogen receptor and progesterone receptor status, HER-2/neu status, surgical resection margins, status of (intramammary) lymph nodes and pTNM stage). If DCIS is present, the type of DCIS, the histological grade, the size of the lesion, and resection margins are reported.
22. Eventually, the histopathological findings can be added to the digital specimen radiograms and these annotated specimen radiograms can be returned to the hospital database.
23. The pathological findings are presented in a multidisciplinary meeting with pathologists, radiologists, medical oncologists, radiotherapists, and surgeons where the (annotated) specimen radiograms are compared with the preoperative radiological examinations.

CLASSIFICATION OF PREMALIGNANT LESIONS

Lobular carcinoma-in-situ (LCIS)

Lobular carcinoma-in-situ can be divided in two main groups, classical LCIS, that is most frequently found, and pleomorphic LCIS. Classical LCIS is defined as a proliferation of small and often loosely cohesive cells originating in the terminal duct-lobular unit, with or without pagetoid involvement of terminal ducts, and with completely distended duct-lobular units. The lobular architecture is maintained. The cells are loosely cohesive and regularly dispersed. The cells are monomorphic, small, round, polygonal, or cuboidal, with a high nuclear-cytoplasmic ratio and frequently intracytoplasmic lumina (Tavassoli and Devilee, 2003) (Figure 6.5A). These cells are also referred to as type A cells (Simpson *et al.*, 2003; Lakhani *et al.*, 2006). Pleomorphic LCIS has the same architecture as the classical type of LCIS, however, the cells show mild to moderate enlarged and pleomorphic nuclei with more abundant cytoplasm. These cells are also referred to as type B cells (Simpson *et al.*, 2003; Lakhani *et al.*, 2006).

Atypical lobular hyperplasia (ALH)

Atypical lobular hyperplasia is defined as a proliferation of small and often loosely cohesive cells originating in the terminal duct-lobular unit, with or without pagetoid involvement of terminal ducts, but without completely distended duct-lobular units (Tavassoli and Devilee, 2003) (Figure 6.5B). The cells of ALH are of type A cells as described under classical LCIS (Simpson *et al.*, 2003). The cells of LCIS and ALH characteristically

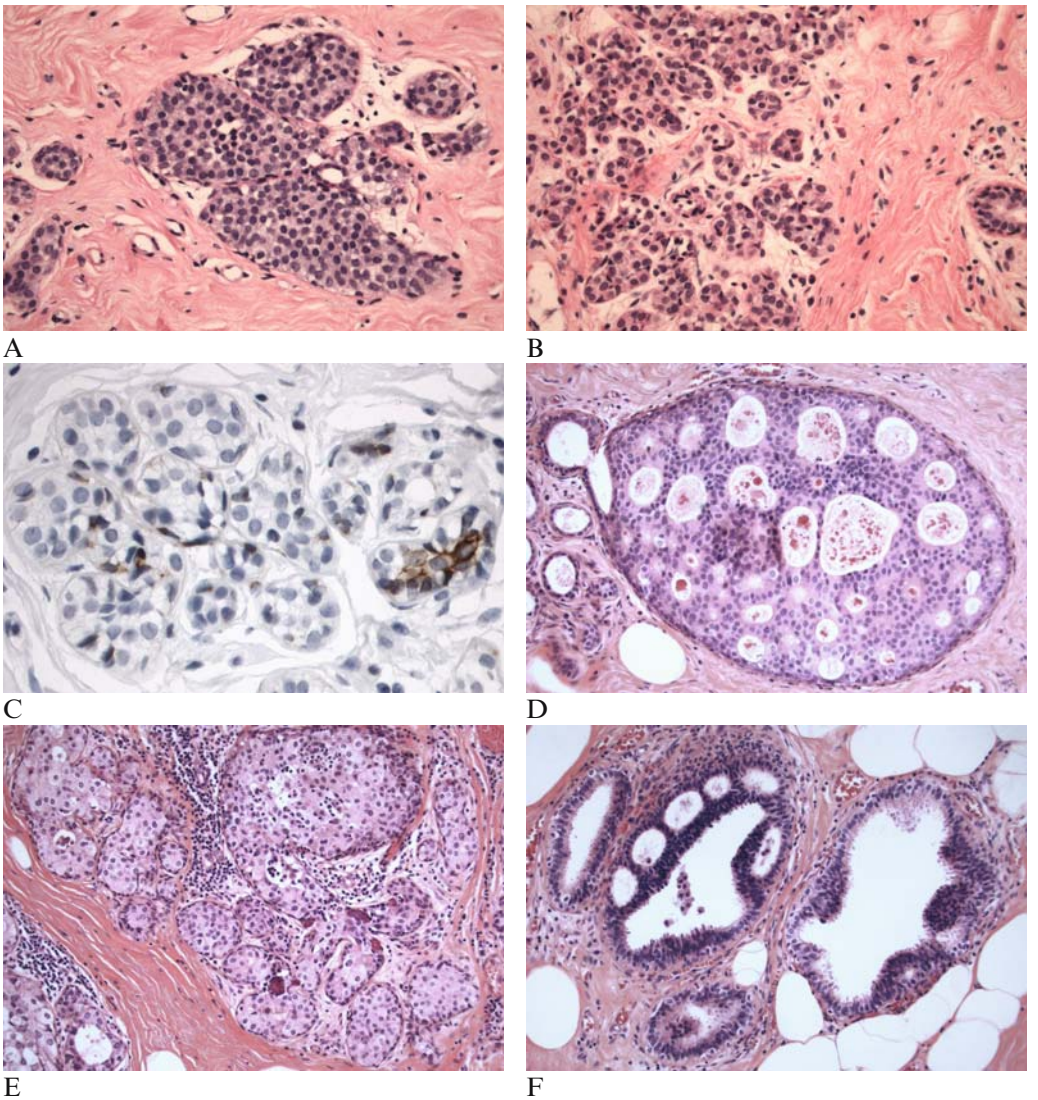


FIGURE 6.5. Premalignant high-risk lesions: lobular carcinoma in situ (A), atypical lobular hyperplasia (B), E-cadherin immunohistochemistry (C), ductal carcinoma in situ; cribriform (D) and solid (E), and atypical ductal hyperplasia (F)

lack expression of E-cadherin, an intercellular adhesion molecule of epithelial cells (Gamallo *et al*., 1993) (Figure 6.5C).

Ductal carcinoma in situ (DCIS)

Ductal carcinoma in situ is defined using the classification of Holland *et al*. (1994), which is based on cytonuclear and architectural differentiation. Three categories are defined; well differentiated, intermediately, and poorly differentiated DCIS. Well differentiated DCIS is composed of cells with monomorphic, regularly spaced nuclei containing fine chromatin, inconspicuous nucleoli, and few mitoses. The cells show pronounced polarization with orientation of their apical border towards

intercellular spaces usually resulting in rigid cribriform (Figure 6.5D), micropapillary, and clinging patterns, although solid growth may also occur. Necrosis is uncommon. Calcifications, when present, are usually psammomatous.

Poorly differentiated DCIS is composed of cells with very pleomorphic, irregularly spaced nuclei, with coarse, clumped chromatin, prominent nucleoli, and frequent mitoses. Architectural differentiation is absent or minimal. The growth pattern is solid or pseudo-cribriform and pseudo-micropapillary (without cellular polarization). Necrosis is usually present. Calcifications, when present, are usually amorphous.

Intermediately differentiated DCIS is composed of cells showing some pleomorphism but not so marked as in the poorly differentiated group, and mitosis is less numerous as in the poorly differentiated group. There is, however, always evidence of polarization around intercellular spaces, although this is not so pronounced as in the well-differentiated group. Solid growth can also be seen (Figure 6.5E). Necrosis can be present. Calcifications, when present, can be psammomatous and amorphous.

Atypical Ductal Hyperplasia (ADH)

Atypical ductal hyperplasia is used in the spectrum of ductal hyperplasia, ADH, and well differentiated DCIS, and is defined as a lesion exhibiting some, but not all features of well differentiated DCIS (Page and Rogers, 1992; Pinder and Ellis, 2003). ADH is formed by a uniform population of small or medium-size, round, cuboidal, or polygonal hyperchromatic cells, which are regularly arranged. The nuclei are evenly distributed and may form a rosette-like pattern. Only single small nucleoli are seen. Mitoses, particularly abnormal forms, is rarely found. Geometric spaces are present and, in cribriform type, the cells are arranged at right angles to the bridges formed (Figure 6.5F). Rigid punched-out lumina are not seen. Micropapillary ADH is also recognized, and a solid pattern may very rarely be seen. Small foci of necrosis may rarely be identified in ADH, and do not indicate that the process should be classified as DCIS.

In case of doubt between classification as ADH or DCIS, ADH is diagnosed.

RESULTS AND DISCUSSION

The prevalence of DCIS and LCIS lesions reported in studies with women at high genetic risk for breast cancer is higher than that reported in the general population. The reported prevalence of undiagnosed DCIS in a review of autopsy studies, using various techniques, of women with an age range between 15–80 years is ≈9% (range 0–15%) (Welch and Black, 1997). For LCIS this prevalence is less extensively studied and reported to range between ≈1–3% (Singletary, 1994).

Controversies exist regarding the role of DCIS in both *BRCA1* and *BRCA2* mutation carriers (Sun *et al*., 1996). From our series we concluded that *BRCA1* and *BRCA2* mutation carriers are equally prone to develop high-risk lesions in their breasts, with ≈8% having occult DCIS (Hoogerbrugge *et al*., 2006) (Table 6.1). The data from recent studies among DCIS patients show that the prevalence of *BRCA* mutations among DCIS patients is similar to that found in patients with invasive breast cancer (Claus *et al*., 2005; Hwang *et al*., 2007), suggesting that DCIS belongs

TABLE 6.1. High risk epithelial lesions in various groups of patients with a prophylactic mastectomy because of a clear autosomal dominant family history for breast cancer. (Hoogerbrugge *et al*., 2006).

	BRCA1	BRCA2	non-BRCA
(N)	(68)	(14)	(24)
Overall presence of high-risk lesions	44%	36%	71%
ALH	26%	21%	67%
ADH	18%	14%	42%
LCIS	16%	7%	29%
DCIS	9%	7%	17%

to the spectrum of inherited breast cancer. As a consequence, in family-histories, DCIS can be considered as equal to the breast-cancer affected status, when considering familial at-risk status for *BRCA*.

The natural course of LCIS is not entirely clear. There is disagreement about whether LCIS is a precursor of invasive disease or merely a marker of subsequent invasive carcinoma risk. In women diagnosed with LCIS, ≈30% will develop an invasive carcinoma (Hutter, 1984), mostly of the ductal type (Rosen *et al*., 1980). It seems that LCIS is merely a risk-indicator for breast cancer, not a true precursor for invasive disease in most patients. This seems true for breast cancer developing in *BRCA1* mutation carriers, as ILC is less frequently found in these patients than in sporadic breast cancer patients (1–16% versus 5–25%, respectively). For *BRCA2* mutation carriers, however, this is less obvious, as the frequency of ILC is the same as in sporadic breast cancer patients (9–29% and 5–25%, respectively) (Honrado *et al*., 2006). So, at least a part of the invasive breast carcinomas (mean ≈15%) originates from LCIS and LCIS is thus the precursor of the invasive cancer. Atypical ductal hyperplasia and ALH are also associated with an increased risk of a subsequent invasive carcinoma. The risk is ≈4–5 times than that of the general population for both ADH and ALH.

We found a high prevalence of high-risk epithelial lesions in *BRCA* mutation carriers and women at high genetic risk tested negative for such a mutation (43% and 71%, respectively). The prevalence was highly correlated with age. Women over 40 years of age had a higher prevalence of high-risk lesions than younger women: 60% versus 38%, respectively (Hoogerbrugge *et al*., 2006). This finding is in concordance with the steep increase of the risk of breast cancer after the age of 40 years with a cumulative risk of developing breast cancer before the age of 50 of 40–50% (Ford *et al*., 1998). These findings strongly suggest that invasive breast cancer in *BRCA* mutation carriers and in women at high genetic risk who are tested negative for such a mutation is preceded by premalignant lesions as in sporadic breast cancer.

In conclusion, extensive pathological examination of a prophylactically removed breast in *BRCA* mutation carriers and in women at high genetic risk tested negative for such a mutation is necessary to exclude an occult invasive carcinoma, and may add plausibility to the concept of breast cancer risk reduction by prophylactic mastectomy when premalignant lesions

are detected. Specimen radiography can be very helpful for adequate sampling of the specimen. Especially on high resolution radiograms, lesions that are likely to be missed by conventional examination, can easily be detected. Nevertheless, a substantial number of premalignant high-risk epithelial lesions can only be identified by examination of "blind" tissue samples of the breast specimen.

REFERENCES

Bramley, M., Clarke, R.B., Howell, A., Evans, D.G.R., Armer, T., Baildam, A.D., and Anderson, E. 2006. Effects of oestrogens and anti-oestrogens on normal breast tissue from women bearing BRCA1 and BRCA2 mutations. *Br. J. Cancer 94*: 1021–1028.

Claus, E.B., Petruzella, S., Matloff, E., and Carter, D. 2005. Prevalence of BRCA1 and BRCA2 mutations in women diagnosed with ductal carcinoma in situ. *J. A. M. A. 293*: 964–969.

Dupont, W.D., and Page, D.L. 1985. Risk factors for breast cancer in women with proliferative breast disease. *New. Eng. J. Med. 312*: 146–151.

Easton, D.F., Deffenbaugh, A.M., Pruss, D., Frye, C., Wenstrup, R.J., Allen-Brady, K., Tavtigian, S.V., Monteiro, A.N., Iversen, E.S., Couch, F.J., and Goldgar, D.E. 2007. A systematic genetic assessment of 1,433 sequence variants of unknown clinical significance in the BRCA1 and BRCA2 breast cancer-predisposition genes. *Am. J. Hum. Genet. 81*: 873–883.

Egan, R.L. 1982. Multicentric breast carcinomas: clinical-radiographic-pathologic whole organ studies and 10-year survival. *Cancer 49*: 1123–1130.

Ford, D., Easton, D.F., Stratton, M., Narod, S., Goldgar, D., Devilee, P., Bishop, D.T., Weber, B., Lenoir, G., Chang-Claude, J., Sobol, H., Teare, M.D., Struewing, J., Arason, A., Scherneck, S., Peto, J., Rebbeck, T.R., Tonin, P., Neuhausen, S., Barkardottir, R., Eyfjord, J., Lynch, H., Ponder, B.A.J., Gayther, S.A., Birch, J.M., Lindblom, A., Stoppa-Lyonnet, D., Bignon, Y., Borg, A., Hamann, U., Haites, N., Scott, R.J., Maugard, C.M., and Vasen, H. 1998. Genetic heterogeneity and penetrance analysis of BRCA1 and BRCA2 genes in breast cancer families. The Breast Cancer Linkage Consortium. *Am. J. Hum. Genet. 62*: 676–689.

Gamallo, C., Palacios, J., Suarez, A., Pizarro, A., Navarro, P., Quintanilla, M., and Cano, A. 1993. Correlation of E-cadherin expression with differentiation grade and histological type in breast carcinoma. *Am. J. Pathol. 142*: 987–993.

Holland, R., Veling, S.H., Mravunac, M., and Hendriks, J.H. 1985. Pathologic multifocality of Tis, T1-2 breast carcinomas. Implications for clinical trials of breast-conserving surgery. *Cancer 56*: 979–990.

Holland, R., Peterse, J.L., Millis, R.R., Eusebi, V., Faverly, D., van de Vijver, M.J., and Zafrani, B. 1994. Ductal carcinoma-in-situ: a proposal for a new classification. *Semin. Diagn. Path. 11*: 167–180.

Honrado, E., Benítez, J., and Palacios, J. 2006. Histopathology of BRCA1- and BRCA2-associated breast cancer. *Crit. Rev. Oncol. Hematol. 59*: 27–39.

Hoogerbrugge, N., Bult, P., de Widt-Levert, L.M., Beex, L.V., Kiemeney, L.A., Ligtenberg, M.J., Massuger, L.F., Boetes, C., Manders, P., and Brunner, H.G. 2003. High prevalence of premalignant lesions in prophylactically removed breasts from women at hereditary risk for breast cancer. *J. Clin. Oncol. 21*: 41–45.

Hoogerbrugge, N., Bult, P., Bonenkamp, J.J., Ligtenberg, M.J., Kiemeney, L.A., de Hullu, J.A., Boetes, C., Niermeijer, M.F., and Brunner, H.G. 2006. Numerous high-risk epithelial lesions in familial breast cancer. *Eur. J. Cancer 42*: 2492–2498.

Hutter, R.V. 1984. The management of patients with lobular carcinoma in situ of the breast. *Cancer 53*: 798–802.

Hwang, E.S., McLennan, J.L., Moore, D.H., Crawford, B.B., Esserman, L.J., and Ziegler, J.L. 2007. Ductal carcinoma in situ in BRCA mutation carriers. *J. Clin. Oncol. 25*: 642–647.

Kauff, N.D., Brogi, E., Scheuer, L., Pathak, D.R., Borgen, P.I., Hudis, C.A., Offit, K., and Robson, M.E. 2003. Epithelial lesions in prophylactic mastectomy specimens from women with BRCA mutations. *Cancer 97*: 1601–1608.

Kriege, M., Brekelmans, C.T.M., Boetes, C., Besnard, P.E., Zonderland, H.M., Obdeijn, I.M., Manoliu, R.A., Kok, T., Peterse, H., Tilanus-

Linthorst, M.M.A., Muller, S.H., Meijer, S., Oosterwijk, J.C., Beex, L.V.A.M., Tollenaar, R.A.E.M., de Koning, H.J., Rutgers, E.J.T., and Klijn, J.G., for the Magnetic Resonance Imaging Screening Study Group. 2004. Efficacy of MRI and mammography for breast-cancer screening in women with a familial or genetic predisposition. *N. Engl. J. Med. 351*: 427–437.

Lakhani, S.R., Audretsch, W., Cleton-Jensen, A.M., Cutuli, B., Ellis, I., Eusebi, V., Greco, M., Houslton, R.S., Kuhl, C.K., Kurtz, J., Palacios, J., Peterse, H., Rochard, F., and Rutgers, E., on behalf of EUSOMA. 2006. The management of lobular carcinoma in situ (LCIS). Is LCIS the same as ductal carcinoma in situ (DCIS)? *Eur. J. Cancer 42*: 2205–2211.

Lostumbo, L., Carbine, N., Wallace, J., and Ezzo, J. 2004. Prophylactic mastectomy for the prevention of breast cancer. Cochrane. Database. Syst. Rev. Oct 18: CD002748.

Meijers-Heijboer, H., van Geel, B., van Putten, W.L.J., Henzen-Logmans, S.C., Seynaeve, C., Menke-Pluymers, M.B.E., Bartels, C.C.M., Verhoog, L.C., van den Ouweland, A.M.W., Niermeijer, M.F., Brekelmans, C.T.M., and Klijn, J.G.M. 2001. Breast cancer after prophylactic bilateral mastectomy in women with a BRCA1 or BRCA2 mutation. *N. Engl. J. Med. 345*: 159–164.

Metcalfe, K., Lynch, H.T., Chadirian, P., Tung, N., Olivotto, V., Warner, E., Olopade, O.I., Eisen, A., Weber, B., McLennan, J., Sun, P., Foulkes, W.D., and Narod, S.A. 2004. Contralateral breast cancer in BRCA1 and BRCA2 mutation carriers. *J. Clin. Oncol. 22*: 2328–2335.

Page, D.L., and Rogers, L.W. 1992. Combined histologic and cytologic criteria for the diagnosis of mammary atypical ductal hyperplasia. *Hum. Pathol. 23*: 1095–1097.

Pinder, S.E., and Ellis, I.O. 2003. The diagnosis and management of pre-invasive breast disease: ductal carcinoma in situ (DCIS) and atypical ductal hyperplasia (ADH) – current definitions and classification. *Breast Cancer Res. 5*: 254–257.

Rebbeck, T.R., Friebel, T., Lynch, H.T., Neuhausen, S.L., van 't Veer, L., Garber, J.E., Evans, G.R., Narod, S.A., Isaacs, C., Matloff, E., Daly, M.B., Olopade, O.I., and Weber, B.L. 2004. Bilateral prophylactic mastectomy reduces breast cancer risk in BRCA1 and BRCA2 mutation carriers: the PROSE Study Group. *J. Clin. Oncol. 22*: 1055–1062.

van Roosmalen, M.S., Stalmeier, P.F., Verhoef, L.C., Hoogerbrugge, N., and van Daal, W.A.J. 2002. Decision analysis of prophylactic surgery or screening for BRCA1 mutation carriers: a more prominent role for oophorectomy. *J. Clin. Oncol. 20*: 2092–3000.

Rosen, P.P., Braun, D.W., and Kinne, D.W. 1980. The clinical significance of pre-invasive breast carcinoma. *Cancer 46*: 35–51.

Simpson, P.T., Gale, T., Fulford, L.G., Reis, J.S., and Lakhani, S.R. 2003. The diagnosis and management of pre-invasive breast disease: pathology of atypical lobular hyperplasia and lobular carcinoma in situ. *Breast Cancer Res. 5*: 258–262.

Singletary, S.E. 1994. Lobular carcinoma in situ of the breast: 31 year experience at the University of Texas, M.D. Anderson Cancer Center. *Breast Dis. 7*: 157–163.

Sun, C.C., Lenoir, G., Lynch, H., and Narod, S.A. 1996. In-situ breast cancer and BRCA1. *Lancet 348*: 408.

Tavassoli, F.A., and Devilee, P., editors. 2003. World Health Organisation Classification of tumours: pathology and genetics of tumours of the breast and female genital organs. Lyon: IARC Press.

Verhoog, L.C., Brekelmans, C.T., Seynaeve, C., Meijers-Heijboer, E.J., and Klijn, J.G. 2000. Contralateral breast cancer risk is influenced by the age at onset in BRCA1 associated breast cancer. *Br. J. Cancer 83*: 384–386.

Welch, H.G., and Black, W.C. 1997. Using autopsy series to estimate the disease "reservoir" for ductal carcinoma in situ of the breast: how much more breast cancer can we find? *Ann. Intern. Med. 127*: 1023–1028.

7
Differentiation Between Benign and Malignant Papillary Lesions of Breast: Excisional Biopsy or Stereotactic Vacuum-Assisted Biopsy (Methodology)

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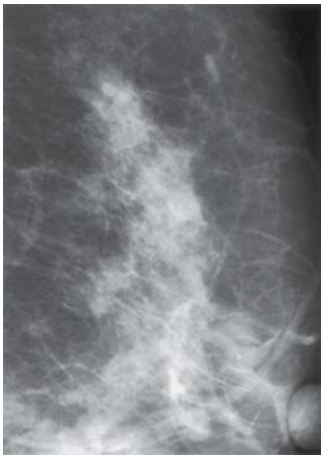
INTRODUCTION

Diagnosis and management of papillary lesions of the breast present a major challenge to the surgeons, radiologists, and pathologists. Papillary lesions of the breast occur in women of all ages with the majority of papillary carcinoma found in the fifth and sixth decades. Malignant papillary lesions are relatively rare, but papillary lesions as a group account for 1–4% of all breast lesions undergoing breast biopsy. The clinical presentation of papillary lesions in the breast is variable, as it may be asymptomatic or may present as a palpable mass with or without nipple discharge. The discharge may be serous, serosanguinous, or bloody.

Microdochectomy, which causes minimal morbidity, remains the mainstay of treatment for benign papillary lesions. As most of the papillary lesions arise from the large ducts, excision in the form of microdochectomy is adequate for benign papilloma. In one large series reported by Burton *et al*. (2003), 36 patients with benign and malignant papillary lesions (33 papillomas and three papillomas with superimposed malignancy or atypical epithelial hyperplasia) were disease free for a significant follow up period after microdochectomy. In the rare instance of papillary carcinoma, which was reported to range from 3% to 6% of all carcinomas, appropriate oncological excision should be pursued. It is, therefore, important to differentiate benign from malignant papillary lesions of the breast, so as to plan the surgery accordingly.

RADIOLOGICAL APPEARANCE OF PAPILLARY LESIONS OF THE BREASTS

Radiologically, the lesion can be small and may be mammographically and sonographically occult (Figure 7.1). The overall sensitivity for detection of papillary lesions on mammography is quite low. Mammographically, they



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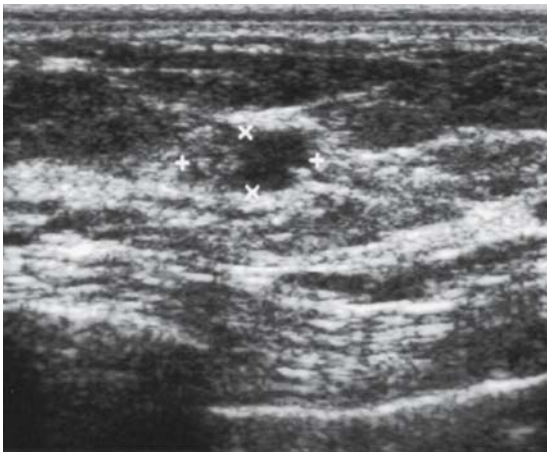

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FIGURE 7.1. (A) Mammography of a 46 year old women presented with bloody nipple discharge shows no definite mass. Microcalcifications of mild pleomorphism are identified. (B) Ultrasound of the same patient shows a small lesion with mixed cystic and solid component. Percutaneous biopsy of the lesion shows papillary carcinoma which is confirmed at surgery

may present as a mass with or without microcalcification, calcification alone, or architectural distortion. The margin of the mass can be circumscribed, indistinct, obscured, or even spiculated. Microcalcification can be pleomorphic, amorphous or punctuate, and the distribution can be clustered, linear, segmental or regional. In patients presenting with nipple discharge, galactography will be useful to confirm the intraductal location of the lesion. Papillary lesions, whether benign or malignant, may present as sessile polypoid filling defects or complete obstruction of a duct. Malignant intraductal papillary growth may cause duct perforation with extravasation of contrast. Ductal ectasia might also be demonstrated on galactography, though ductal ectasia usually occurs late.

As compared to mammography, sonography is more sensitive in detecting papillary lesions. Three main types of intraductal papillary lesions have been detected by sonography. These include intraductal mass with or without ductal dilatation, intracystic mass and a predominantly solid lesion when the mass totally fills the duct and intraductal location of the lesion is then difficult to be determined. Papillary lesions are highly vascular tumors, and on color doppler imaging, flow signal representing the fibrovascular core can be demonstrated (Figure 7.2). Though commonly detected, the absence of the color flow signal does not exclude papillary lesion. Benign papillomas tend to have single vessel while the presence of multiple vessels may suggest a malignant lesion. However, the presence or absence of color flow signal and the number of vessels detected are not sensitive or specific to differentiate benign from malignant lesions. Both benign papillomas and carcinomas may undergo hemorrhagic infarction and no flow can then be demonstrated. Most intracystic papillary lesions appear echogenic and coarse in echo-texture. In women of older age group, the presence of a large solid component and evidence of spontaneous intracystic bleed are more suggestive of papillary carcinomas than benign papillomas. Ultrasound might not

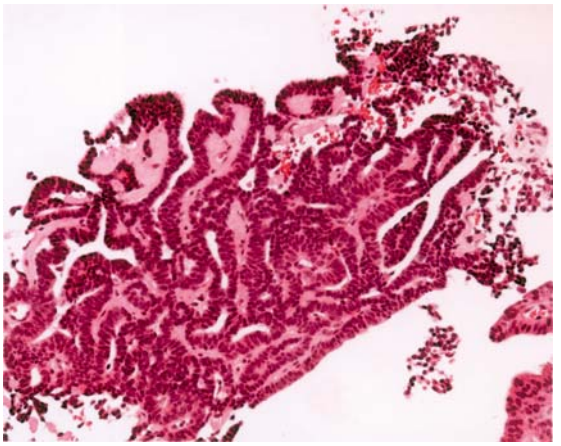

FIGURE 7.2. Needle core biospy of a papillary carcinoma showing elaborate fibrovascular cores that are lined by neoplastic epithelial cells with rather bland morphology and underrepresentation of myoepithelial cells

be able to detect the presence of small lesions, particularly when the ducts are not dilated. Sometimes post galactography ultrasound will detect small intraductal growth not previously demonstrated in simple sonography alone and is useful for ultrasound guided biopsy of the suspicious lesion.

Both mammographically and sonographically, there is significant overlap between benign and malignant papillary lesions, and differentiation between them is difficult. Imaging is neither sensitive nor specific enough for making accurate diagnosis. Lam *et al*. (2006) showed a sensitivity of 61% and a specificity of 33% in diagnosing malignancy when the authors analyzed both the mammography and sonography findings of 65 papillary lesions of the breast. Similar sensitivity and specificity are reported by Puglisi *et al*. (2003), who classified papillary lesions according to their index of suspicion based on imaging findings and correlated the findings of surgical excision with the index of suspicion. Four papillary lesions were confirmed to be malignant out of a total of 11 lesions in the low suspicion group, giving a false-negative rate of 36%. Ten lesions were confirmed to be malignant out of 32 lesions in the moderate suspicion group and five out of eight lesions in the group with high suspicion index. It is therefore a real challenge for the radiologists to differentiate benign from malignant papillary lesions.

PATHOLOGICAL FINDINGS

Papillary lesions of the breast comprise a wide spectrum of benign, atypical, and malignant lesions whose hallmark is the presence of papillary tufts composed only of epithelium or true papillary structures with fibrovascular support and epithelium. The spectrum includes papilloma, sclerosing papilloma, atypical papilloma, carcinoma arising in a papilloma, intraductal papillary carcinoma, and invasive papillary carcinoma. Papillary lesions are classified according to the cellular pattern and the cytological features seen within the papillary epithelial proliferation.

To differentiate benign from malignant papillary lesion can sometimes be a challenge, even to the experienced pathologists. Pathologically, papillary carcinoma consists of complex papillary process that often displays multi-layer epithelial proliferations supported by a fibrovascular stalk. The following criteria for diagnosing of papillary carcinoma have been suggested: the presence of hyperchromatic nuclei, marked nuclear atypia, cribriform pattern, absence of supporting stroma, and a monotonous cell population. The cells

resemble those of intermediate grade ductal carcinoma *in situ*, solid or cribriform type, and have a solid or cribriform architecture. Some lesions consist of sheets of somewhat elongated, hyperchromatic cells or multiple layers of elongated cells covering the papillary fronds. Stromal or vascular invasion indicates an infiltrative component. In practice, many papillary carcinomas appear deceptively benign because of the absence of pleomorphism. Moreover, benign papillomas and papillary carcinoma may coexist in biopsy samples. The diagnosis of papillary carcinoma arising from a papilloma is particularly problematic. These lesions typically retain areas of benign papilloma. It is the presence of cellular proliferation (which might occur in small foci in these lesions) that allows the diagnosis of carcinoma. The presence of myoepithelial cells within a papillary lesion may be helpful for making diagnosis. Tse *et al*. (2006) reported that unlike invasive ductal carcinoma, papillary ductal carcinoma *in situ* may show an attenuated layer rather than total absence of myoepithelial cells. Thus, it is prudent to note that the presence of this layer does not preclude malignancy, whereas its absence is highly suggestive of malignancy. The use of the atypical category, including the terminology of 'atypical papilloma', 'papilloma with atypical duct hyperplasia' in the diagnostic paradigm reflects the difficulty in the differentiation. In the pathology literature, the approach to increase the diagnostic accuracy has been a subject of investigation.

To date, most authors recommend the use of immunohistochemistry, available in most diagnostic laboratories, as a useful adjunct to assist differentiation between benign and malignant papillary lesions. The most commonly recommended markers are myoepithelial markers and epithelial markers. The use of the former is based on the premise that in papillary ductal carcinoma *in situ*, myoepithelial cells may either be absent or present in diminished number, and staining for myoepithelial cells can be useful. In addition, staining for high molecular weight cytokeratin has also been reported to be useful to allow for correct differentiation between benign and malignant papillary lesions. High molecular weight cytokeratin is expressed in the benign or hyperplastic epithelium in the benign papilloma, but not in the neoplastic epithelium of the papillary ductal carcinoma *in situ*. CD44, an adhesion molecule involved in lymphocyte homing, has also been reported by Tse *et al*. (2005) to be useful.

ASSOCIATION OF PAPILLARY LESIONS WITH DUCTAL CARCINOMA

To make the pathologists' life even more difficult, both papillary carcinomas and benign papillomas have been reported to be associated with ductal carcinoma of the breasts. Additional ductal carcinoma *in situ* (DCIS) can be associated with papillary carcinoma at the same quadrant, or it can be found in a different quadrant at the time of mastectomy. There is evidence that incidence of recurrence is tied to the presence of DCIS in adjacent ducts. Therefore, thorough examination of the surrounding breast tissue is important.

Stout (1954) studied benign intraductal papillomas from 125 patients treated in New York Hospital. Carcinoma was subsequently detected in the ipsilateral breast of

7 patients, typically in close proximity to the papilloma diagnosed previously. Ciatto *et al*. (1991) followed up 339 women with benign intraductal papillomas of the breast for an average of 6.6 years and suggested that intraductal papilloma was a precancerous lesion which might slowly progress into cancer. More recent data proposes that there is an increased risk of subsequent malignancy when proliferative epithelial changes are present. The risk of development of subsequent cancer is up to double for women with papillomas as compared with the general female population without papillomas. A slightly increased cancer risk, up to two-folds has also been reported in patients with proliferative breast disease. Other authors found that atypical hyperplasia was the most important factor predicting malignancy. The relative risk of malignancy among patients with papilloma containing atypical hyperplasia has been reported to be 7.5 times higher than those with papilloma without atypia. Other authors reported that the most important predictor was the number of papillomas. Solitary, central papillomas were not associated with a significantly increased risk of subsequent development of invasive breast carcinoma, whereas a significant increase in risk was found in patients with multiple papillomas. According to these studies, the necessity of follow up for patients with papillary lesions of the breasts is therefore obvious.

FINE NEEDLE ASPIRATION

Pathological assessment of papillary lesions of the breast includes cytology assessment i.e., fine needle aspiration and nipple discharge cytology, stereotactic directed biopsy, and excision. The value of fine needle aspiration cytology in the assessment of papillary lesions of the breast remains unclear. In the literature, the results of various reports vary significantly. Gendler *et al*. (2004) analyzed 153 (2%) papillary lesions out of 9,310 image-guided biopsies or image guided aspirates performed. Eighty-seven patients underwent subsequent excision biopsy. Fifty-three (61%) underwent fine needle aspiration with 22-gauge needle. Thirty-four patients (39%) underwent core biopsies with 11 gauge vacuum-assisted biopsies or 14-gauge automated gun. For 11-gauge vacuum-assisted biopsy, 5–15 core biopsy specimens were obtained for each lesion. Thirteen out of 53 (25%) aspirates originally diagnosed as papillary lesion had breast cancer or atypical ductal hyperplasia. Eighteen of 34 core needle biopsies (53%) specimen diagnosed as papillary lesions subsequently had breast cancer or atypical ductal hyperplasia on excision biopsy. From the results, the diagnostic accuracy of an image-guided biopsy was not improved by use of large-core needle biopsy compared with fine needle aspiration. Despite the encouraging results of fine needle aspirates in a few reports, in general, the diagnoses of papillary lesions (papilloma or papillary carcinoma) are usually made with less degree of certainty than most other breast lesions. In one such study reported by Ariga *et al*. (2002), the patients were stratified according to age. In the younger age group with > 200 patients (40 years or younger), two papillomas were diagnosed cytologically as suspicious. One atypical papilloma was diagnosed cytologically as malignant, and this was a false-positive. In the older age group with > 900 patients (41 years or older), four papillomas were diagnosed

cytologically as suspicious and one atypical papilloma was diagnosed cytologically as malignant. The malignant case was also a false-positive. This study fully illustrates the difficulty in establishing the diagnosis of papillary lesion, and more so in the differentiation of benign and malignant papillary lesions in fine needle aspirates. In one series reported by Saad *et al*. (2006), all six such cases reported as malignant at fine needle aspirates were subsequently found to represent false-positives. Much has been written about the cytological criteria, and Mosunjac *et al*. (2000) suggested that immunohistochemical staining with myoepithelial marker calponin in the cell block material might be useful. So far the result is still far from definite, and papillary lesions still account for significant inaccuracies in fine needle aspirate cytology. This is particularly so for variant papillary lesions such as sclerosing papillary lesions. In addition to fine needle aspirates, another less commonly used cytological approach, namely nipple discharge cytology, has been recently reported to be as specific but less sensitive in diagnosing papilloma.

CORE NEEDLE BIOPSY

Although surgical excision is still considered as the gold standard for diagnosis, needle biopsy has its own distinct advantages. Needle biopsies are usually less invasive. The procedure can be performed in shorter period of time and at a lower cost. With the introduction of core needle biopsy since 1980, many centers have replaced fine needle aspiration with core needle biopsy. In general, both stereotactic core needle biopsy and ultrasound guided core biopsy have been reported to have very good accuracy for diagnosis of all breast lesions. However, when one examines the assessment of papillary lesions at core biopsy, the accuracy is less consistent as compared to other breast lesions.

Valdes *et al*. (2006) reviewed 120 biopsies samples from 109 patients with papillary lesions identified at image-guided biopsy. Surgical excision of 80 lesions was available for correlation. Out of these 80 papillary lesions, 33 lesions had biopsy performed with an 11-gauge vacuum-assisted device; 17 with 14-gauge needle and 30 had aspirates collected with a 20- or 22-gauge needle. The original diagnoses of aspirates or core needle biopsy included 36 papilloma or papillomatosis, 28 papillary lesions, nine papillary lesions with atypia and seven lesions with atypical papillomatosis or papilloma with atypical ductal hyperplasia. Malignancy was found in 6 (17%) of the papilloma or papillomatosis, 9 (32%) of the papillary lesions, two (22%) of papillary lesions with atypia and 2 (29%) of the papillomatosis or papillomas with atypical duct hyperplasia. Malignancies was found in 4 (12%) out of 33 stereotactic biopsy papillary lesions, six (35%) of 17 core biopsy papillary lesions and 9 (30%) of 30 fine-needle biopsy papillary lesions. Therefore, malignancy was missed significantly less frequently with stereotactic 11-gauge vacuum-assisted biopsy. It is also important to note that in this study, 15 out of 64 papillary lesions without atypia were found to be associated with malignancy at surgical excision.

Masood *et al*. (2003) correlated surgical result with fine needle aspiration and core needle biopsy in the diagnosis of papillary lesions. A total of 36 papillary lesions

were analyzed. Out of the 36 fine needle aspirates, 21 aspirates were diagnosed as benign, ten as atypical and five as malignant. When the diagnoses of fine needle aspirates were compared with surgical histology, invasive papillary carcinoma was diagnosed in 4 out of 5 cases diagnosed cytologically as malignant. The other case was a micropapillary ductal carcinoma *in situ*. Of the ten cases diagnosed as atypical, surgical excision showed invasive carcinoma in three. Of the 21 benign aspirates, micropapillary DCIS was diagnosed in three cases ultimately. Eleven cases of core needle biopsy were also evaluated, including a total of four atypical papillary lesions, one papillary carcinoma and six benign papillomas. Surgical evaluation showed three additional malignancies. One lesion with original diagnosis of intraductal papilloma was upgraded to DCIS. Two other lesions had original diagnoses of atypical papilloma, one lesion was upgraded to invasive carcinoma and the other one was upgraded to micropapillary ductal carcinoma *in situ*. In this study, the authors concluded that the overall diagnostic accuracy of fine needle aspiration in distinguishing between benign versus malignant papillary lesion was superior to the core needle biopsy.

Apart from false-negative diagnosis, false-positives for core biopsies have also been found. One false-positive was reported by Rosen *et al*. (2002), in which one lesion that was labeled as papillary carcinoma on core biopsy was subsequently diagnosed to be benign papilloma at excision. The accuracy of needle core biopsy can be improved by ancillary tests, particularly immunohistochemical staining techniques. Tse *et al*. (2005) reported that the needle core biopsy have the ability to pick up foci of atypical epithelial proliferation in cases of carcinoma.

CURRENT ROLE OF EXCISIONAL BIOPSY

The indications for excisional biopsy differ in different centers. In view of the difficulty in differentiating benign and malignant papillary lesions based on core needle biopsy, some centers advocate that excisional biopsy should be performed once the biopsy yield papillary lesion. Other authors such as Ivan *et al*. (2004) advocate that excisional biopsy should only be performed when the needle biopsy yield papillary lesion with atypical ductal hyperplasia. Lesions yielding benign papilloma can be managed conservatively with clinical follow up and radiological follow up. Some centers manage the patients by a combined clinical, radiological, and pathological approach. Any patients with core biopsy yielding papillary lesion of the breast will have the core biopsy and the imaging findings reviewed. Experienced radiologists will review all mammography and ultrasound findings. The lesions detected will be categorized according to BI-RADS criteria. For the group of patients with concordant benign imaging and pathological findings, patients will be clinically monitored and followed up with imaging. For patients with concordant malignant imaging and pathological findings, surgical intervention will be planned accordingly. For patients with discordant imaging and pathological findings, surgical biopsy will be performed and the disease management will then be planned accordingly.

DIFFICULTY IN PATHOLOGICAL DIFFERENTIATION BETWEEN MALIGNANT AND BENIGN LESIONS AFTER NEEDLE BIOPSY

After needle biopsy and localization, normal anatomy of the duct wall might be disrupted and dislodgement of the tumor cells into the surrounding stroma mimicking an invasive carcinoma is a concern. This is particularly a problem when the papillary lesion is malignant and the needle tract is not readily apparent in the specimen. The tumor cells in these settings often appear to be loosely floating in the stroma, particularly if the excision biopsy has been performed shortly after the needle insult. The presence of recent or old hemorrhage associated with reactive changes and the absence of a classic pattern of invasion will create difficulty in diagnosis.

VACUUM-ASSISTED BIOPSY

To minimize the risk of under-sampling, vacuum-assisted biopsy probes have gained an additional significant role in breast imaging. Directional vacuum-assisted biopsy instrument is a special device, using vacuum to pull tissue into the probe and removing the specimen without withdrawing the probe each time. It permits directional, contiguous tissue acquisition. The vacuum-assisted biopsy can be performed under either stereotactic guidance or sonographic guidance. The mode of imaging guidance depends on the type of lesions. For most masses, biopsy is performed under ultrasound guidance. For most microcalcification and asymmetrical density, biopsy is usually done under stereotactic guidance.

TECHNIQUE OF STEREOTACTIC VACUUM-ASSISTED BIOPSY

In stereotactic guided biopsy, the patient is instructed to lie on the probe table device. As standard stereotactic localization, a scout and two stereotactic scout views are obtained. The lesion is then targeted. It is best to target at the anterior or posterior aspect of the lesion so that the probe will not obscure the lesion.

Skin is liberally infused with local anesthetic. As recommended by Parker and Klaus (1997), 1% lignocaine mixed with small amount of 8.4% sodium bicarbonate solution in a ratio of 1:9 is used for skin infusion. Sodium bicarbonate is added to neutralize the relative acidic lignocaine and it helps to decrease the stingy sensation. For deep infusion, mixture of 1% lignocaine with epinephrine (1:100,000) is used to decrease blood loss.

A vertical incision of ~ 5 mm is made on the skin. The probe is inserted and advanced manually through the aperture in the 12 o'clock position. For biopsy of calcification cluster, slight change in position of calcification may occur after infusion of anesthetics. This is not a problem in practice as most calcifications still lie within the accessibility of the probe.

The probe is then fired to the designated depth. A single set of stereotactic positioning view is obtained to confirm the correct

positioning of the probe. For thin breast or superficial lesion, the proximal part of the probe aperture may reside outside the breast, resulting in failure of the vacuum suction. Under these circumstances, injection of a large amount of 1% lignocaine into the dermis and subcutaneous layer will help to increase the depth of the compressed breast at the skin entry point. Alternatively, the radiologists can use a short 25-gauge needle as a hook to pull the skin to cover the aperture to ensure good suction force. Tissue is obtained in increments of 45 degrees to achieve contiguous sampling. Direction of sampling depends on the relative position of the probe to the lesion. Multiple samples are obtained, an average of 15 contiguous samples are generally obtained with 11-gauge probe. After tissue sampling, the probe is rotated around the biopsy cavity with vacuum applied and aperture opened. This helps to remove any residual blood or anesthetic; thus, leaving a clean, air-filled cavity. A pair of stereotactic view is then taken, with the probe stayed in place, for the assessment of adequacy of removal of the targeted lesion.

If the post-biopsy stereotactic view shows near or total lesion removal, a percutaneous clip will then be placed to mark the site. This will facilitate post-biopsy localization or therapeutic lumpectomy, if the lesion is proved to be cancerous. It also helps for surveillance of the exact position of previous biopsy. The clips are available in titanium and stainless steel. Clips of stainless steel are preferred in some centers, as they are available at a cheaper cost. Both types of clip do not create significant problem for magnetic resonance imaging. Only a small signal void focus is identified at the site of the stainless steel clip.

TECHNIQUE OF ULTRASOUND-GUIDED VACUUM-ASSISTED BIOPSY

Patient lies supine on the ultrasound couch with the skin prepared in the usual fashion. The skin is anesthetized with 1% lignocaine mixed with bicarbonate. With a 20-gauge spinal needle, ~ 15–20 ml 1% lignocaine with 1/100,000 epinephrine is then injected along the proposed course of the vacuum-assisted biopsy probe. The injection is targeted at the inferior aspect of the targeted lesion, as this will help to lift up the lesion for subsequent probe insertion. A 4 mm incision is made with a scalpel. The probe, preferably attached to an articulating arm, is inserted under continuous sonographic guidance. The probe is best positioned posterior to the lesion so as not to obscure the lesion. Continuous monitoring of the lesion sampling progress in real time is, therefore, possible. The aperture can be clearly delineated and its position is marked by the discontinuity of the anterior wall of the probe and the ring down artifact of the vacuum holes on the opposite site of the aperture. Tissue samples are acquired between 9 and 3 o'clock position, traveling through the 12 o'clock position, under continuous sonographic guidance. If the lesion is superficially located, a generous lignocaine injection is given between the lesion and skin surface, to serve as a safety cushion and to minimize the chance of injury of the skin. If the lesion involves the nipple or nipple areolar complex, biopsy might be taken from the side or above to avoid skin transgression.

ADVANTAGES OF DIRECTIONAL VACUUM-ASSISTED DEVICE

There are multiple factors accounting for improved performance of the directional vacuum-assisted device as compared with the conventional automated gun biopsy needle. These factors include the ease of obtaining a larger number of specimens, greater average specimen weights, a higher percentage of breast tissue (versus blood clot) per specimen, and contiguous (or nearly contiguous) breast tissue acquisition.

An increase in the number of specimens might improve the diagnosis. Lomoschitz *et al*. (2004) found that highest diagnostic yield was achieved when 12 specimens per lesion were obtained, yielding a correct diagnosis in 96% of mass lesions and 92% of microcalcifications. According to their findings, further increasing specimen acquisition did not improve the diagnostic yield. Jackman *et al*. (2001) evaluated > 1,200 lesions and found that in the evaluation of DCIS, the chance of missing the diagnosis would be 17.5% if only ten or less specimens were obtained with a vacuum-assisted biopsy device. The missing rate would then decrease to 11.5% when > ten specimens per lesion were obtained. A large number of specimens per lesion are therefore obtained for every single vacuum-assisted biopsy. During the procedure, the vacuum-assisted biopsy needle is simply inserted into the site of interest. Through rotation of the biopsy knob, multiple tissue samplings can then be obtained with a single needle puncture. Suction helps to pull tissue towards the biopsy cub, the pinpoint accuracy for targeting the biopsy needle at the lesion is therefore not necessary. It is therefore much easier for the operator to acquire the skill as compared to operating an automated biopsy gun. The sampling time by vacuum-assisted biopsy is also much shorter. Parker *et al*. (1996) found a tissue harvesting rate of 14 specimens per minute with vacuum-assisted biopsy. An average of 26.5 specimens per lesion could be obtained in short period of time. On the other hand, it took an average of 17 min for five specimens per lesion when automated needle device was used, giving a tissue harvesting rate of only 0.3 specimens per minute.

The total volume of tissue harvested depends on the total number of specimen obtained and the average weight of each specimen. The utilization of larger caliber needles such as 8-gauge or 11-gauge needle allows harvest of specimen with greater average weight and length. Berg *et al*. (1997) evaluated 14- and 11-gauge directional vacuum-assisted biopsy probes and 14-gauge biopsy guns with a fresh turkey breast parenchyma model. They demonstrated that directional, vacuum-assisted biopsy yielded bigger tissue specimens, up to 36.8 mg per core specimen for the 14-gauge and 94.4 mg per core specimen for the 11-gauge probe, as compared with 14-gauge handheld device with maximum yield of 17.7 mg per core specimen. The specimen length using directional, vacuum-assisted biopsy of 14-gauge was 20.7 mm +/– 3.0 mm, with a mean diameter of 1.9 mm. The corresponding measurements of the specimen yielded by an 11-gauge were 26.9 +/– 7.1 mm and 2.6 mm respectively. With a 14-gauge biopsy gun, the length of the specimens was 6.2–10.1 mm, with a diameter of 1.5–1.9 mm. The specimen lengths were directly correlated to the specimen weights. Specimens obtained

with the directional, vacuum-assisted biopsy probe averaged more than twice as long as those obtained with any of the handheld devices.

Moreover, bleeding is effectively controlled during the procedure. One percent lignocaine with epinephrine is commonly administered through the probe intermittently during the procedure to reduce bleeding. Blood is sucked out with the probe and therefore there is no accumulation of blood at the biopsy site. Vacuum-assisted biopsy specimens are nearly free of blood. Under microscopic observation, blood composed < 14% of the directional vacuum-assisted biopsy specimens, and pathologists could evaluate more tissue from each specimen.

To summarize, vacuum-assisted biopsy provides a much larger tissue volume, which might allow the pathologists to have better assessment of the lesion. As compared to needle biopsy, a lower frequency of imaging-histological discordance will be expected. As reported by Riedl *et al*. (2005), the chance of missing DCIS and atypical ductal hyperplasia was significantly lower as compared with needle sampling; similar findings were also observed by many other authors. Philpotts *et al*. (2000) found that the mammography pathological discrepancy was only 0.85% when mammotome technique was used for obtaining biopsy whereas the incidence was up to 3.4% when automated biopsy gun was used. Liberman *et al*. (1998) reported successful calcification retrieval in 95% of cases with the 11 gauge mammotome. A much lower frequency of underestimation of diagnosis of atypical ductal hyperplasia and DCIS was found. Pfarl *et al*. (2002) found a false-negative rate of 3.5% only when calcified lesions were evaluated and a false-negative rate of 3.0% only was found for malignant mass when biopsy were performed by vacuum-assisted device. Burbank (1997) also confirmed the superiority of the vacuum-assisted biopsy in the assessment of breast lesions.

Vacuum-assisted biopsy specimens can be obtained contiguously (or nearly so). Henceforth, for small lesions, vacuum-assisted biopsy is more than just tissue sampling, it can also be used to achieve radiological excision of the lesions. Complete removal is usually practical in small lesions < 1 cm and only partial removal of lesion is achieved with lesions > 12 mm in maximum diameter. The incidence of complete removal of lesion depends on the size of the lesion, the size of the needle used and when a large number of specimens are taken Jackman *et al*. (1998) reported higher incidence of complete removal of the mammographic lesion when mammotome instead of automated biopsy gun was used. Calcified lesions were removed completely in 7%, 26% and 69% of cases when 14-guage automated biopsy gun, 14-gauge mammotome and 11-gauge mammotome were employed, respectively. Burbank *et al*. (1996) reported 48% complete excision of lesion when the average diameter of the lesion was 9 mm. Liberman *et al*. (1997) reported complete removal of 11 (58%) out of 19 lesions with maximum diameter of 5 mm or smaller.

Similarly, complete removal of cluster of microcalcifications can be achieved by vacuum-assisted biopsy. The incidence of complete excision depends on the size of the needle used, the number of the specimen taken, and the maximum span of the microcalcifications. With a cluster of microcalcification no > 5 mm in span;

complete excision can be achieved in 79% of biopsy.

There are other advantages of using vacuum-assisted biopsy device. The target area can be imaged immediately after the procedure; thus, adequacy of the biopsy can be ensured. A clip can be placed at the biopsy site to mark the area, helping the surgeons for surgical planning if subsequent wire localization excision is necessary. Vacuum-assisted biopsy also allows easier targeting and biopsy at smaller cluster of microcalcifications. Meyer *et al*. (1999) reported a minimum diameter of microcalcification cluster of 5 mm was required if automated gun biopsy was used. When mammotome was used, a minimum diameter of 3 mm would be practically feasible. With vacuum-assisted biopsy, earlier cancer detection and removal of the lesion might therefore be possible. Moreover, with vacuum-assisted biopsy device, only single needle puncture is required. There is less anatomical disruption and less epithelial displacement during breast needling procedures performed by vacuum-assisted device, rendering diagnosis by pathologists less difficult.

Patients with papillary lesions of the breast require close monitoring due to higher chance of subsequent malignancy. Follow up mammography and ultrasound would be difficult if biopsy causes significant scarring and anatomical distortion. Huber *et al*. (2003) reported little mammographical or sonographical alteration of the breast when biopsy was performed by vacuum-assisted device. In this study, only one out of 91 patients showed an area of increased focal density in mammography at 6-month follow up after biopsy performed with vacuum-assisted biopsy.

The incidence of re-biopsy is also lower when mammotome is compared with automated biopsy gun. Philpotts *et al*. (2000) found a re-biopsy (excision) rate of 9% after 11-gauge mammotome biopsy compared with 14.9% after 14-gauge automated gun biopsy. They found significantly fewer cases of insufficient sampling and therefore re-biopsy was less frequently required. This is definitely a significant advantage of vacuum-assisted biopsy as most patients would be very reluctant to go through the same procedure again.

With larger volume of tissue removed and complete radiological excision of the intraductal lesion, biopsy with vacuum-assisted device might achieve symptomatic relief, similar to microdochectomy. Dennis *et al*. (2000) reported alleviation of symptomatic nipple discharge in the majority of the patients with intraductal papillomas when vacuum-assisted biopsy was used. The incidence of symptomatic relief would again depend on the size of the lesion, the number of specimens taken and size of the needle used.

COMPLICATIONS OF VACUUM-ASSISTED BIOPSY

Complications of stereotactic vacuum-assisted breast biopsy are relatively uncommon. Major complications are those expected for any needling procedure, including bleeding, wound bruising, vasovagal reaction. With the use of a relatively large needle, post-biopsy site often shows various degrees of hematoma and ecchymosis. Significant ecchymosis (defined as the maximum diameter of ecchymosis greater than 5 cm) was encountered in 4.3% of patients. As reported by Dennis *et al*. (2000), although clinically it might

be alarming to the patient, significant ecchymosis was usually self limiting and required no intervention. In less than 1% of the patients, development of significant hematoma caused a delay in operating time. Yet, most of the hematoma in patients suffering from this complication will resolve gradually and the majority of these patients do not require surgical evacuation of the hematoma. Post-biopsy discomfort occurs in 5.4% of the patients, prescription of anti-inflammatory analgesic is not usually required. Wound infection is infrequently encountered, in < 0.1% of the patients and usually these patients do not require antibiotic treatment.

CURRENT EXPERIENCE OF VACUUM-ASSISTED BIOPSY AND PERCUTANEOUS CORE BIOPSY IN THE MANAGEMENT OF PAPILLARY LESIONS

In the past most percutaneous needle biopsy diagnosis refers to tissue biopsy obtained by percutaneous automated gun. With the advantages of vacuum-assisted biopsy device, more and more centers are now taking breast biopsy with vacuum-assisted biopsy device. Recent data shows the superiority of vacuum-assisted biopsy over automated gun biopsy in assessment of other breast lesions such as ductal carcinoma and carcinoma *in situ*. In principle, one would expect better diagnosis of papillary lesions by vacuum-assisted biopsy device as compared to automated gun needles. With significantly increase in number of tissue cores and tissue weight yielded by vacuum-assisted biopsy needles; one would expect a smaller chance of missing small foci of papillary carcinoma. With almost complete removal of small lesions, pathologists should be more confident in diagnosis of invasiveness of papillary carcinoma. Excision biopsy might therefore be replaced by vacuum-assisted biopsy. Such hypothesis, however, has not been supported by literature due to the relative low incidence of papillary lesions of the breast. When one reviews the current literature, one would realize that most studies are retrospective review of breast biopsies performed in a certain period of time. In most studies, up to a few thousands of breast biopsies are reviewed. Take Liberman *et al*. (2006)'s study as an example, up to 3,864 breast biopsies are reviewed. The yield of papillary lesions, however, is often low, accounting for only 1.9–4% of all breast biopsies. The number of papillary lesions for analysis is therefore small. Studies such as those reported by Liberman *et al*. (1999), Ioffe *et al*. (2000), Mercado *et al*. (2001), Philpotts *et al*. (2000), Rosen *et al*. (2002), Agoff and Lawton (2004), Ivan *et al*. (2004), Gendler *et al*. (2004), Sydnor *et al*. (2006), Liberman *et al*. (2006) and Valdes *et al*. (2006) are considered as large series on the topic of papillary lesions of the breasts. In these studies, reported number of papillary lesions range from 23 to 87. In many other studies, only a few papillary lesions are included in the analysis. With these figures in mind, it is therefore not difficult to understand why most authors tend to report the overall accuracy of diagnosis of percutaneous biopsy, taking both automated guns and vacuum-assisted device as one homogenous entity. It is also very understandable why most authors do not give detail analysis of the false-negative,

false positive and accuracy of individual type of biopsy needle as the very small number does not carry much statistical significance.

Hopefully, one might get the necessary information from individual study and combining the results of all these large and small series, one might then be able to draw some useful conclusion about the accuracy of different needles. Disappointingly, one would find many authors have adopted a combined approach and have reported the overall accuracy of diagnosis of papillary lesion by both automated gun needle and vacuum-assisted biopsy device. In the study by Sydnor *et al*. (2006), a total of 63 papillary lesions were diagnosed by needle biopsies. Out of these 63 lesions, 15 lesions had biopsy performed with a 14 gauge core needle and the remaining 48 lesions had biopsy performed with vacuum-assisted device. They reported an overall 3% risk of missing a malignant process in benign papilloma, without detail breakdown of the missing rate of individual type of needle. Similarly, in the study by Agoff and Lawton (2004) the needle biopsy results of 51 papillary lesions were reported, with the biopsy performed by both vacuum-assisted and non-vacuum-assisted biopsy needles. The number of biopsy performed by different types of biopsy needles were not specified in the article. Data presented in the article is often incomplete and from the figures provided, one cannot dig out the number of lesions diagnosed or missed by individual type of needles. Only a few studies have specified the needles used in each lesion and unfortunately most of these studies consist of small number of papillary lesions, and even smaller number of malignant papillary lesions. This explains the difficulty in assessment of accuracy in differentiation of benign and malignant papillary lesions.

When one reviews the literature on papillary lesions of the breast, it is also important to understand that in many studies, only a portion of the lesions have surgical excision for correlation. Most papillary lesions of the breast are benign at biopsy and surgical excision is very often performed only when there are discordant imaging findings or due to patients' request. Most of the authors would therefore take no interval change in long term mammography follow up as signs of benignity. In most of these studies, these benign papillary lesions diagnosed at biopsy very often have imaging follow up for a period of 14 months to 2 years. As discussed before, Ciatto *et al*. (1991) suggest that intraductal papilloma might be a precancerous lesion which slowly progress into cancer. In their study, the patients had an average follow up of 6.6 years. If the results by Ciatto *et al*. (1991) are taken into consideration, one might then challenge whether no interval change in 14 month or even 2 years mammographic follow up is sufficient to be considered as signs of benignity. One should therefore note that the false positive, false-negative rates and the accuracy quoted in most studies have their own practical limitations and constraints. These issues can be well demonstrated when one takes a close look at any of the large series on this topic. For example, Rosen *et al*. (2002) concluded in their study that papillary lesion could be safely managed with imaging follow up when benign pathology was yielded at biopsy. They drew the conclusion based on the following findings. They had reviewed a total of 1,374 patients with large-core needle

breast biopsy. Fifty-seven papillary lesions were identified (4%), of which 46 papillary lesions were analyzed. Surgical excision was performed in 17 lesions, including four benign papillary lesions, ten atypical papillary lesions and three malignant papillary lesion diagnosed at core biopsy. For three carcinoma diagnosed by core biopsy, surgical excision showed papillary carcinoma *in situ* with no evidence of invasion in two lesions. There was one false positive, of which original diagnosis at core biopsy was invasive ductal carcinoma. The lesion was subsequently diagnosed to be benign papilloma with extensive adjacent apocrine metaplasia. Of the ten lesions with cellular atypia diagnosed either in or adjacent to the papillary lesion undergoing surgical excision, three out of the ten lesions (30%) were upgraded to papillary carcinoma *in situ*. Out of these three lesions, two had the original diagnosis of atypical ductal hyperplasia and the other one was an atypical papilloma. Another one lesion had original diagnosis of benign papilloma with atypical papilloma demonstrated at excision. Imaging follow up was performed in the remaining 29 lesions. All lesions were stable or had decreased in size on follow up. The negative predictive value for core needle biopsy for excluding malignancy was therefore calculated to be 93% in this series.

Many other authors also believe that core needle biopsy is accurate for excluding malignancy of papillary lesions of the breast. In the study reported by Agoff and Lawton (2004), a total of 25 benign papillary lesions and 26 papillary lesions with atypical ductal hyperplasia were retrieved. Out of these 25 benign papillary lesions, 11 had surgical excision. No discordant findings of excision biopsy and core biopsy were found in these 11 lesions. The authors therefore concluded that core needle biopsy was an effective and accurate way to evaluate papillary lesion of the breasts.

Some authors have emphasized the significance of imaging histologic concordance. In Liberman *et al*. (1999)'s study, they identified a total of 34 papillary lesions, accounting for 3% of all biopsies. Out of the 34 papillary lesions, two papillomatosis were found at core needle biopsy, one lesion showed mammographic histologic discordance with a spiculated mass demonstrated on mammography. Surgical biopsy revealed a radial sclerosing lesion and ductal carcinoma *in situ*. The authors therefore concluded that diagnosis by percutaneous core biopsy of benign papillary lesions was accurate when concordant with imaging findings. They also suggested that surgical excision was required only when diagnosis of atypical papillary lesions were yielded at percutaneous biopsy. Similarly, in the study by Mercado *et al*. (2006), the significance of imaging histologic concordance has also been demonstrated in two cases. Mercado *et al*. (2006) reviewed 43 benign papillary lesions in 42 patients diagnosed at core needle biopsy. Excision was performed in seven sclerosing papillomas diagnosed at core needle biopsy. Out of these seven lesions, surgical excision showed four papillomas, one DCIS and two with no residual lesions. The DCIS lesion diagnosed by surgical excision showed discordant imaging and pathology findings. This lesion was classified as BI-RADS category 5 with cluster of pleomorphic microcalcification in linear distribution while core biopsy revealed intraductal papilloma with sclerotic fronds. In the same series, six benign papillary lesions not otherwise specified diagnosed

at core biopsy had excision for correlation. Surgery revealed one papillomatosis, four papillomas and one DCIS. In the papillary lesion subsequently diagnosed to be DCIS, it had mammography classified as BI-RADS category 4 because new calcifications were found in this elderly woman and therefore it was regarded as discordant imaging findings. Many authors from different centers have therefore emphasized histologic pathological discordance as an indication for surgical excision even when benign papillary lesion is yielded at core needle biopsy.

IS EXCISION BIOPSY NEEDED IN ASSESSING PAPILLARY LESIONS OF THE BREAST?

Despite the reassuring results of core biopsy by Liberman *et al*. (1999), Rosen *et al*. (2002) and other authors, some other investigators do not agree with conservative management of benign papillary lesions diagnosed at biopsy. In Ioffe *et al*. (2000)'s study, they reviewed 1,327 core biopsies. Twenty-eight (2.1%) benign papillomas were identified. Eight lesions had surgical excision. Of these eight lesions, two (25%) had adjacent DCIS at excision. Excision of the remaining six cases revealed adjacent atypical ductal hyperplasia in two lesions, adjacent atypical ductal hyperplasia, and lobular carcinoma *in situ* in one lesion and non-atypical benign finding in three lesions. Henceforth, out of these eight lesions, there was a 25% false-negative rate and the remaining six excisions showed high risk lesions. The accuracy of the diagnosis of papillary lesions at core biopsy was therefore questioned. It is important to note that image concordance or discordance has not specifically stated in these eight lesions. One might then question the reasons for performing surgical excision in these eight lesions. If imaging discordance is the indication for these surgical excisions, one might then argue that the findings of this study has no conflicts with the many other studies in that papillary lesions can be safely followed up once biopsy yield benign pathology and no imaging discordance is found. It might appear that different authors hold different views on the management of these lesions based on similar findings. In the study reported by Mercado *et al*. (2006), despite the findings that negative predictive value of core needle biopsy for excluding malignancy among benign papillary lesions of the breast was up to 94%, the authors suggested that papillary lesions diagnosed as benign at biopsy should be surgically excised because a substantial number of lesions were upgraded to atypical ductal hyperplasia and DCIS at excision. In their series, a total of 43 benign papillary lesions diagnosed at core needle biopsy were reviewed. Out of these 43 lesions, surgical excision was available in 36 lesions (84%). A total of eight papillomas with atypical ductal hyperplasia and two DCIS were diagnosed at surgical excision. The two DCIS had suspicious mammographic features. In this series, the findings suggest that discordant pathologic imaging findings will prompt surgical excision and early diagnosis of the two DCIS. However, the cases with atypical ductal hyperplasia would have been missed as these lesions show concordant imaging pathologic findings.

The findings of the study by Liberman *et al*. (2006) also challenge the current management protocol. They reviewed 3,864 percutaneous imaging-guided biopsies. Out of these 3,864 lesions, 50 lesions showed concordant histologic and imaging diagnosis of benign papillary lesions. Thirty-five lesions were analysed. Out of these 35 lesions, 25 had surgical pathology and ten had or a minimum of 2 year mammographic follow up. All 35 lesions had biopsy performed using either 11-gauge vacuum-assisted (n = 20) or 14 gauge automated (n = 15) needles. Twenty lesions had surgical excision due to preference of clinician or patients. The time lapsed between the surgery and the biopsy was 1–15 weeks with a median of 5 weeks. Another five surgical excisions were performed as there was evidence of interval growth in follow up mammography. Of all these 35 lesions, DCIS was ultimately found in four lesions (14%) and node negative invasive cancer was found in one lesion. In 6 (17%) of 35 lesions, surgery revealed high-risk lesions, these included atypical ductal hyperplasia in three, radial scar in two, and lobular carcinoma *in situ* in one. The authors then concluded that surgery revealed up to 14% cancer and 17% high risk lesions in percutaneously diagnosed papillomas. Surgical excision was therefore recommended in papillary lesions even though they had benign concordant imaging-biopsy findings. The findings of this study have major impact on the current belief. In this series, not only high risk lesions have been missed, cancer has also been missed in papillary lesion with concordant benign histologic and imaging findings. One should be reminded that in 1999, Liberman *et al*. have concluded that conservative management of benign papillary lesions diagnosed at core biopsy with concordant imaging findings is safe. Liberman *et al*. have obviously changed their thinking when a larger number of benign papillary lesions with concordant imaging histologic findings are analysed 7 years later.

One might then argue that in both the studies by Liberman *et al*. (2006) and Mercado *et al*. (2006), biopsies were performed with both 14 gauge automated needles and vacuum-assisted biopsy device. With better tissue yields, conservative management of benign papillary lesion diagnosed at vacuum-assisted biopsy device might be safe. Chances of missing atypical ductal hyperplasia might be significantly decreased. Imaging histological concordance might not be an indication for surgical excision anymore if sufficient tissue could be yielded by vacuum-assisted biopsy device and diagnosis of papillary lesions could be made confidently and accurately. Results of preliminary study of Mercado *et al*. (2001) might suggest the role of vacuum-assisted biopsy in the management of the papillary lesion.

Mercado *et al*. (2001) reviewed 734 consecutive stereotactic directional vacuum-assisted biopsies. A total of 36 papillary lesions were detected. All stereotactic biopsies were performed with an 11-gauge directional vacuum biopsy, with an average of eleven core samples (range 6–18 samples). Out of these 36 lesions, ten lesions were excluded from analysis due to incomplete information. Correlation with subsequent surgical excision was available in 20 lesions. The remaining six lesions had follow up by mammography

for a period of up to 2 years. The histopathologic diagnoses of these 26 lesions by vacuum-assisted biopsy included 12 benign lesions (46%), atypia in six lesions (23%) and papillary carcinoma in eight lesions (31%). One benign lesion showed discordant imaging and pathological findings. Excision of this lesion showed atypia with adjacent papillary intraductal carcinoma. At surgical excision, the six atypical lesions revealed atypical ductal hyperplasia in three cases, atypical papilloma in one case and papilloma with adjacent focus of atypical ductal hyperplasia in one case. For the sixth case, the lesion was completely removed by vacuum-assisted device and residual lesion could not be identified at surgical excision. For all eight malignant lesions, the diagnoses were confirmed by surgical excision. In addition, two of the eight lesions also revealed concomitant invasive ductal carcinoma. This is the first article in which all papillary lesions had biopsy performed with stereotactic directional vacuum-assisted device. The authors concluded that both benign and malignant papillary lesions of the breast could be reliably diagnosed at vacuum-assisted biopsy, though the extent of malignant papillary lesion might be underestimated. It should be noted that papillary intraductal carcinoma was ultimately diagnosed for the only case with discordant imaging and pathological findings. From the very preliminary experience, it would therefore appear that imaging pathologic concordance remains an important concept in the management of papillary lesion of the breast, even when the biopsy was performed with a vacuum-assisted biopsy device.

The findings of the study by Irfan and Brem (2002) might be in conflict to that by Mercado *et al*. (2001). Their study, however, consisted of only a very small number of papillary lesions. Irfan and Brem (2002) reviewed 212 stereotactic vacuum-assisted biopsies. Out of these 212 biopsies, six (2.8%) demonstrated intraductal papilloma. Out of these six lesions, one was reported to have features suggestive of radial scar. The remaining five lesions were diagnosed to have no evidence of atypia or unusual histological features. Only three of these six lesions were surgically excised. One lesion showed fibrocystic change with a radial scar, one showed benign fibroglandular tissue, the other one showed foci of atypical ductal hyperplasia. Therefore, in such a small series, despite the fact that surgical excision was only available in three cases, one atypical ductal hyperplasia was diagnosed at surgical excision. When one reviewed the technique of biopsy in detail, all biopsies were performed with an 8 gauge vacuum-assisted probe with 11–17 core samples taken per lesion. That means that in this small series, all biopsies were taken with an extremely large gauge vacuum-assisted probe. With 11–17 cores taken per lesion, it would be usually regarded as adequate. Henceforth, in this very small series, upgrading of the benign papillary lesion to high risk lesion at surgical excision has been demonstrated despite adequate tissue sampling by vacuum-assisted biopsy device.

These studies have obviously brought new impact and challenges to current thinking and management of papillary lesions of the breast. Based on the limited data available in current literature, the role of excision biopsy when vacuum assisted biopsy yields atypical papilloma and benign papillary is explored in the following discussion.

ATYPICAL PAPILLOMA/ PAPILLARY LESION WITH ATYPICAL DUCTAL HYPERPLASIA AT VACUUM-ASSISTED BIOPSY

Atypical papilloma should be regarded as high risk lesion. Analysis of the series reported by Ciatto *et al*. (1991) and Czernobilsky (1967) shows a high proportion of atypical papilloma and papilloma with atypical duct hyperplasia (ranging from 30% to 70%) have final diagnosis of malignancy. Review and summary of the current literature has been performed. In the literature search, a total of 114 atypical papilloma/papillary lesions with atypical ductal hyperplasia diagnosed at either 14-gague large core technique or 14- or 11-gauge vacuum technique are found to have surgical excision correlation. The results of these lesions are tabulated (Table 7.1). Taking all the findings of these studies together, carcinoma is subsequently diagnosed on surgical excision in 46 lesions, including five invasive carcinomas. The rate of underestimation among these studies is therefore up to 40.4%. It is therefore clear from this analysis that atypical papilloma is associated with a very high risk of malignancy.

As discussed before, details of the needle used in each of these lesions were not indicated in many of these studies, the rate of underestimation by vacuum assisted biopsy device cannot be assessed. In the study by Mercado *et al*. (2001) no upgrade at surgery for the six atypical papillomas diagnosed at vacuum-assisted biopsy were reported. However, from other articles, sporadic cases of underestimation of malignancy by vacuum-assisted device could be identified. In the series reported by Liberman *et al*. (1999), one case diagnosed as papilloma with atypical ductal hyperplasia had specimens taken by a 14 gauge vacuum-assisted biopsy was subsequently upgraded to DCIS at surgery. In the series reported by Philpotts *et al*. (2000), out of 15 atypical papilloma diagnosed by 11 gauge vacuum-assisted biopsy, four lesions were upgraded, the incidence of upgrade was therefore up to 26.7% in this series. In the study reported by Rosen *et al*. (2002), two out of eight atypical papilloma sampled by 11 gauge probe were upgraded to ductal

TABLE 7.1. Number of malignancy missed in atypical papilloma/papillary lesions with atypical ductal hyperplasia diagnosed by percutaneous core biopsy.

Study	Needle gauge (11G- vacuum-assisted, 14G-automated gun or not specified)	Number of lesions diagnosed to be atypical papilloma/papillary lesions with atypical ductal hyperplasia by percutaneous biopsy	Number of lesions diagnosed to be malignant by surgical excision (%)
Ivan *et al*. (2004)	Various include 11G/14G, 16,18,20G	8	5 (63%)
Syndor *et al*. (2006)	11G/14G	15	10 (67%)
Agoff *et al*. (2004)	11G/14G	26	12 (46%)
Mercado *et al*. (2001)	11G	6	0 (0%)
Philpotts *et al*. (1999)	11G	15	4 (26.7%)
Liberman *et al*. (1999)	11G/14G	10	3 (30%)
Rosen *et al*. (2002)	11G/14G	10	3 (30%)
Ioffe *et al*. (2000)	Not specified	3	3 (100%)
Rajendiran *et al*. (2001)	Not specified	10	2 (20%)
Renshaw *et al*. (2004)	11G/14G	7	2 (28.6%)
Masood *et al*. (2003)	Not specified	4	2 (50%)
Total		114	46 (40.4%)

carcinoma *in situ*, the incidence of upgrade was therefore up to 25% in this series. In the series reported by Sydnor *et al*. (2006), a total of 15 atypical papillomas were diagnosed at core biopsy performed by either a 14 gauge core needle or an 11 or 14 gauge vacuum-assisted device. Ten out of these 15 lesions were malignant at excision biopsy. Details of the needle type of these 15 atypical papillomas were not given. However, as stated in their results, they found no significant relationship between the type of needle and the presence of malignancy at excision. Therefore it was reasonable to presume that a significant percentage of these 15 atypical papillomas had biopsy performed with an 11gauge vacuum-assisted device. When only the results of Mercado *et al*. (2001), Rosen *et al*. (2002), Philpotts *et al*. (2000) and Liberman *et al*. (1999) are considered, correlation of surgical excision with a total of 30 atypical papilloma diagnosed at vacuum-assisted device is available. Seven out of these lesions were finally upgraded to malignancy. The missing rate of malignancy was therefore up to 23.3%. Despite a small total number of lesions available for analysis is small, a high incidence of missing malignancy has been found. From the results of this preliminary analysis, it is clear that it is unsafe not to perform surgical excision when atypical papilloma is yielded at biopsy, even if the biopsy is performed by vacuum-assisted device and adequate tissue sampling has been performed.

BENIGN PAPILLOMA AT VACUUM-ASSISTED BIOPSY

The management of the benign papillary lesion is more controversial. As discussed above, many investigators and clinicians think that the incidence of malignancy among papillary lesion is low. Once a core biopsy has diagnosed benign papillary lesion and especially if there is concordant imaging findings, it might not be cost-effective to perform surgical excision. The findings of Gendler *et al*. (2004)'s study obviously does not support such thinking. In their study, the upgrading rate can be as high as 52.9%. Gendler *et al*. (2004) evaluated 87 papillary lesions with excision biopsy. Out of these 87 lesions, 34 benign papillomas had biopsy with either an 11 gauge vacuum-assisted device or a 14 gauge biopsy needle. The remaining 53 lesions had image guided fine needle aspiration. When only core needle biopsy was considered, a total of 18 lesions diagnosed as papillary lesions subsequently were upgraded to breast cancer or atypical ductal hyperplasia at excision biopsy. Rather than relying on just one study, a review of the current literature on benign papilloma and analysis of the results available from major studies would provide a clearer picture of the outcome of benign papillary lesion diagnosed at biopsy.

In the following analysis, sclerosing papilloma and benign non-specified papillary lesions have been excluded. The results and summary of the major studies involving benign papilloma diagnosed at vacuum- assisted/core needle biopsy and the number of malignancy missed are tabulated (Table 7.2). As discussed above, a significant portion of the benign papilloma yielded at biopsy has mammographic follow up. Surgical excision is only carried out if the sample is proved to be insufficient, due to pathologists' or patients' recommendation or preference, or when there are discordant imaging findings. Out of 322 benign papillary lesions diagnosed

TABLE 7.2. Number of malignancy missed in benign papilloma diagnosed by percutaenous biopsy (FNA: fine needle aspiration, G: gauge, NS: not specified, VA: vacuum-assisted device, A: automated gun).

				No. of papillomas with	
Study	Needle gauge type of biopsy	Number of malignant	Number of papillary lesions with follow-up	Surgical follow-up	Mammography follow up
Liberman *et al*. (1999)	14G A 11G VA	0	7	4	3
Ioffe *et al*. (2000)	NS	2	18	8	10
Mercado *et al*. (2006)	11G VA	1	12	6	6
Rosen *et al*. (2002)	14G A 14G VA 11G VA	0	27	4	23
Masood *et al*. (2003)	NS	1	6	6	0
Irfan and Brem (2002)	8G VA	0	6	3	3
Valdes *et al*. (2006)	14G A 11G VA	6 4	17 33	50	0
Philpotts *et al*. (2000)	14G A 11G VA	1	12	12	0
Sydnor *et al*. (2006)	A (unknown G) VA (unknown G)	3	43	18	25
Ivan *et al*. (2004)	Variable	0	30	6	24
Agoff and Lawton (2004)	14G A 11G VA 9G VA	0	16	11	5
Renshaw *et al*. (2004)	11G A 14G A	0	18	18	0
Gendler *et al*. (2004)	14G A 11G VA	2	13	13	0
Mercado *et al*. (2006)	14G A 11G VA	0	29	23	6
Liberman *et al*. (1998)	14G A 11G VA	5	35	25	10
Total		25 (12.1%)	322	207	115

at core biopsy performed by both automated gun and vacuum-assisted device, 25 lesions ultimately demonstrated malignancy, accounting for 7.8% of all benign papilloma. The incidence of malignancy among papilloma is not low, especially when one considers the fact that all these lesions have benign papilloma diagnosed at core needle biopsy. Naturally, one would then raise the question, whether vacuum-assisted biopsy can replace surgical excision in the management of benign papilloma diagnosed at biopsy. Valdes *et al*. (2006) reported a total of 120 papillary lesions diagnosed by different types of biopsy needles. Out of these 120 papillary lesions, 80 lesions underwent surgical excision. Sixteen lesions were diagnosed as atypical papilloma and the remaining 64 lesions were diagnosed as papilloma/papillomatosis or pure papillary lesions. Out of these 64 lesions, a total of 15 malignancies were found at surgical excision. The missing rate of malignancy in benign papillary lesion was up to 23.4% in this series. In this series, details of types of needles used in 64 lesions were not given. However, when all 80 papillary lesions were taken as a group, the investigators found that vacuum-assisted device had a lower incidence of missing malignant lesions as compared with fine needle aspiration and ultrasound guided 14G core biopsy needles. In the series reported by Liberman *et al*. (1999),

out of the seven benign papillomas diagnosed at percutaneous biopsy, only one case had the biopsy performed by 11 gauge vacuum-assisted device and surgery confirmed benign pathology in that case. With the apparently encouraging results of vacuum-assisted device in management of benign papilloma, it is important to note that 23.4% quoted in Valdes *et al*. (2006)'s study refers to missing rate of malignancy, the upgrading rate of benign papilloma to atypical ductal hyperplasia or other high risk lesions are not given in this series. Missing malignancy is obviously unacceptable, it is also important to know the chance of missing high risk lesion such as atypical ductal hyperplasia before a logical and cost effective management protocol could be established. As discussed before, in Irfan and Brem (2002)'s study, the authors reported six intraductal papillomas with biopsy performed by an 8 gauge vacuum- assisted biopsy. Out of the three intraductal papillomas with surgical excision, one lesion had demonstrated atypical ductal hyperplasia at surgery.

The series reported by Liberman *et al*. (2006) obviously gives much impact to the management of benign papillary lesion. In this study, a total of 35 papillary lesions with concordant pathologic imaging findings were analysed. Out of these 35 lesions, 15 lesions had biopsy taken with 14 gauge automated gun, the remaining 20 lesions had biopsy taken with 11gauge vacuum-assisted probe. With these 20 lesions, two lesions were upgraded from papilloma at core biopsy to DCIS after excision. One lesion had 29 cores of tissue obtained and the other lesion had 14 cores of tissue obtained. The number of cores taken would again be considered as adequate. In the series reported by Mercado *et al*. (2006) study, a total of 17 lesions had biopsy taken with an 11 gauge vacuum-assisted device. Out of these 17 lesions, one lesion showing benign papilloma not otherwise specified at biopsy was upgraded to DCIS after surgical excision. For this lesion, nine cores of tissue were taken. This lesion had surgical excision because mammographically the lesion was classified as BIRADS category 5.

Without knowing the total number of benign papillomas diagnosed at vacuum-assisted device in these studies, one cannot calculate the missing rate of malignancy or the missing rate of high risk lesions. It is however clear that carcinoma is still being mis-diagnosed in cases of benign papilloma diagnosed at 11 gauge vacuum-assisted biopsy. In the study by Liberman *et al*. (2006) no statistically significant difference in the likelihood of missing cancer as a function of percutaneous biopsy method was found. This study is extremely important because it shows that benign concordant imaging biopsy findings do not preclude malignancy, even if the biopsy is performed with 11 gauge vacuum-assisted device.

Moreover, in the study by Liberman *et al*., six high risk lesions including three atypical ductal hyperplasia, two radial scars and one lobular carcinoma *in situ* were identified at surgical excision, accounting for an incidence of 17.1% of all 35 benign papillomas diagnosed at biopsy. The number of high risk lesion missed by vacuum-assisted biopsy was not stated in the study. However, from the incidence of missing cancer in the same study, one would expect similar missing rate for high risk lesions. The presence of such high risk lesion may alter the management of the patients. More intensive surveillance

and consideration of risk reducing measures such as tamoxifen therapy might be required. Some authors consider atypical ductal hyperplasia in or around a papilloma as pre-malignant, early removal of the papilloma with atypical ductal hyperplasia might therefore decrease the likelihood of development of subsequent breast cancer. Following the same line of thought and taking one step further, if there is a high incidence of high risk lesions among benign papillomas, early surgical excision of benign papillomas might be justified. Whether the benign papilloma shows concordant imaging findings or not might not be important, as concordant imaging findings do not predict the presence of high risk lesion, as suggested by Mercado *et al*. (2006). A low incidence of cancer at surgery will be expected for such practice, but failure to excise the papilloma may allow evolution of premalignant change to cancer development.

In conclusion, vacuum-assisted biopsy has proved itself to be advantageous over core needle biopsy in the diagnosis of various malignant and precancerous breasts lesions such as DCIS and atypical ductal hyperplasia. In the management of the papillary lesion, the role of vacuum-assisted biopsy is less certain. Current literature shows that cancer and high risk lesions are still missed in biopsy taken with vacuum-assisted device. Atypical papillomas or papillary lesions with unusual histologic findings at vacuum-assisted device biopsy still warrant surgical excision. The role of surgical excision with vacuum-assisted device in benign papillomas is less certain and larger data set is required for a definite answer. With relative small number of lesions managed in each center, multicenter collaboration is required so that critical analysis of data is possible for setting up a cost effective management protocol for these patients.

REFERENCES

Agoff, S.N., and Lawton, T.J. 2004. Papillary lesions of the breast with and without atypical ductal hyperplasia. *Am. J. Clin. Pathol. 122*: 440–443.

Ariga, R., Bloom, K., Reddy, V.B., Kluskens, L., Francescatti, D., Dowlat, K., and Siziopikou, P. 2002. Gattuso P. Fine-needle aspiration of clinically suspicious palpable breast masses with histopathologic correlation. *Am. J. Surg. 184*: 410–413.

Berg, W.A., Krebs, T.L., Campassi, C., Magder, L.S., and Sun, C. C. 1997. Evaluation of 14- and 11-gauge directional, vacuum-assisted biopsy probes and 14-gauge biopsy guns in a breast parenchymal model. *Radiology 205*: 203–208.

Burbank, F., Parker, S., and Fogarty, T. 1996. Stereotactic breast biopsy: improved tissue harvesting with the Mammotome. *Am. Surg. 62*: 738–744.

Burbank, F. 1997. Stereotactic breast biopsy: comparison of 14- and 11-gauge mammotome probe performance and complication rates. *Am. Surg. 63*: 988–995.

Burton, S., Li, W.Y., Himpson, R., Sulieman, S., and Ball, A. 2003. Microdochectomy in women aged over 50 years. *Ann. R. Coll. Surg. Engl. 85*: 47–49.

Ciatto, S., Andreoli, C., Cirillo, A., Bonardi, R., Bianchi, S., Santoro, G., Farante, G., Magni, A., Campa, T., and Costa, A. 1991. The risk of breast cancer subsequent to histologic diagnosis of benign intraductal papilloma: follow-up study of 339 cases. *Tumori 77*: 41–43.

Czernobilsky, B. 1967. Intracystic carcinoma of the female breast. *Surg. Gynecol. Obstet. 124*: 93–98.

Dennis, M.A., Parker, S., Kaske, T.I., Stavros, A.T., and Camp, J. 2000. Incidental treatment of nipple discharge caused by benign intraductal papilloma through diagnostic mammotome biopsy. *AJR Am. J. Roentgenol. 174*: 1263–1268.

Gendler, L.S., Feldman, S.M., Balassanian, R., Riker, M.A., Frencher, S.K., Whelan, D.B., Anne, S., Gross, J.D., Cohen, J.M., and Boolbol, S.K. 2004. Association of breast cancer with

papillary lesions identified at percutaneous image-guided breast biopsy. *Am. J. Surg. 188*: 365–370.

Huber, S., Wagner, M., Medl, M., and Czembirek, H. 2003. Benign breast lesions: minimally invasive vacuum-assisted biopsy with 11-gauge needles patient acceptance and effect on follow-up imaging findings. *Radiology 226*: 783–790.

Ioffe, O.B., Berg, W.A., and Silverberg, S.G. 2000. Analysis of papillary lesions diagnosed on core needle biopsy of the breast: management implications. [Abstr] *Mod. Pathol. 13*: 23A.

Irfan, K., and Brem, R.F. 2002. Surgical and mammographic follow-up of papillary lesions and atypical lobular hyperplasia diagnosed with stereotactic vacuum-assisted biopsy. *Breast J. 8*: 230–233.

Ivan, D., Selinko, V., Sahin, A.A., Sneige N., and Middleton, L.P. 2004. Accuracy of core needle biopsy diagnosis in assessing papillary breast lesions: histologic predictors of malignancy. *Mod. Pathol. 17*: 165–171.

Jackman, R.J., Burbank, F., Parker, S.H., Evans, W.P.3rd., Lechner, M.C., Richardson, T.R., Smid, A.A., Borofsky, H.B., Lee, C.H., Goldstein, H.M., Schilling, K.J., Wray, A.B., Brem, R.F., Helbich, T.H., Lehrer, D.E., and Adler, S.J. 2001. Stereotactic breast biopsy of nonpalpable lesions: determinants of ductal carcinoma in situ underestimation rates. *Radiology 218*: 497–502.

Jackman, R.J., Marzoni, F.A. Jr., and Nowels, K.W. 1998. Percutaneous removal of benign mammographic lesions: comparison of automated large-core and directional vacuum-assisted biopsy techniques. *AJR Am. J. Roentgenol. 171*: 1325–1350.

Lam, WW., Chu, W.C., Tang, A.P., Tse, G., and Ma, T.K. 2006. Role of radiologic features in the management of papillary lesions of the breast. *AJR Am. J. Roentgenol. 186*: 1322–1327.

Liberman, L., Bracero, N., Vuolo, M.A., Dershaw, D.D., Morris, E.A., Abramson, A.F., and Rosen, P.P. 1999. Percutaneous large-core biopsy of papillary breast lesions. *AJR Am. J. Roentgenol. 172*: 331–337.

Liberman, L., Hann, L.E., Dershaw, D.D., Morris, E.A., Abramson, A.F., and Rosen, P.P. 1997. Mammographic findings after stereotactic 14-gauge vacuum biopsy. *Radiology 203*: 343–347.

Liberman, L., Smolkin, J.H., Dershaw, D.D., Morris, E.A., Abramson, A.F., and Rosen, P.P. 1998. Calcification retrieval at stereotactic, 11-gauge, directional, vacuum-assisted breast biopsy. *Radiology 208*: 251–260.

Liberman, L., Tornos, C., Huzjan, R., Bartella, L., Morris, E.A., and Dershaw, D.D. 2006. Is surgical excision warranted after benign, concordant diagnosis of papilloma at percutaneous breast biopsy? *AJR Am. J. Roentgenol. 186*: 1328–1334.

Lomoschitz, F.M., Helbich, T.H., Rudas, M., Pfarl, G., Linnau, K.F., Stadler, A., and Jackman, R.J. 2004. Stereotactic 11-gauge vacuum-assisted breast biopsy: influence of number of specimens on diagnostic accuracy. *Radiology 232*: 897–903.

Masood, S., Loya, A., and Khalbuss, W. 2003. Is core needle biopsy superior to fine-needle aspiration biopsy in the diagnosis of papillary breast lesions? *Diagn. Cytopathol. 28*: 329–334.

Mercado, C. L., Hamele-Bena D, Singer, C., Koenigsberg, T., Pile-Spellman, E., Higgins, H., and Smith, S.J. 2001. Papillary lesions of the breast: evaluation with stereotactic directional vacuum-assisted biopsy. *Radiology 221*: 650–655.

Mercado, C.L., Hamele-Bena, D., Oken, S.M., Singer, C.I., and Cangiarella, J. 2006. Papillary lesions of the breast at percutaneous core-needle biopsy. *Radiology 238*: 801–808.

Meyer, J.E., Smith, D.N., Lester, S.C., Kaelin, C., DiPiro, P.J., Denison, C.M., Christian, R.L., Harvey, S.C., Selland, D.L., and Durfee, S.M. 1999. Large-core needle biopsy of nonpalpable breast lesions. *JAMA. 281*: 1638–1641.

Mosunjac, M.B., Lewis, M.M., Lawson, D., and Cohen, C. 2000. Use of a novel marker, calponin, for myoepithelial cells in fine-needle aspirates of papillary breast lesions. *Diagn. Cytopathol. 23*: 151–155.

Parker, S.H., Dennis, M.K., Stavros, A.T., and Johnson, K.K. 1996. Ultrasound-guided mammotomy: a new breast biopsy technique. *J. Diagn. Med. Sonogr. 12*: 113–118.

Parker, S.H., and Klaus, A.J. 1997. Performing a breast biopsy with a directional, vacuum-assisted biopsy instrument. *RadioGraphics 17*: 1233–1252.

Pfarl, G., Helbich T.H., Riedl, C.C., Wagner, T., Gnant, M., Rudas, M., and Liberman, L.

2002. Stereotactic 11-gauge vacuum-assisted breast biopsy: a validation study. *AJR Am. J. Roentgenol. 179*: 1503–1507.

Philpotts, L.E, Lee, C.H., Horvath, L.J., Lange, R.C., Carter, D., and Tocino, I. 2000. Underestimation of breast cancer with 11-gauge vacuum suction biopsy. *AJR Am. J. Roentgenol. 175*: 1047–1050.

Philpotts, L.E., Shaheen, N.A., Jain, K.S., Carter, D., and Lee, C.H. 2000. Uncommon high-risk lesions of the breast diagnosed at stereotactic core-needle biopsy: clinical importance. *Radiology 216*: 831–837.

Puglisi, F., Zuiani, C., Bazzocchi, M., Valent, F., Aprile, G., Pertoldi, B., Minisini, A.M., Cedolini, C., Londero, V., Piga, A., and Di Loreto, C. 2003. Role of mammography, ultrasound and large core biopsy in the diagnostic evaluation of papillary breast lesions. *Oncology 65*: 311–315.

Rajendiran, S., Gupta, D., and Sumkin, J. 2001. Correlation of imaging guided core and excision biopsy of papillary lesion of the breast [Abstract]. *Mod. Pathol. 14*: 34A.

Renshaw, A.A., Derhagopian, R.P., Tizol-Blanco, D.M., and Gould, E.W. 2004. Papillomas and atypical papillomas in breast core needle biopsy specimens: risk of carcinoma in subsequent excision. *Am. J. Clin. Pathol. 122*: 217–221.

Riedl, C.C., Pfarl, G., Memarsadeghi, M., Wagner, T., Fitzal, F., Rudas, M., and Helbich, T.H. 2005. Lesions miss rates and false-negative rates for 1115 consecutive cases of stereotactically guided needle-localized open breast biopsy with long-term follow-up. *Radiology 237*: 847–853.

Rosen, E.L., Bentley, R.C., Baker, J.A., and Soo, M.S. 2002. Imaging-guided core needle biopsy of papillary lesions of the breast. *AJR Am. J. Roentgenol. 179*: 1185–1192.

Rubin, E., Dempsey, P.J., Pile, N.S., Bernreuter, W.K., Urist, M.M., Shumate, C.R., and Maddox, W.A. 1995. Needle-localization biopsy of the breast: impact of a selective core needle biopsy program on yield. *Radiology 195*: 627–631.

Saad, R.S., Kanbour-Shakir, A., Syed, A., and Kanbour, A. 2006. Sclerosing papillary lesion of the breast: a diagnostic pitfall for malignancy in fine needle aspiration biopsy. *Diagn. Cytopathol. 34*: 114–118.

Stout, A.P. 1954. The relationship of benign lesions of the breast to cancer. *J. Natl. Med Assoc. 46*: 375–381.

Sydnor, M.K., Wilson, J.D., Hijaz, T.A., Massey, H.D., and Shaw de Paredes, E.S. 2006. Underestimation of the presence of breast carcinoma in papillary lesions initially diagnosed at core-needle biopsy. *Radiology 242*: 58–62.

Tse, G.M., Tan, P.H., Ma, T.K., Gilks, C.B., Poon, C.S., and Law, B.K. 2005. CD44s is useful in the differentiation of benign and malignant papillary lesions of the breast. *J. Clin. Pathol. 58*: 1185–1188.

Tse, G.M., Tan, P.H., Lui, P.C., Gilks, B., Poon, C.S., Ma, T.K., Law, B.K., and Lam, W.W. 2006. The role of immunohistochemistry for smooth muscle actin, p63, CD10 and CK 14 in the differential diagnosis of papillary lesions of the breast. *J. Clin. Pathol. 12*: [Epub ahead of print]

Valdes, E.K., Tartter, P.I., Genelus-Dominique, E., Guilbaud, D.A., Rosenbaum-Smith, S., and Estabrook, A. 2006. Significance of papillary lesions at percutaneous breast biopsy. *Ann. Surg. Oncol. 13*: 480–482.

8
Multicentric Breast Cancer: Sentinel Node Biopsy as a Diagnostic Tool

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INTRODUCTION

The sentinel lymph node (SN) is the first lymph node in a lymph node basin to drain a primary tumor. This sentinel node can be removed after labeling with blue dye and/or a radiocolloid by limited surgery. Compared to routine axillary lymph node dissection (ALND), sentinel node biopsy causes less morbidity, lower costs, and is more reliable for axillary staging due to multiple section analysis (Haid *et al*., 2002). First described by A. Giuliano 12 years ago, sentinel node biopsy (SNB) has become clinical routine, and the standard of care for axillary staging of breast cancer patients in many centers (Giuliano *et al*., 1994). The 2003 St. Gallen Consensus Panel also accepted it as a diagnostic tool for axillary staging. Many validation studies showed it to be accurate and feasible for unicentric invasive breast cancer (Giuliano *et al*., 1995; Haid *et al*., 2003; Veronesi *et al*., 1999). Accepted indications for SNB, in addition to unicentric breast cancers, are larger ductal carcinomas in situ as well as multifocal cancers up to 3–4 cm in diameter (Lyman *et al*., 2005). Sentinel Node Biopsy continues to be controversial after preoperative chemotherapy, although the evidence is getting stronger in favour of its usefulness (Haid *et al*., 2001; Mamounas *et al*., 2005). Further contraindications are inflammatory breast cancer, palpable axillary lymph nodes, and previous surgery of the breast and axilla.

Most authors considered SNB to be contra-indicated in multicentric breast cancer, because tumors located in different quadrants of the breast were thought to drain into different sentinel nodes. This may result in an inaccurate lymph node staging and high false-negative rates (Veronesi *et al*., 1999). Evidence obtained in the past few years regarding the functional anatomy of the lymphatic drainage of the breast supports the theory that all quadrants of the breast drain into the same lymph node(s) (Borgstein *et al*., 2000; Klimberg *et al*., 1999). But, if SNB proved to be a valid

concept in multicentric breast cancer as in unifocal lesions, this patient group would also benefit from less morbidity and more accurate lymph node staging. In the ASCO Guidelines published in JCO in October 2005, SNB was also rated as an acceptable procedure for multicentric breast cancer, although the available evidence is limited to some small-scale trials (Lyman *et al*., 2005). This rating was supported by our data, which were derived from the largest series reported to date in a multicenter setting (Knauer *et al*., 2006).

TECHNIQUE OF SENTINEL NODE BIOPSY IN MULTICENTRIC BREAST CANCER

In the beginning SNB in unicentric breast cancer was performed after peritumoral (PT) dye or radiocolloid injection. This approach was dictated by the assumption that every lymph node was associated with a specific body region. Estourgie *et al*. (2004) even injected the radioactive tracer directly into the tumor suspecting that tumors in different quadrants drained into different SNs. Because peritumoral injection was technically difficult in nonpalpable breast cancers, a search for alternative application methods began. Borgstein *et al*. (2000) and Klimberg *et al*. (1999) showed that tumors located in different quadrants of the breast drained into the axilla via one subareolar lymphatic plexus connected to communicating superficial and deep lymphatic systems. With intradermal radiocolloid and peritumoral dye injections in the same quadrant or in a different breast quadrant of 119 patients, Nathanson *et al*. (2001) found a statistically identical concordance of blue staining and hot lymph nodes in both groups (95.5% versus 93.9%). These results led to the conclusion that tumors located in different quadrants drained into the same SN.

Multicentric breast cancer is defined as two or more physically separate invasive tumors in two or more breast quadrants as shown in Figure 8.1. Multicentricity of these tumors in our study was suspected from clinical and radiological findings, i.e., mammography and sonography were performed on all patients, completed by MRI in some unclear cases. The diagnosis

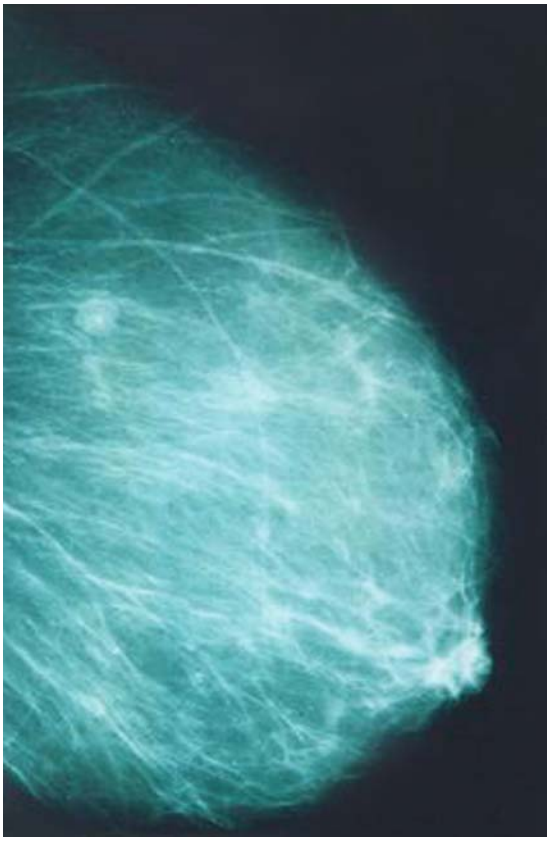

FIGURE 8.1. Multicentric breast cancer with suspicious lesions in different quadrants seen by mammography

of multicentric cancer is confirmed histologically in all patients by fine-needle aspiration or core-needle biopsy prior to surgery or by frozen sections of the tumor specimen intraoperatively. Peritumoral injection of dye and radiocolloid have also been tried for multicentric lesions, but proved to be unfeasible in patients with nonpalpable lesions or produced high false-negative rates (Bergkvist *et al*., 2001). In contrast to the preoperative imaging that is necessary for multiple peritumoral injections, subareolar injection offers a dual advantage: the blue dye or radiocolloid is drained into the axillary lymph nodes rapidly and time is saved. Several studies have established that SA injections were at least equal to PT injections in unicentric breast cancer (Klimberg *et al*., 1999; Reitsamer *et al*., 2003; Smith *et al*., 2000). In an analysis of 307 patients from our institution, SA injections were found to be even superior to PT blue dye injections. We found statistically significant better detection rates of 99% (194 out of 196 patients) for the SA technique versus 90% (100 out of 111 patients) for the PT technique (Knauer *et al*., 2004). All of these studies showed high rates of concordance between subareolar and peritumoral injections suggesting that both techniques detect the same SNs. The feasibility and accuracy of SNB in multicentric breast cancer using subareolar injection techniques was described by some authors (Layeeque *et al*., 2003; Schrenk *et al*., 2001; Tsunoda *et al*., 2002). In our study population of 106 patients with multicentric lesions, the false-negative rates were 3.2% for SA dye or radiocolloid injections versus 4.0% for all patients including varying techniques.

Technique of Lymphatic Mapping

For lymphatic mapping of multicentric breast cancer either vital blue dye as shown in Figure 8.2a (Isosulfan blue 1%, Lymphazurin®, or Patent blue V Guerbet 2.5%), 2–5 ml, or Tc^{99m}-nanocolloid (Nanocoll®), 20–85 MBq, alone or combinations of blue dye and radiocolloid are used. The distribution of different techniques in our study of 142 patients with multicentric cancer is shown in Table 8.1: Radionuclide alone was injected in 7%, blue dye alone in 38% and a combination of both in 55%. Preoperative lymphoscintigraphy should be performed in all patients who have a radiocolloid injected previously. Intraoperatively, sentinel nodes are identified by retrieving blue staining lymph nodes as shown in Figure 8.2b and hot nodes using a handheld gamma camera. The search for SNs

TABLE 8.1. Lymphatic mapping techniques and concordance of blue dye and radiocolloid in 142 patients with multicentric breast cancer.

Lymphatic mapping technique	n (%)
Blue dye total	132
Subareolar	115 (87.1)
Peritumoral	8 (6.1)
Others	9 (6.8)
Radiocolloid total	88
Subareolar	10 (11.4)
Peritumoral	20 (22.7)
Sucutaneous above tumor	53 (60.2)
Others	5 (5.7)
Concordance	
n (blue dye + radiocolloid)	78
Blue nodes and (different) hot nodes	63 (80.8)
"Blue and hot" nodes	53 (67.9)
Blue only	7 (9.0)
Hot only	3 (3.8)
No SN found	5 (6.4)

n: number of patients, SN: sentinel node

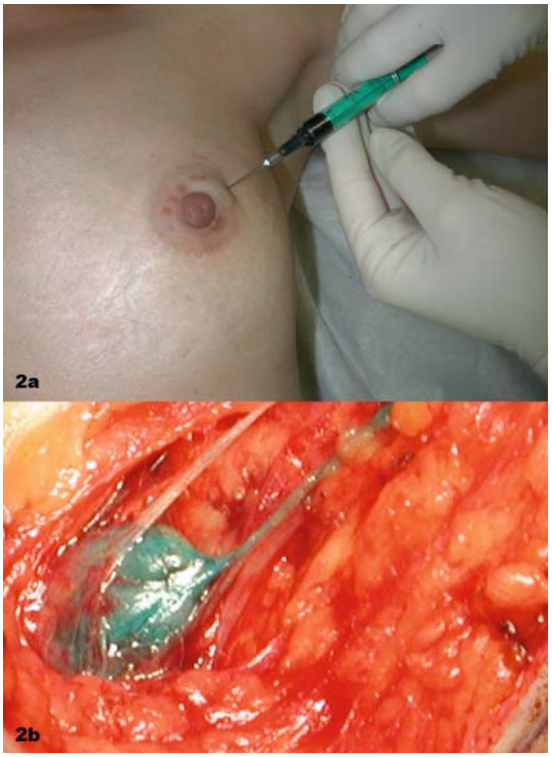

FIGURE 8.2. Subareolar application of blue dye (2a) detects a blue sentinel node (2b)

is completed, when the background activity in the axilla is < 10–30% of the most active SN. Every SNB is followed by visual inspection and palpation for further suspect lymph nodes. Because the impact of extra-axillary SNs is controversial, in our study these nodes were not removed. Levels I and II ALND were completed in 125 of the 142 cases in this study. One participating surgical department in the Austrian Sentinel Node Study Group has started to avoid routine ALND in patients with negative SNB after they had fulfilled the quality criteria of identification and false-negative rate. Sentinel and non-sentinel nodes (NSNs) were separately submitted for histology.

Histologic Evaluation of Sentinel Nodes

As recommended by the Austrian Society of Pathologists (Rudas *et al.*, 2002), sentinel nodes < 5 mm in size are cut in half paracentrally. One half is used for frozen section analysis in three planes, the other is embedded in paraffin for further analysis (sections spaced 200 µm apart). Of SNs ~ 5 mm in size a central slice of 2 mm is used for frozen section analysis in three planes. The remaining material is cut in 2 µm-slices (spaced 200 µm apart) and embedded in paraffin for further analysis. From all slices one specimen is stained with H&E. In all H&E-negative cases immunohistochemical staining is used. All SNs are classified by the UICC criteria.

Surgical Procedures

The standard surgical procedure for multicentric breast cancer is the modified radical mastectomy. Despite this, after completion of SNB, subsequent surgery of the breast cancer in our study consisted of mastectomy in 91% of the 142 patients and breast conserving procedures in 9% (13 selected cases), when assumed reasonable by the surgeon and patients refused to undergo mastectomy.

RESULTS OF THE AUSTRIAN SENTINEL NODE STUDY GROUP

The purpose of our validation study was to confirm the feasibility and accuracy of SNB in multicentric breast cancer. Designed as a multi-institutional trial, it was conducted by the Austrian Sentinel Node Study Group

(ASNSG) and supported by the Austrian Society of Senology (Knauer *et al*., 2006).

Patients and Data

Between 1996 and 2004, 3,730 clinically node-negative patients with operable and biopsy-proven invasive or in situ breast cancer underwent SNB at 15 member hospitals of the Austrian Sentinel Node Study Group (ASNSG). Patient data were entered prospectively in the multicenter database (Microsoft Access®) (Konstantiniuk *et al*., 2001). Regular checks of the entered data were made to assure high data quality and regular postoperative follow-up. One hundred and forty-two patients with multicentric breast cancer prospectively underwent SNB. Their data were compared with those of 3,216 patients with unicentric cancer. The SNB results of the ASNSG in unicentric breast cancer were reported elsewhere (Pichler-Gebhard *et al*., 2002). Further inclusion criteria for the study were a negative axilla by clinical and sonographic evidence and the absence of distant metastases. The mean age of the study patients was 56 years. About 40% of the patients were premenopausal. Detailed patient and tumor characteristics of the study population are shown in Table 8.2. Written informed consent to participate in the lymphatic mapping procedure was obtained from all patients.

In total a number of 142 patients (1 to 44 per participating surgical unit) with multicentric invasive breast cancer on final histopathologic examination fulfilled the criteria for this study. The detection rate in these patients was 91.5% (130 of 142 patients). Intra-operatively, a mean number of 1.67 sentinel nodes (range 1–5 nodes, SD 0.9) was successfully excised. The concordance rate of blue dye and radiocolloid in 78 patients with combined applications was 67.9% for "blue and hot" nodes (Table 8.1).

TABLE 8.2. Demographic and tumor characteristics of study population.

Characteristic	n = 142 (%)
Age (years)	
Mean (SD)	56.6 (13.2)
Range	28–89
Menopausal status	
Premenopausal	58 (40.8)
Postmenopausal	84 (59.2)
Tumor stage	
T1mic	4 (2.8)
T1a	3 (2.1)
T1b	14 (9.9)
T1c	51 (35.9)
T2	63 (44.4)
T3	5 (3.5)
T4b	2 (1.4)
Pathologic tumor size (mm)	
Mean (SD)	21.9 (12.9)
Range	1–70
Tumor histology	
Ductal ± DCIS	110 (77.5)
Lobular	25 (17.6)
Other	4 (2.8)
DCIS + Microinvasion	3 (2.1)
Hormone receptor status	
Positive	128 (90.1)
Negative	14 (9.9)

n: number of patients, yrs: years, SD: standard deviation, mm: millimeter, DCIS: ductal carcinoma in situ

The overall incidence of SN metastases was 60.8% (79/130 patients), which is higher than in unicentric breast cancer. Fifty-one patients (39.2%) had a negative SN. 83.5% out of the positive SNs were found by regular H&E staining, the remaining 16.5% of the metastases were detected by immunohistochemical staining, which was routinely performed in all H&E-negative sentinel nodes. The operation was completed by level I and II axillary dissection in only 125 of the 142 patients, because one department had started to omit routine ALND in patients with negative SNB from January 2003. The mean number

of resected axillary lymphnodes in ALND was 16.1 (4–46 lymph nodes per patient).

In 39.2% (31/79 patients) only the SN harboured metastatic tumor cells, while in the other 60.8% (48/79 patients) other lymph nodes were also involved. We also found tumor cells in axillary lymph nodes of three patients who had a negative SN indicating a false-negative rate of 4%. While the rates of positive SNs and NSNs were significantly higher than in the unicentric breast cancer, there was no statistical difference in detection and false-negative rates. However, there was a non-significant lower false-negative rate of 4.0% versus 6.4% in unicentric cancer. The detailed results of SNB in unicentric and multicentric cancer are shown in Table 8.3.

Sensitivity, negative predictive value and overall accuracy in our study population were 96.0%, 93.3% and 97.3%, respectively. The likelihood ratio for negative test results was 0.04, indicating an excellent test outcome. Subareolar injection of blue dye or radiocolloid appeared to be as effective and accurate as peritumoral application. Consistent with the existing literature (Bauer *et al.*, 2002; Kern, 2002; Klimberg *et al.*, 1999) in 106 patients who underwent SA injection of blue dye or radiocolloid and ALND the acceptable false-negative rate of 4.0% in all patients dropped to 3.1%. The adjuvant treatment after definitive pathologic staging was distributed as follows: Hormonal therapy in 70% of patients, chemotherapy in 48%, adjuvant radiation therapy in another 48%, and immunotherapy with trastuzumab in 3%.

Follow-Up

After a mean follow-up time of 17 months (4–49 months) 17 SNB-negative patients, who were not subjected to axillary dissection, have so far not shown any regional, i.e., axillary recurrence. One patient had to be reoperated because of local relapse after 36 months. Distant metastases have so far been absent and this patient is well at 49 months of follow-up. Of the 34 patients with negative SNs, who underwent ALND, 3 were found to have metastatic NSNs. At a mean follow-up time of 35.4 months (4–66 months) four events were recorded: one local recurrence at 16 months, distant metastases in two patients after 21 and 48 months, respectively, and one patient died after 11 months of

TABLE 8.3. Study results of SNB in multicentric versus unicentric breast cancer, Austrian Sentinel Node Study Group.

	Multicentric cancer	Unicentric cancer	p-value
n	142	3216	
Detection rate	91.5% (130/142)	91.2% (2934/3216)	1.0
Number of SNs	1.67	2.01	< 0.001
Positive SNs	60.8% (79/130)	34.5% (1013/2934)	< 0.001
T1	46.2% (31/67)	27.6% (568/2061)	
T2, T3, T4	76.2% (48/63)	51.6% (444/861)	
Positive NSNs	60.8% (48/79)	38.1% (358/940)[a]	< 0.001
False-negative rate	4.0% (3/75)	6.4% (15/233)[a]	0.58
Sensitivity	96.0% (72/75)	93.6% (218/233)[a]	
Negative predictive value	93.3% (42/45)	97.9% (707/722)[a]	
Overall accuracy	97.3% (110/113)	98.4% (925/940)[a]	

n: number of patients, SN: sentinel node, NSN: non-sentinel node, [a]: ALND not performed in all patients

follow-up. In the largest group of 79 patients (60.8%) with positive SNs, who underwent ALND, 11 events were recorded during a mean follow-up time of 28.5 months (0–73 months). These recurrences consisted of two local recurrences at 19 and 41 months, eight patients with distant metastases after 3–36 months and four patients died after 10–38 months. In summary, after a total follow-up time of 28.8 months none of the women in our study who underwent sentinel node biopsy for multicentric breast cancer developed recurrent axillary disease.

DISCUSSION

Different authors have investigated the feasibility of SNB for multifocal and multicentric lesions with controversial results and conclusions. On one hand Ozmen *et al*. (2002) reported a false-negative rate of 33% in patients with multifocal lesions (4 out of 12 patients) and attributed these to the multifocality. The exclusive peritumoral dye injection without the use of any radiocolloids is likely to have been a contributing factor. On the other hand, Goyal *et al*. (2004) reported a detection rate of 95% and a false-negative rate of 8.8% in 75 patients with multifocal cancer. The authors concluded that SNB was an alternative to routine axillary dissection. The other two studies in this field were carried out by Tousimis *et al*. (2003) and Layeeque *et al*. (2003). They documented the data for 70 and 40 patients, respectively, some of them with multicentric cancer. In view of a detection rate of 97% and a false-negative rate of 8%, the group at the Memorial Sloan-Kettering Cancer Center concluded that the accuracy of SNB was comparable to that in unicentric lesions, but that formal ALND should be performed for all T3 lesions. The Little Rock group reported a detection rate of 100% and 0% false-negative findings and also concluded that subareolar radiocolloid or dye injection may be an alternative to complete ALND. In Table 8.4 the reported studies of SNB in patients with multicentric and multifocal cancer are set against those we recorded in strictly multicentric breast cancer patients. In our study, the mode of dye and radiocolloid application were deliberately left to the involved departments. This explains the multiple methods shown in Table 8.1. Significantly, a strict concordance of "blue and hot" SNs was limited to 68% of the cases with combined injections of a blue dye and a radiocolloid. 81% showed both blue nodes and sometimes different hot nodes, 9% only blue nodes and 4% only hot nodes. We underscore the need for

TABLE 8.4. Studies on SNB in multifocal and multicentric breast cancer.

Source	Goyal, 2004	Touisimis, 2003	Layeeque, 2003	Knauer, 2006
n	75 MF	70: 26 MF, 44 MC	40: 19 MF, 21 MC	142 MC
Detection rate	95% (71/75)	97%	100%	91.6% (130/142)
Number of SNs	2.4	2.7	2.3	1.67
Positive SNs	48% (34/71)	52% (35/67)	63% (25/40)	60.8% (79/130)
Positive NSNs	42% (13/31)	63% (24/38)	28% (7/25)	60.8% (48/79)
False-negative rate	8.8% (3/34)	8% (3/38)	0%	4.1% (3/75)
Sensitivity	91.2%	92%	100%	96.0%
Negative predictive value	92.5%	92%	100%	93.3%

n: number of patients, SN: sentinel node, NSN: non-sentinel node, MF: multifocal cancer, MC: multicentric cancer

combined injections especially in multicentric cancer. This rate of 68% strict concordance is lower than the rates observed in unicentric breast cancer.

In the series of Goyal *et al*. (2004), Tousimis *et al*. (2003), and Layeeque *et al*. (2004) the high rate of tumor-positive SNs and NSNs (48–63% and 28–63%) was a significant finding. Our data confirmed these highly significant differences. This implicates that the actual definition of the tumor size by the largest lesion is unsuited for T staging. Andea *et al*. (2002) proposed that the sum total of the diameters of all lesions would be more accurate for predicting the likelihood of positive SNs in multicentric cancer. In another study, however, the authors computed the sum total of multicentric cancer volumes and concluded that multicentric tumors have distinctly different biologies and that even small lesions tended to metastasize to the lymph nodes (Andea *et al*., 2004).

Clinical Advantage of Sentinel Node Biopsy in Multicentric Breast Cancer

In our patient population the percentage of SN-positive patients turned out to be higher than in unicentric cancer (61% versus 35%). Involvement of the sentinel node was also associated with involvement of other lymph nodes in over 60% of cases versus 38% in unicentric cancer. This implies, on the other hand, that more than every third woman with multicentric cancer benefits from SNB and can at least be spared the morbidity of axillary dissection if not mastectomy.

Quality Control of Sentinel Node Biopsy Procedure

Many different modes of Sentinel Node Biopsy exist in the different centers. This aspect of procedural SNB quality is in need of improvement not only for multicentric breast cancer but also for unicentric cancer. The mode of dye and radionuclide application is a case in point as well as compliance with certain quality criteria (detection rate ~ 95%, FNR < 5%). Collecting patient data in a database such as the one kept by the Austrian Sentinel Node Study Group is an excellent approach because it ensures an ongoing outcome quality control by clearing up the data at regular intervals and monitoring post-SNB patient follow-ups.

The outcome quality of diagnostic SNB should, however, not be confined to high detection rates and low FNRs. The long-term prognosis is equally important. None of the patients, including the 17 without ALND, developed axillary recurrence during the follow-up times listed. However, the follow-up times are yet too short for recommending SNB without ALND for all patients with multicentric breast cancer in every institution. The follow-up times were 35 months for the patients with negative SNs who underwent ALND and 17 months for those with negative SNBs who were spared ALND. Still, the evidence available to date is encouraging. In conclusion, in our opinion multicentric breast cancer constitutes a new indication for SNB only without axillary dissection. Presently, it should still be restricted to controlled trials given a defined interdisciplinary structure, and compliance with the quality criteria addressed above.

Acknowledgements. We thank the Austrian Society of Senology for supporting the study and all the contributing institutions for the continuous high-quality data collection, as follows:

General Hospital Linz, Department of Surgery II

University Medical Center Salzburg, Department of Gynecology
General Hospital Feldkirch, Department of Surgery
Wilhelminenspital, Department of Gynecology, Vienna
Krankenhaus der Barmherzigen Schwestern, Department of Surgery, Linz
Lainz Hospital, Department of Gynecology, Vienna
General Hospital Hainburg, Department of Surgery
General Hospital Klagenfurt, Department of Surgery
General Hospital Kufstein, Department of Surgery
Clinic Chirurgie Tausch, Linz
General Hospital Vienna, Department of Surgery

REFERENCES

Andea, A.A., Wallis, T., Newman, L.A., Bouwman, D., Dey, J., and Visscher, D.W. 2002. Pathologic analysis of tumor size and lymph node status in multifocal/multicentric breast carcinoma. *Cancer 94*: 1383–1390.

Andea, A.A., Bouwman, D., Wallis, T., and Visscher, D.W. 2004. Correlation of tumor volume and surface area with lymph node status in patients with multifocal/multicentric breast carcinoma. *Cancer 100*: 20–27.

Bauer, T.W., Spitz, F.R., Callans, L.S., Alavi, A., Mick, R., Weinstein, S.P., Bedrosian, I., Fraker, D.L., Bauer, T.L., and Czerniecki, B.J. 2002. Subareolar and peritumoral injection identify similar sentinel nodes for breast cancer. *Ann. Surg. Oncol. 9*: 169–176.

Bergkvist, L., Frisell, J., Liljegren, G., Celebioglu, F., Damm, S., and Thorn, M. 2001. Multicentre study of detection and false-negative rates in sentinel node biopsy for breast cancer. *Br. J. Surg. 88*: 1644–1648.

Borgstein, P.J., Meijer, S., Pijpers, R.J., and van Diest, P.J. 2000. Functional lymphatic anatomy for sentinel node biopsy in breast cancer: echoes from the past and the periareolar blue method. *Ann. Surg. 232*: 81–89.

Estourgie, S.H., Nieweg, O.E., Olmos, R.A., Rutgers, E.J., and Kroon, B.B. 2004. Lymphatic drainage patterns from the breast. *Ann. Surg. 239*: 232–237.

Giuliano, A.E., Kirgan, D.M., Guenther, J.M., and Morton, D.L. 1994. Lymphatic mapping and sentinel lymphadenectomy for breast cancer. *Ann. Surg. 220*: 391–398.

Giuliano, A.E., Dale, P.S., Turner, R.R., Morton, D.L., Evans, S.W., and Krasne, D.L. 1995. Improved axillary staging of breast cancer with sentinel lymphadenectomy. *Ann. Surg. 222*: 394–399.

Goyal, A., Newcombe, R.G., Mansel, R.E., Chetty, U., Ell, P., Fallowfield, L., Kissin, M., and Sibbering, M. 2004. Sentinel lymph node biopsy in patients with multifocal breast cancer. *Eur. J. Surg. Oncol. 30*: 475–479.

Haid, A., Tausch, C., Lang, A., Lutz, J., Fritzsche, H., Peschina, W., Breitfellner, G., Sega, W., Aufschnaiter, M., Sturn, H., and Zimmermann, G. 2001. Is sentinel lymph node biopsy reliable and indicated after preoperative chemotherapy in patients with breast carcinoma? *Cancer 92*: 1080–1084.

Haid, A., Koberle-Wuhrer, R., Knauer, M., Burtscher, J., Fritzsche, H., Peschina, W., Jasarevic, Z., Ammann, M., Hergan, K., Sturn, H., and Zimmermann, G. 2002. Morbidity of breast cancer patients following complete axillary dissection or sentinel node biopsy only: a comparative evaluation. *Breast Cancer Res.Treat. 73*: 31–36.

Haid, A., Koeberle-Wuehrer, R., Offner, F., Jasarevic, Z., Fritzsche, H., and Zimmermann, G. 2003. Clinical usefulness and perspectives of sentinel node biopsy in the management of breast cancer. *Chirurg 74*: 657–664.

Kern, K.A. 2002. Concordance and validation study of sentinel lymph node biopsy for breast cancer using subareolar injection of blue dye and technetium 99 m sulfur colloid. *J. Am. Coll. Surg. 195*: 467–475.

Klimberg, V.S., Rubio, I.T., Henry, R., Cowan, C., Colvert, M., and Korourian, S. 1999. Subareolar versus peritumoral injection for location of the sentinel lymph node. *Ann. Surg. 229*: 860–864.

Knauer, M., Haid, A., Köberle-Wührer, R., and Zimmermann, G. 2004. Stellenwert der subareolären Farbstoffinjektion zur Sentinel Node Biopsy: Eigene Erfahrungen und Literaturübersicht. *Eur. Surg. 36*: 41

Knauer, M., Konstantiniuk, P., Haid, A., Wenzl, E., Riegler-Keil, M., Postlberger, S., Reitsamer, R., and Schrenk, P. 2006. Multicentric breast cancer: a new indication for sentinel node biopsy–a multi-institutional validation study. *J. Clin. Oncol. 24*: 3374–3380.

Konstantiniuk, P., Kowatsch, E., Haid, A., Bauer, Ch., and Steindorfer, P. 2001. Das Multicenter Database Project (MCDBP). *Acta Chir. Austr. 33*: 200–201.

Layeeque, R., Henry-Tillman, R., Korourian, S., Kass, R., and Klimberg, V.S. 2003. Subareolar sentinel node biopsy for multiple breast cancers. *Am. J. Surg. 186*: 730–735.

Lyman, G.H., Giuliano, A.E., Somerfield, M.R., Benson, A.B., III, Bodurka, D.C., Burstein, H.J., Cochran, A.J., Cody, H.S., III, Edge, S.B., Galper, S., Hayman, J.A., Kim, T.Y., Perkins, C.L., Podoloff, D.A., Sivasubramaniam, V.H., Turner, R.R., Wahl, R., Weaver, D.L., Wolff, A.C., and Winer, E.P. 2005. American Society of Clinical Oncology guideline recommendations for sentinel lymph node biopsy in early-stage breast cancer. *J. Clin. Oncol. 23*: 7703–7720.

Mamounas, E.P., Brown, A., Anderson, S., Smith, R., Julian, T., Miller, B., Bear, H.D., Caldwell, C.B., Walker, A.P., Mikkelson, W.M., Stauffer, J.S., Robidoux, A., Theoret, H., Soran, A., Fisher, B., Wickerham, D.L., and Wolmark, N. 2005. Sentinel node biopsy after neoadjuvant chemotherapy in breast cancer: results from National Surgical Adjuvant Breast and Bowel Project Protocol B-27. *J. Clin. Oncol. 23*: 2694–2702.

Nathanson, S.D., Wachna, D.L., Gilman, D., Karvelis, K., Havstad, S., and Ferrara, J. 2001. Pathways of lymphatic drainage from the breast. *Ann. Surg. Oncol. 8*: 837–843.

Ozmen, V., Muslumanoglu, M., Cabioglu, N., Tuzlali, S., Ilhan, R., Igci, A., Kecer, M., Bozfakioglu, Y., and Dagoglu, T. 2002. Increased false negative rates in sentinel lymph node biopsies in patients with multi-focal breast cancer. *Breast Cancer Res. Treat. 76*: 237–244.

Pichler-Gebhard, B., Konstantiniuk, P., Tausch, Ch., Jörg, L., Haid, A., Schrenk, P., Peters-Engl, C., and Roka, S. 2002. Factors affecting Identification rate and positivity of the sentinel node in breast cancer in 1567 patients, using blue dye and 99mTc-labelled colloid, based on a multicenter databased project in Austria. *Eur. Surg. 34*: 272–276.

Reitsamer, R., Peintinger, F., Rettenbacher, L., Prokop, E., and Sedlmayer, F. 2003. Subareolar subcutaneous injection of blue dye versus peritumoral injection of technetium-labeled human albumin to identify sentinel lymph nodes in breast cancer patients. *World J. Surg. 27*: 1291–1294.

Rudas, M., Konstantiniuk, P., Horvat, R., Niedermoser, P., Bogner, S., Pichler-Gebhard, B., Hoffmann, B., and Offner, F. 2002. Sentinel lymph node examination: balancing between high workload and low metastasis detection rate. *Eur. Surg. 34*: 277–279.

Schrenk, P. and Wayand, W. 2001. Sentinel-node biopsy in axillary lymph-node staging for patients with multicentric breast cancer. *Lancet 357*: 122

Smith, L.F., Cross, M.J., and Klimberg, V.S. 2000. Subareolar injection is a better technique for sentinel lymph node biopsy. *Am. J. Surg. 180*: 434–437.

Tousimis, E., Van Zee, K.J., Fey, J.V., Hoque, L.W., Tan, L.K., Cody, H.S., III, Borgen, P.I., and Montgomery, L.L. 2003. The accuracy of sentinel lymph node biopsy in multicentric and multifocal invasive breast cancers. *J. Am. Coll. Surg. 197*: 529–535.

Tsunoda, N., Iwata, H., Sarumaru, S., Mizutani, M., Iwase, T., and Miura, S. 2002. Combination of subareolar blue dye and peritumoral RI for sentinel lymph node biopsy. *Breast Cancer 9*: 323–328.

Veronesi, U., Paganelli, G., Viale, G., Galimberti, V., Luini, A., Zurrida, S., Robertson, C., Sacchini, V., Veronesi, P., Orvieto, E., De Cicco, C., Intra, M., Tosi, G., and Scarpa, D. 1999. Sentinel lymph node biopsy and axillary dissection in breast cancer: results in a large series. *J. Natl. Cancer Inst. 91*: 368–373.

9
Breast Cancer Recurrence: Role of Serum Tumor Markers CEA and CA 15-3

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INTRODUCTION

Breast cancer is a heterogeneous, most frequent disease in women. All patients with breast cancer may develop progression or recurrence of the disease, and thus they need an effective lifelong follow-up (Lumachi *et al*., 2001). Breast cancer recurrence is a significant problem for clinicians. Even patients with early stage of the disease (stage I–II) have a recurrence rate of ~ 30%, and local recurrences after conserving therapy have been reported from 6% to 12% at 5 and 10 years, respectively (Temple *et al*., 1999). In spite of advances in the diagnosis and therapy achieved over the past decade, a share of patients with breast cancer still develop relapse and metastases, from which they will ultimately die (Cheung *et al*., 2000).

An early detection and treatment of recurrence may improve the quality of life of patients who have undergone curative surgery and adjunctive therapy, although it does not seem to have a significant impact on long-term survival. In patients with breast cancer, the axillary node status still remains the main prognostic factor, especially in those with early-stage disease, but different factors, enclosing tumor markers serum levels, are available and potentially useful (Lumachi *et al*., 2004).

SERUM TUMOR MARKERS AND BREAST CANCER

Several molecules or substances can be detected in the serum of patients with malignancy: tumor-associated proteins penetrated into the bloodstream, cytokines, stimulating or inhibiting factors, and antibodies against antigenic tumor-associated substances (Stearns *et al*., 1998). Their potential use concerns different aspects, such as determination of cancer risk, screening, diagnosis, prognosis, prediction of response to therapy, and monitoring the course of disease (Stearns *et al*., 1998; Lumachi and Basso, 2004). Serum tumor markers measurements are currently used in clinical practice, because they reflect the dynamic evolution of the disease, and can be easily repeated when required (Lumachi and Basso, 2004). They are currently used in detecting recurrence and monitoring response to therapy, especially in patients with advanced disease, although their sensitivity varies widely and the real value of serial observation of serum tumor

marker levels remains unclear (Bartsch *et al*., 2006).

The most common serum markers used for postoperative monitoring of breast cancer are carcinoembryonic antigen (CEA) and cancer antigen 15-3, although several other markers have been tested. Unfortunately, aspecific elevation of both CEA and cancer antigen 15-3 may be found also in patients with inflammatory (i.e., diverticulitis, bronchitis), autoimmune (i.e., sarcoidosis) and other benign diseases (i.e., hepatitis, cirrhosis, hypothyroidism), in the presence of lung, gastrointestinal or neuroendocrine tumors, as well as in smokers and in the elderly (Lumachi and Basso, 2004; Duffy, 2006).

Breast cancer may still relapse after 15 years or more from surgery, and therefore follow-up of these women should not be stopped. Many strategies of surveillance have been proposed, but an intensive and costly program of periodical use of both imaging techniques (i.e., chest X-ray, liver ultrasound, bone scan, CT-scan) and biochemical assessments has not demonstrated to impact on hard clinical endpoints such as overall survival and quality of life (Khatcheressian *et al*., 2006). Data reviewed by the American Society of Clinical Oncology (ASCO) showed elevated CEA and cancer antigen 15-3 serum levels in 10–64% and 9–75% of women with stage I–IV breast cancer, respectively. The ASCO panel also established and updated clinical practice guidelines for breast cancer management and follow-up, and confirmed the importance of performing regular history, physical examination, and mammography for early detection of breast cancer recurrence. Serum tumor markers measurement was not recommended by the ASCO panel for routine breast cancer follow-up (Khatcheressian *et al*., 2006).

CARCINOEMBRYONIC ANTIGEN

The carcinoembryonic antigen is a glycoprotein discovered in human colon carcinomas in 1965. It has been the most investigated marker in breast cancer and several other malignancies enclosing lung, ovarian, liver, pancreas, and prostate cancer.

Carcinoembryonic antigen is historically the first antigenic glycoprotein studied in breast cancer, and has been considered a reference to which are compared the new serum tumor markers. Carcinoembryonic antigen can be measured by several commercially available kits, both chemiluminescence and radioimmunoassay methods. Usually the cut-off value is 5 ng/ml. Nevertheless, the dichotomous division into a positive-to-negative cut-off is often inadequate and differs between the laboratories. This method does not emphasize the kinetic parameter of CEA, which is known to be particularly important in breast cancer. Moreover, the assay, increasing the range of effective measurement, is inevitably affected by imprecision near the lower detection limit, leading to changes in sensitivity and specificity due to different cut-off limits, which likely cause the variability of results.

Low sensitivity of CEA in both early and advanced breast cancer was shown, when compared with other serum tumor markers. Moreover, measurement of CEA together with other serum markers only lead to a slight increase in sensitivity (Cheung *et al*., 2000). We did not find any significant relationship between preoperative CEA serum

levels and tumor burden, type of recurrence, surgical procedure, and hormone receptor rate (Lumachi *et al*., 2000).

Carcinoembryonic antigen is usually considered a poor predictor of breast cancer recurrence. Elevated CEA serum levels as first sign of recurrence have been observed, but unfortunately the reported sensitivity varies widely, and false-positive results are also found. Its reported sensitivity ranges from 30% to 70% for visceral and bone metastases, with a positive predictive value ranging from 18% to 26%, respectively (Given *et al*., 2000). Circulating anti-CEA antibodies may represent a more sensitive serum tumor marker than CEA, and their presence seems to be associated with improved recurrence-free survival (Haidopoulos *et al*., 2000).

CANCER ANTIGEN 15-3

Cancer antigen 15-3 (CA 15-3) is the most widely used serum tumor marker in patients with breast cancer. The monoclonal antibody of the CA 15-3 immunoassay recognizes an epitope localized in a mucine glycoprotein encoded by the gene MUC1. This protein is normally located on the apical surface of epithelial cells, but is overexpressed in transformed cells and shed into the bloodstream. Members of the same mucine glycoprotein family may be assessed by other tests such as CA 27–29, MCA, and CA 549, but because they gave similar diagnostic sensitivity and specificity, the use of more than one MUC1 antigen is unlikely to confer any advantage (Molina *et al*., 2005).

Due to low sensitivity in the presence of small tumor burden, CA 15-3 has a limited role in the management of early breast cancer, but it is always advisable to perform a preoperative assessment of this marker in order to have a baseline value against which all future results should be compared (Lumachi and Basso, 2004; Duffy, 2006). In a cohort of 255 women with early breast cancer, CEA and CA 15-3 serum levels were above the cut-off in 17% and 29% of the cases, respectively, and the overall sensitivity of Serum tumor markers was 38% (Lumachi *et al*., 2004). In patients receiving neoadjuvant chemotherapy, the persistence of high concentrations of CA 15-3 may be associated not only with poorer clinical and pathological response, but also with increased risk of recurrence (Al-azawi *et al*., 2006).

Many attempts have been made in the past to provide evidence of the ability of CA 15-3 elevation at diagnosis to predict shorter disease-free and overall survival, but results were conflicting, and statistical significance was often lost at multivariate analysis in the available studies (Lumachi *et al*., 2001; Lumachi and Basso, 2004; Duffy, 2006). Therefore, CA 15-3 is not an independent prognostic factor in the prediction of risk of recurrence, and also it has no clinical value in the early detection of local relapse or second tumor because of low sensitivity in the presence of localized disease.

PREDICTION OF RISK OF RECURRENT AND LOCOREGIONAL RELAPSE

Increase of CA 15-3 can be found in 50–70% of patients with advanced breast cancer, especially when the site of relapse is liver or bone, and a rise of serum tumor marker levels may precede radiological alterations in up to 50% of

the cases (Lumachi and Basso, 2004; Molina *et al*., 2005). In a retrospective analysis made by the International Breast Cancer Study Group upon 784 recurrences of breast cancer, only 35% of patients had increase of CA 15-3. The hazard ratio of recurrence in women with elevated serum tumor marker levels was 1.3, but reached 4.7 if a concomitant elevation of alkaline phosphatase was present (Keshaviah *et al*., 2007). The Authors confirmed that treatment was not decided upon CA 15-3 increase, and survival benefit of marker testing remained undeterminable. At the same time, there are reports that metastatic patients who are always serum tumor marker negative may have a favorable prognosis, but data from prospective studies are lacking (Duffy, 2006; Keshaviah *et al*., 2007). Increasing the upper normal value, or considering positive only a serum tumor marker elevation confirmed by two serial measurements, may improve specificity to almost 100%, but then the sensitivity falls below 5% (Molina *et al*., 2005; Nicolini *et al*., 2000).

Potential cost savings of biochemical response evaluation compared to radiological criteria have been suggested. Yet, standardized criteria for response or progression of serum tumor markers are not available, and the psychological distress of patients alarmed by insignificant changes of marker values has to be taken into account (Molina *et al*., 2005). Unfortunately, periodic measurements of serum tumor markers may be influenced by several biases: intra-individual biological variations and analytical inaccuracy of the assay and paradoxical increases due to tumor lysis lasting as long as 90 days since chemotherapy administration or treatment with G-CSF (Molina *et al*., 2005).

MONITORING THE RESPONSE TO THERAPY OF RECURRENCES

Because serum tumor marker levels may reflect the extent of tumor burden both in the localized and in the advanced disease, serial monitoring of CEA and CA 15-3 has been proposed as a surrogate test for tumor response. Moreover, some studies have shown that CA 15-3 changes correlate with response according to radiological and physical examination findings, and that venous puncture is much more advantageous for the patient compared to imaging studies (Duffy, 2006). It has also been advocated that CA 15-3 should be assessed before each chemotherapy cycle in metastatic breast cancer in order to monitor accurately the status of disease, and to enable oncologists to take earlier treatment decisions regarding continuation of effective drugs or suspension of ineffective ones. However, up to 20% of patients with progressive disease do not show a relevant increase in serum tumor markers, and in >30% of those responsive to therapy a reduction of CA 15-3 serum levels is not observed (Lumachi and Basso, 2004).

Unfortunately the assumption that an early change of therapy before radiological or clinical evidence of progression might improve overall survival of patients has not been documented in randomized trials. Moreover, the tumor response may sometimes be achieved after several cycles of chemotherapy, and there is the risk that changing treatment too early deprives patients of active drugs. For all these reasons, the variation of CA 15-3 is not comprised within international response evaluation guidelines, and serum tumor marker measurements are rarely quoted

in phase II or III studies of chemotherapy in advanced breast cancer. However, in patients with bone metastases, neoplastic effusions and irradiated lesions, CA 15-3 may be the only measurable tumor-related parameter.

CONCLUSIONS

The measurement of serum tumor markers may give relevant but not conclusive information in patients with breast cancer. From early studies it was clear that no single blood marker would suffice for diagnosing disease relapse and monitoring the therapeutic response of therapy (Cheung *et al*., 2000). CA 15-3 has a higher sensitivity than CEA, but a similar specificity. However, combining different markers has been shown to be better than any single marker, and the most widely adopted recommendation is the combination of CEA and CA 15-3 (Cheung *et al*., 2000; Duffy, 2006; Lumachi *et al*., 2000).

The idea that earlier diagnosis improves survival is closely dependent on the biological conviction that the smaller the tumor, the more likely will be the response to systemic chemotherapy, due to reduced probability of genetic alterations leading to multidrug resistance. Yet, proof that early detection and treatment of metastatic breast cancer ultimately improves survival is still lacking. The most probably explanation is that, apart from occasional long-term remissions achieved with aggressive polychemotherapy and/or surgical consolidation, metastatic breast cancer remains an incurable disease, and in these patients usefulness of serum tumor markers is limited, especially when distant metastases have already been suspected by clinical, radiological, and biochemical data. The improved survival reported in some studies performed on patients in whom the relapse is found after CA 15-3 elevation, might reflect a lead-time bias rather than a real improvement of tumor control achieved by earlier start of chemotherapy (Lumachi and Basso, 2004; Duffy, 2006). Moreover, treatment decisions are rarely modified according to the way in which the relapse is diagnosed (marker elevation *versus* patient's symptoms).

Taking all these data together, ASCO Technology Assessment does not recommend periodical serum tumor marker testing in the follow-up of women operated of breast cancer, and although they recognize that this guideline does not supplant physician judgment and cannot account for individual variations among patients, they also underline the potential risks of psychological distress in cases of false-positive test. However, the European Group on Tumor Markers suggests that CEA and CA 15-3 testing should be performed even in asymptomatic women despite the impact of the lead time on patients' survival is not clear (Molina *et al*., 2005). Methodologically, the easiest approach in detecting a serum tumor markers should be the sequential use of fixed thresholds as a means to determine an abnormal test. Because serum tumor marker may have an heterogeneous behavior across patients, the use of a longitudinal algorithm to tailor the threshold to the individual screening history could be proposed (McIntosh *et al*., 2002).

In conclusion, there is a general agreement concerning the little clinical usefulness of CEA and CA 15-3, both in early diagnosis of cancer and in detecting breast cancer recurrence. However, serum tumor

marker measurements during follow-up can occasionally assist the clinician in the managements of patients. Probably, multicentric studies design and high-throughput novel technology discovering highly specific serum tumor markers will be validated in the future (Lumachi and Basso, 2004; Li *et al*., 2005).

Acknowledgements. A special thank you to Dr. Annamaria Grigio for help in writing the manuscript and reviewing the English.

REFERENCES

Al-azawi, D., Kelly, G., Myers, E., McDermott, E.W., Hill, A.D.K., Duffy, M.J., and O'Higgins, N. 2006. CA 15-3 is predictive of response and disease recurrence following treatment in locally advanced breast cancer. *BMC Cancer 6*: 220–226.

Bartsch, R., Wenzel, C., Pluschnig, U., Hussian, D., Sevelda, U., Altojai, G., Locker, G.J., Mader, R., Zielinski, C.C., and Steger, G.G. 2006. Prognostic value of monitoring tumour markers CA 15-3 and CEA during fulvestrant treatment. *BMC Cancer 6*: 81–87.

Cheung, K.L., Graves, C.R.L., and Robertson, J.F.R. 2000. Tumour marker measurement in the diagnosis and monitoring of breast cancer. *Cancer Treat. Rev. 26*: 91–102.

Duffy, M.J. 2006. Serum tumor markers in breast cancer: are they of clinical value ? *Clin. Chem. 52*: 345–351.

Given, M., Scott, M., Mc Grath, J.P., and Given, H. 2000. The predictive of tumour markers CA 15-3, TPS and CEA in breast cancer recurrence. *Breast 9*: 277–280.

Haidopoulos, D., Konstadoulakis, M.M., Antonakis, P.T., Alexiou, D.G., Manouras, A.M., Katsaragakis, S.M., and Androulakis, G.F. 2000. Circulating anti-CEA antibodies in the sera of patients with breast cancer. *Eur. J. Surg. Oncol. 26*: 742–746.

Keshaviah, A.S., Dellapasqua, S., Rotmensz, N., Lindtner, J., Crivellari, D., Collins, J., Colleoni, M., Thurlimann, B., Mendiola, C., Aebi, S., Price, K.N., Pagani, O., Simoncini, E., Castiglione Gertsch, M., Gelber, R., Coates, A.S., and Goldhirsch, A. 2007. CA 15-3 and alkaline phosphatase as predictors for breast cancer recurrence: a combined analysis of seven International Breast Cancer Study Group trials. *Ann. Oncol. 18*: 701–708.

Khatcheressian, J.L., Wolff, A.C., Smith, T.J., Grunfeld, E., Muss, H.B., Vogel, V.G., Halberg, F., Somerfield, M.R., and Davidson, N.E. 2006. American Society of Clinical Oncology 2006 update of the breast cancer follow-up and management guidelines in the adjuvant setting. *J. Clin. Oncol. 24*: 5091–5097.

Li, J., Zhao, J., Yu, X., Lange, J., Kuerer, H., Krishnamurthy, S., Schilling, E., Khan, S.A., Sukumar, S., and Chan, D.W. 2005. Identification of biomarkers for breast cancer in nipple aspiration and ductal lavage fluid. *Clin. Cancer Res. 11*: 8312–8320.

Lumachi, F., and Basso, S.M.M. 2004. Serum tumor markers in patients with breast cancer. *Expert Rev, Anticancer Ther. 4*: 921–931.

Lumachi, F., Brandes, A.A., Ermani, M., Bruno, G., and Boccagni, P. 2000. Sensitivity of serum tumor markers CEA and CA 15-3 in breast cancer recurrences and correlation with different prognostic factors. *Anticancer Res. 20*: 4751–4755.

Lumachi, F., Ermani, M., Brandes, A.A., Basso, S.M., Basso, U., and Boccagni, P. 2001. Predictive value of different prognostic factors in breast cancer recurrences: multivariate analysis using a logistic regression model. *Anticancer Res. 21*: 4105–4108.

Lumachi, F., Basso, S.M.M., Brandes, A.A., Pagano, D., and Ermani, M. 2004. Relationship between tumor markers CEA and CA 15-3, TNM staging, estrogen receptor rate and MIB-1 index in patients with pT1-2 breast cancer. *Anticancer Res. 24*: 3221–3224.

McIntosh, M.W., Urban, N., and Karlan, B. 2002. Generating longitudinal screening algorithms using novel biomarkers for disease. *Cancer Epidemiol. Biomarkers Prev. 11*: 159–166.

Molina, R., Barak, V., van Dalen, A., Duffy, M.J., Einarsson, R., Gion, M., Hoike, H., Lamerz, R., Nap, M., Sölétormos, G., and Stieber, P. 2005. Tumor markers in breast cancer-European

Group on tumor markers recommendation. *Tumor Biol. 26*: 281–293.

Nicolini, A., Carpi, A., Ferrari, P., Anselmi, L., Spinelli, C., Conte, M., and Miccoli, P. 2000. The role of tumour markers in improving the accuracy of conventional chest X-ray and liver echography in the post-operative detection of thoracic and liver metastases from breast cancer. *Br. J. Cancer 83*: 1412–1417.

Stearns, V., Yamauchi, H., and Hayes, D.F. 1998. Circulating tumor markers in breast cancer: accepted utilities and novel prospects. *Breast Cancer Res. Treat. 52*: 239–259.

Temple, L.K., Wang, E.E., and McLeod, R.S. 1999. Preventive health care, 1999 update: 3. Follow-up after breast cancer. Canadian Task Force on Preventive Health Care. *CMAJ 161*: 1001–1008.

10
Breast Cancer Patients Before, During or After Treatment: Circulating Tumor Cells in Peripheral Blood Detected by Multigene Real-Time Reverse Transcriptase-Polymerase Chain Reaction

Barbara K. Zehentner

INTRODUCTION

Disseminated tumor cells are considered the main cause for disease progression and metastatic relapse in breast cancer. Histological and immunological protocols are routinely used to detect metastatic cancer cells in lymph node and more recently in bone marrow specimens. In addition to conventional pathology procedures, polymerase chain reaction (PCR) has already been proposed over 10 years ago as a sensitive tool to detect micrometastatic cells by Ghossein and Rosai (1996). A continuously growing number of studies have demonstrated the use of reverse transcriptase polymerase chain reaction (RT-PCR) to detect neoplastic mammary cells in sentinel and axillary lymph nodes, in bone marrow and peripheral blood. Several RNA markers have been described, including tumor associated transcripts (e.g., carcinoembryonic antigen (*CEA*) Gerhard *et al*., 1994), transcripts of epithelial tissue-specific genes (e.g., *cytokeratin 19 and 20* Slade *et al*., 1999), mucin family members (e.g., *MUC1* de Cremoux *et al*., 2000) and the breast tissue-specific gene Mammaglobin (*hMAM*) by Zach *et al*. (1999).

Mammaglobin is a promising molecular marker for breast cancer due to its high specificity and absence of background expression in normal hematopoietic tissues (Zach *et al*., 1999; Corradini *et al*., 2001). *Mammaglobin* was first identified by Watson and Fleming (1996) as a mammary-specific member of the *uteroglobin* gene family. The uteroglobin/Clara Cell protein family consists of small epithelial, secretory proteins, and has recently been named secretoglobins with currently 23 known family members (Klug *et al*., 2000). All six human member genes are localized on chromosome 11 and form a dense cluster (Ni *et al*., 2000). *Mammaglobin* overexpression has been

reported in 70–80% of primary breast cancer (Min *et al*., 1998; Colpitts *et al*., 2001) and in metastatic breast tumor specimens (Watson *et al*., 1999; Fleming and Watson, 2000; Leygue *et al*., 1999; Houghton *et al*., 2001).

This chapter will outline the application of a multigene real-time RT-PCR assay detecting *mammaglobin* and additional marker genes to increase detection sensitivity for circulating breast tumor cells in peripheral blood. Three genes, *B305D*, γ-aminobutyrate type A receptor π subunit (*GABAπ*) and *B726P*, which complement the expression profile of *mammaglobin* in breast cancers were identified by Houghton *et al*. (2001) and Jiang *et al*. (2002). The novel gene designated as *B305D* is predicted to be a type II membrane protein. *GABAπ* has been reported as a member of the $GABA_A$ receptor family by Hedblom and Kirkness (1997). *B726P* is a novel gene located on chromosome 10 with several different putative open reading frames yielded by mRNA splicing. One of these splice forms has been identified by Jager *et al*. (2001) using reactivity with autologous breast cancer patient sera and referred to as NY-BR-1.

The development of a multigene RT-PCR assay detecting the expression profiles of *mammaglobin* and these three genes simultaneously was described by Zehentner *et al*. (2002). High specificity and sensitivity were demonstrated for this assay in breast cancer lymph node analysis. Two additional studies demonstrated the application of this assay using peripheral blood specimens in a clinical oncology setting and its ability to detect and monitor patient specimens before, during, and after adjuvant therapy treatment. The methodology and results of these two studies will be summarized in this chapter.

MATERIALS AND METHODS

Tumor Cell Enrichment from Peripheral Blood

Ten milliliters patient blood were drawn into EDTA containing vaccutainers and processed within 3 h. Next, RosetteSep™ CD45 depletion cocktail for enrichment of circulating epithelial tumor cells (StemCell Technologies Inc., Vancouver, Canada) was added (at a concentration of 50 µl/ml of whole blood) to the vaccutainer and incubated at room temperature for 20 min. The antibody-treated blood was then transferred into Sigma Accuspin System-Histopaque-1077 tubes (Cat. No. A6929, Sigma Chemical Co., St. Louis, MO) and centrifuged for 10 min at 1,000 × *g* to separate the human epithelial cells from the hematopoietic cells antibody cross-linked to red blood cells. The cell layer was collected, washed once with PBS, and cell pellets were re-suspended with 1.5 ml mRNA isolation (Roche, Indianapolis, IN) lysis buffer.

RNA Extraction and cDNA Synthesis

The tumor cell enriched blood cell lysates were processed according to the manufacturer's manual (Roche mRNA isolation kit). mRNA was eluted with 25 µl nuclease-free H_2O and reverse transcribed into cDNA using oligo(dT) primers (Gibco) and 8 µl Superscript Reverse Transcriptase (Invitrogen, Carlsbad, CA) in a final volume of 120 µl.

Multigene Real-Time RT-PCR

Specific primers and 6-carboxy-fluorescein (FAM)-labeled TaqMan® were designed to cross intron-exon junctions in order to exclude genomic DNA from amplification. Expression levels were measured by

quantitative real-time PCR using the ABI 7700 Prism™ sequence detection system (Applied Biosystems, Foster City, CA). Primer sequenes used for *mammaglobin* were Forward 5′-tgccatagatgaattgaaggaatg at 100 nM, Reverse 5′-tgtcatatattaattgcataaacacctca at 100 nM and Probe 5′-tcttaaccaaacggatgaaactctgagcaatg at 4 pmol. Primer sequences for *GABAπ* were Forward 5′-caatttttggtggagaacccg at 300 nM, Reverse 5′-gctgtcggaggtatatggtg at 50 nM and Probe 5′-catttcagagagtaacatggactacaca at 4 pmol. Primer sequences for *B305D* were Forward 5′-tctgataaaggccgtacaatg at 300 nM, Reverse 5′-tcacgacttgctgtttttgctc at 50 nM and Probe 5′-atcaaaaaacaagcatggcctcacaccact at 4 pmol. Primer sequences for *B726P* were Forward 5′-gcaagtgccaatgatcagagg at 100 nM, Reverse 5′-atatagactcaggtatacacact at 100 nM and Probe 5′-tcccatcagaatccaaacaagaggaaga at 4 pmol.

Actin expression was measured in separate singleplex reactions as a quality control for blood cDNA samples. *Actin* primers used were Forward 5′-actggaacggtgaaggtgaca at 300 nM, Reverse 5′-cggccacattgtgaactttg at 300 nM and Probe 5′-cagtcggttggagcgagcatccc at 4 pmol. Specimens with *actin* expression < 50 copies were excluded from analysis.

Fifty PCR cycles were performed with TaqMan® 1,000 Rxn PCR Core Reagents (Part. No. 430 4439, Applied Biosystems, Foster City, CA) using 0.0375 U/µl TaqGold, 1x Buffer A, 5 mM MgCl, 0.2 mM dCTP, 0.2 mM dATP, 0.4 mM dUTP, 0.2 mM dGTP, 0.01 U/µl AmpErase UNG, 8% (v/v) glycerol, 0.05% (v/v), gelatin, 0.01% (v/v) Tween20. PCR conditions were one cycle at 50° for 2 min, one cycle at 95° for 10 min, 95° for 15 s and 60° for 1 min and 68° for 1 min for 50 cycles. Multigene copy numbers were calculated by constructing a standard curve using TaqMan® SDS analysis software from serial dilutions of four plasmids containing target gene cDNA sequences. Final copy numbers were determined as medians of triplicate reactions. Triplicate reactions were pooled and a 20 µl aliquot was separated by agarose electrophoresis using a 4% E-Gel (Invitrogen). Gene identities were determined according to amplicon size.

RESULTS AND DISCUSSION

In the study reported by Zehentner *et al.* (2004) peripheral blood samples from 147 untreated Senegalese women with biopsy-confirmed breast cancer were tested by the multigene real-time RT-PCR assay. In 77% of the breast cancer blood samples, a positive signal was obtained. The associations between detection of gene transcripts in peripheral blood and tumor characteristics were examined. Interestingly, the detection of the four gene transcripts was associated with stage of disease ($p = 0.03$), being present in 53% of those with stage II, 82% of those with stage III, and 87% of those with stage IV breast cancer. The detection of any of the four genes included in the multigene assay in peripheral blood was not significantly associated with increasing tumor size ($p = 0.4$). Likewise, detection of *mammaglobin* transcripts, alone, were not associated with tumor size ($p = 0.4$). However, peripheral blood detection of *GABAπ* alone was associated with tumor size ($p < 0.001$, test for trend) with no expression in patients with small tumors (2–5 cm, n = 30), expression in 14% of medium tumors (6–9 cm, n = 22) and in 40% of largest tumors (10 cm or greater, n = 30). In addition, *GABAπ* was not detected in stage I, II, or IIIA cancers, but was expressed in blood samples from 29% of those with stage IIIB or IV disease ($p = 0.004$, test for trend). *Mammaglobin*

(p = 0.07) and *GABAπ* (p = 0.05) were each marginally associated with increased nodal involvement; only 20% of those with N0 disease were positive for *mammaglobin*, compared to 60–70% of those with N1 or N2 disease. Similarly, 10% of women with N0 or N1, as compared to 29% of N2 had *GABAπ* detected in blood samples. The sensitivity of the multigene assay was increased with increasing cancer stage, but the detection of *mammaglobin* transcript alone was only marginally associated with increased nodal involvement and not to other tumor or patient characteristics. Our findings agree with a recent study by Lin *et al*. (2003), which evaluated the correlation between *mammaglobin* expression in peripheral blood and known prognostic factors for breast cancer patients. Whereas *mammaglobin* mRNA expression was frequently shown to be increased in patients with unfavorable prognostic factors (tumor size, stage), no significant differences could be confirmed. The same group also reported that the combination of *mammaglobin* mRNA detection with *CEA* or *CA15.3* increased the sensitivity from 54% of 33 metastatic breast cancer patients to 81% and 90% respectively, suggesting *mammaglobin* mRNA as a potential adjunct to routinely used serum markers.

The patient population of this study consisted of a large number of Senegalese women with untreated breast cancer, almost all of whom had breast cancer which had already metastasized to regional lymph nodes. Given this, it was anticipated that these women had a high likelihood of having circulating tumor cells present. On the other hand, the study population evaluated is not representative for newly diagnosed breast cancer patients in the United States. Although *mammaglobin* tissue expression has been shown in ~ 80% of breast cancers, previous studies reported by Zach *et al*. (1999), Gruenewald *et al*. (2000) and Bossolasco *et al*. (2002) in patient blood reported detection of *mammaglobin* transcript in 25–54% of those with, and in 10–25% of patients without metastatic breast cancer. In this study we found *mammaglobin* transcripts in 61% of single blood samples from breast cancer patients. This increased rate of detection may be related to the fact that the patients examined were untreated while many of the women examined in previous studies had undergone chemotherapy. Treatment may lower the number of circulating tumor cells. The addition of the three transcripts detected in the Multigene RT-PCR assay increased blood based detection of circulating cells from 61% to 77% of women with breast cancer.

Demographic and behavioral characteristics were also examined in association with detection of gene transcripts in peripheral blood. Detection of *mammaglobin* or other transcripts did not vary significantly with the age or gravidity of the patient; however, detection of transcripts of *GABAπ* was inversely associated with menopausal status, as 28% of premenopausal compared to 9% of postmenopausal women with breast cancer had transcripts of *GABAπ* detected in peripheral blood (p = 0.02). Interestingly, *GABAπ* transcripts were also more often present in breast cancer patients with larger tumors, nodal involvement, and advanced overall tumor stage. These findings demonstrate a possible application of this marker to monitor disease progression and treatment efficacy in particular in pre-menopausal patients.

An additional study reported by Zehentner *et al*. (2006) evaluated the utility of this multigene real-time RT-PCR

assay for detecting circulating tumor cells in peripheral blood specimens of United States breast cancer patients during and after treatment. 172 blood specimens from 82 breast cancer patients for the presence of circulating tumor cells were examined using the multigene real-time RT-PCR assay. In 63.4% of the blood samples a positive signal for *mammaglobin* and/or three breast cancer associated gene transcripts was detected. 75.6% of breast cancer patients had at least one positive blood sample. Different levels of expression signal were detected in the patient categories stage I and stage II–IV with no evidence of metastasis in comparison to patients with current metastatic disease. The average real-time multigene signals detected in patients with metastatic disease was eight times higher than in stage II–IV patients with no evidence of metastasis and thirty times higher than in stage I patients with no metastasis. In addition, detection rate was also increased with stage and presence of metastasis. A positive expression signal indicating circulating tumor cells was detected in 65% of stage I with no current disease, in 72% of stage II–IV patients with no metastasis, and in 88.5% of patients with current metastatic spread. Analyzing multiple blood draws from single patients increased the sample reactivity by 12%. One reason could be that multiple blood samples increase the likelihood of detecting micrometastasis in the blood. In addition, response to treatment could cause fluctuating levels of disseminated tumor cells in the blood stream.

In addition, peripheral blood specimens of 53 healthy, female donors were tested as normal controls. Only two samples tested positive for *mammaglobin* expression by gel electrophoresis with very low multigene RT-PCR copy numbers. One of the positive donors was pregnant in her first trimester. It is possible that hormonal changes influencing cell proliferation in the breast tissue during pregnancy might lead to upregulation of *mammaglobin* expression and in addition to cell dissemination in the blood stream. Further specimens from pregnant and nursing women have to be examined to investigate this relationship.

During this study two patients were monitored over the time course of 17 months during treatment. The first three blood draws from a breast cancer patient with stable disease on hormone therapy treatment tested negative for circulating tumor cells using the multigene RT-PCR assay. However, a signal was detected by the multigene RT-PCR in a peripheral blood specimen 8 months prior to clinical diagnosis of liver metastasis.

Blood samples from another patient with stage IV invasive ductal carcinoma were analyzed for breast cancer specific gene expression by the multigene RT-PCR assay. The patient received several courses of chemotherapy for the treatment of liver and bone metastasis and was initially diagnosed with stable disease. No signal was detected by the multigene RT-PCR assay in the first peripheral blood specimen; however, the following specimen drawn 1 month prior to diagnosed disease progression tested positive by the multigene assay. Chemotherapy was changed and the clinical disease status was reported as stable. No multigene expression signal was detected by real-time PCR in the next two subsequent blood samples. However, a later specimen, 6 months prior to additional clinical disease progression tested positive.

In these two patients, the occurrence of circulating tumor cells preceded dis-

ease progression and observation of additional metastasis. This finding demonstrates the potential application of circulating tumor cell detection to evaluate treatment response. In a recent study, Weigelt *et al.* (2003) showed that the presence of circulating tumor cell mRNA in peripheral blood predicts significantly shortened survival. Ninety-four breast cancer patients with metastatic disease were studied using real-time RT-PCR assays for four genes (*CK19*, *p1B*, *PS2*, *EGP2*), whereas in 31% of the patients circulating tumor cells could be detected.

Moreover, it is interesting to note that in two patients a change of gene expression in the circulating tumor cells was detected after the chemotherapy regimen was altered. Potential clonal selection due to therapy could also cause changes or specific loss in the tumor marker expression profile. Therefore, the detection and molecular analysis of micrometastatic cells could provide an important tool to assess the status of residual disease for future individualized antibody and vaccine therapy applications.

In summary, the two studies outlined in this chapter demonstrate the potential utility of a *mammaglobin* multigene RT-PCR assay to detect circulating tumor cells in breast cancer patients before, after, or undergoing treatment and its potential application for monitoring therapy. It can be concluded that optimized RT-PCR assays might be valuable tools to detect and monitor circulating breast cancer tumor cells. As shown by Cristofanilli *et al.* (2004) in a prospective, multicenter study, circulating tumor cells are the most significant predictors of progression-free and overall survival in metastatic breast cancer.

REFERENCES

Bossolasco, P., Ricci, C., Farina, G., Soligo, D., Pedretti, D., Scanni, A., and Deliliers, G.L. 2002. Detection of micrometastatic cells in breast cancer by RT-PCR for the mammaglobin gene. *Cancer Detect. Prev. 26*: 60–63.

Colpitts, T.L., Billing-Medel, P., Friedman, P., Granados, E.N., Hayden, M., Hodges, S., Menhart, N., Roberts, L., Russell, J., and Stroupe, S.D. 2001. Mammaglobin is found in breast tissue as a complex with BU101. *Biochemistry 40*: 11048–11059.

Corradini, P., Voena, C., Astolfi, M., Delloro, S. Pilotti, S., Arrigoni, G., Bregni, M., Pileri, A., and Gianni, A.M. 2001. Maspin and mammaglobin genes are specific markers for RT-PCR detection of minimal residual disease in patients with breast cancer. *Ann. Oncol. 12*: 1693–1698.

Cristofanilli, M., Budd, G.T., Ellis, M.J., Stopeck, A., Matera, J., Miller, M.C., Reuben, J.M., Doyle, G.V., Allard, W.J., Terstappen, L.W., and Hayes, D.F. 2004. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *N. Engl. J. Med. 351*: 781–791.

de Cremoux, P., Extra, J.M., Deni, M.G., Pierga, J.Y., Bourstyn, E., Nos, C., Clough, K.B., Boudou, E., Martin, E.C., Muller, A., Pouillart, P., and Magdelenat, H. 2000. Detection of MUC1-expressing mammary carcinoma cells in the peripheral blood of breast cancer patients by real-time polymerase chain reaction. *Clin. Cancer Res. 6*: 3117–3122.

Fleming, T.P., and Watson, M.A. 2000. Mammaglobin, a breast-specific gene, and its utility as a marker for breast cancer. *Ann. NY Acad. Sci. 923*: 78–89.

Gerhard, M., Juhl, H., Kalthoff, H., Schreiber, H.W., Wagener, C., and Neumaier, M. 1994. Specific detection of carcinoembryonic antigen-expressing tumor cells in bone marrow aspirates by polymerase chain reaction. *J. Clin. Oncol. 12*: 725–729.

Ghossein, R.A., and Rosai, J. 1996. Polymerase chain reaction in the detection of micrometastases and circulating tumor cells. *Cancer. 78*: 10–16.

Gruenewald, K., Haun, M., Urbanek, M., Fliegl, M., Mueller-Holzner, E., Gunsilius, E., Dunser, M., Marth, C., and Gastl, G. 2000. Mammaglobin gene expression: a superior marker of breast

cancer cells in peripheral blood in comparison to epidermal-growth-factor receptor and cytokeratin-19. *Lab. Invest. 80*: 1071–1077.

Hedblom, E., and Kirkness, E.F. 1997. A novel class of GABAA receptor subunit in tissues of the reproductive system. *J. Biol. Chem. 272*: 15346–15350.

Houghton, R.L., Dillon, D.C., Molesh, D.A., Zehentner, B.K., Xu, J., Jiang, J., Schmidt, C., Frudakis, A., Repasky, E., Maltez Filho, A., Nolasco, M., Badaro, R., Zhang, X., Roche, P.C., Persing, D.H., and Reed, S.G. 2001. Transcriptional complementarity in breast cancer: application to detection of circulating tumor cells. *Mol. Diagn. 6*: 79–91.

Jager, D., Stockert, E., Gure, A.O., Scanlan, M.J., Karbach, J., Jager, E., Knuth, A., Old, L.J., and Chen, Y.T. 2001. Identification of a tissue-specific putative transcription factor in breast tissue by serological screening of a breast cancer library. *Cancer Res. 61*: 2055–2061.

Jiang, Y., Harlocker, S.L., Molesh, D.A. Dillon, D.C., Stolk, J.A., Houghton, R.L., Repasky, E.A., Badaro, R., Reed, S.G., and Xu, J. 2002. Discovery of differentially expressed genes in human breast cancer using subtracted cDNA libraries and cDNA microarrays. *Oncogene 21*: 2270–2282.

Klug, J., Beier, H.M., Bernard, A., Chilton, B.S., Fleming, T.P., Lehrer, R.I., Miele, L., Pattabiraman, N., and Singh, G. 2000. Uteroglobin/Clara cell 10-kDa family of proteins: nomenclature committee report. *Ann. NY Acad. Sci. 923*: 348–354.

Leygue, E., Snell, L., Dotzlaw, H., Hole, K., Troup, S., Hiller-Hitchock, T., Murphy, L.C., and Watson, P.H. 1999. Mammaglobin, a potential marker of breast cancer nodal metastasis. *J. Pathol. 189*: 28–33.

Lin, Y.C., Chen, S.C., Hsueh, S., Lo, Y.F., Chow-Wu, Y.H., Liaw, I.C., and Cheng, A.J. 2003. Lack of correlation between expression of human mammaglobin mRNA in peripheral blood and known prognostic factors for breast cancer patients. *Cancer Sci. 94*: 99–102.

Min, C.J., Tafra, L., and Verbanac, K.M. 1998. Identification of superior markers for polymerase chain reaction detection of breast cancer metastases in sentinel lymph nodes. *Cancer Res. 58*: 4581–4584.

Ni, J., Kalff-Suske, M., Gentz, R., Schageman, J., Beato, M., and Klug, J. 2000. All human genes of the uteroglobin family are localized on chromosome 11q12.2 and form a dense cluster. *Ann. N. Y. Acad. Sci. 923*: 25–42.

Slade, M.J., Smith, B.M., Sinnett, H.D., Croxx, N.C., and Coombes, R.C. 1999. Quantitative polymerase chain reaction for the detection of micrometastases in patients with breast cancer. *J. Clin. Oncol. 17*: 870–879.

Watson, M.A., and Fleming, T.P. 1996 Mammaglobin, a mammary-specific member of the uteroglobin gene family, is overexpressed in human breast cancer. *Cancer Res. 56*: 860–865.

Watson, M.A., Dintzis, S., Darrow, C.M., Voss, L.E., DiPersio, J., Jensen R., and Fleming, T.P. 1999. Mammaglobin expression in primary, metastatic, and occult breast cancer. *Cancer Res. 59*: 3028–3031.

Weigelt, B., Bosma, A.J., Hart, A.A., Rodenhuis, S., and 't Veer, L.J. 2003. Marker genes for circulating tumour cells predict survival in metastasized breast cancer patients. *Br. J. Cancer 88*: 1091–1094.

Zach, O., Kasparu, H., Krieger, O., Hehenwarter, W., Girschikofsky, M., and Lutz, D. 1999. Detection of circulating mammary carcinoma cells in the peripheral blood of breast cancer patients via a nested reverse transciptase polymerase chain reaction for mammaglobin mRNA. *J. Clin. Oncol. 17*: 2015–2019.

Zehentner, B.K., Dillon, D.C., Jiang, Y., Xu J., Bennington, A., Molesh, D.A., Zhang, X., Reed, S.G., Persing, D., and Houghton, R.L. 2002. Application of a multigene reverse transcription-PCR assay for detection of mammaglobin and complementary transcribed genes in breast cancer lymph nodes. *Clin. Chem. 48*: 1225–1231.

Zehentner, B.K., Persing, D.H., Deme, A., Toure, P., Hawes, S.E., Brooks, L., Feng, Q., Hayes, D.C., Critichlow, C.W., Houghton, R.L., and Kiviat, N.B. 2004. Mammaglobin as a novel breast cancer biomarker: multigene reverse transcription-PCR assay and sandwich ELISA. *Clin. Chem. 50*: 2069–2076.

Zehentner, B.K., Secrist, H., Hayes, D.C., Zhang, X., Ostenson, R.C., Loop, S., Goodman, G., Houghton, R.L., and Persing, D.H. 2006. Detection of circulating tumor cells in peripheral blood of breast cancer patients during or after therapy using a multigene real-time RT-PCR assay. *Mol. Diagn. Ther. 10*: 41–47.

11
Breast Cancer Patients: Diagnostic Epigenetic Markers in Blood

Qinghua Feng and Nancy B. Kiviat

INTRODUCTION

Epigenetic changes are defined as changes of gene expression without changes in the DNA sequence. Both DNA methylation and histone modifications are essential in normal mammalian development. Recently, it has been shown that epigenetic alterations play an important role in tumorigenesis and represent alternative means of activation of tumor oncogenes, inactivation of tumor suppressor genes, and induction of genomic instability (Jones and Baylin, 2007). During the last 2 decades, much effort has been focused on the role of DNA methylation changes in cancer development and their utilities in cancer detection. It is now clear that histone modifications are an integral part of the epigenetic programming.

DNA methylation, referring here to the addition of a methyl group to the cytosine in the cytosine-phosphate-guanine (CpG) dinucleotides, plays an important role in normal development, genomic stability, and regulation of gene expression. In normal cells, the majority of CpG dinucleotides is located in the repetitive sequences of the genome and methylated. Approximately, 50% of housekeeping genes contains a CpG rich region (called the CpG island) in their promoter region, which is not methylated regardless of whether the gene is transcribed or not. On the contrary, tumor cells are characterized by both global hypomethylation and gene-specific hypermethylation (Jones and Baylin, 2007). Hypomethylation of repetitive sequences leads to genome instability, while promoter hypermethylation leads to transcriptional silencing of many tumor suppressor genes, with similar consequence as genetic mutations.

Histone modifications, referring to acetylation, methylation, phosphorylation, ubiquitination, and sumoylation of amino acids in histone tails, collectively form histone codes that link various chromatin structures to gene expression states. Accumulating evidence suggests that acetylation and methylation of different lysine and arginine residues in histones H3 and H4 are associated with either transcriptionally active or transcriptionally repressed states of gene expression. For example, acetylated histones are associated with transcribed genes, while deacetylated histones are associated with silenced genes. Methylated lysine 9 (K9) or K27 on H3 and K20 on H4 are generally associated with transcription repressed genes, whereas methylated K4, K36, and K79 are found mainly in active chromatin (Kouzarides,

2007). Global profiling of histone modifications identified loss of acetylation of lysine 16 (K16) and trimethylation of lysine 20 (K20) on H4 a common hallmark of human tumor cells, and these changes appear early and accumulate during tumor development. Further, emerging data suggests that chromatin states can directly affect DNA methylation, either inducing de novo DNA methylation or demethylation (D'Alessio and Szyf, 2006). Therefore, specific combinations of histone modifications at specific loci might have use for cancer diagnosis. However, there are currently no available technologies to detect histone modifications for clinical assays.

UTILITY OF DNA METHYLATION MARKERS IN CANCER DETECTION

There is a great deal of interest in the potential of aberrant DNA methylation as a cancer biomarker for early detection of cancer, cancer staging, and response to therapy (Jones and Baylin, 2002). Aberrant DNA methylation has been reported in virtually every tumor type tested and is an early event in tumorigenesis, occurring in precursor lesions of many cancer types (Esteller *et al*., 2000).

Technically, there are advantages of using DNA methylation changes as biomarkers. First, DNA is relatively stable during specimen collection, storage, and manipulation. DNA can be readily amplified by polymerase chain reaction (PCR) and therefore a minimal amount of clinical material often provides a sufficient quantity of DNA for analysis. Second, detection of DNA methylation has the highest analytical sensitivity against a background of normal DNA, compared to gene mutations and loss of heterozygosity (LOH) markers (Goessl *et al*., 2002). Finally, unlike gene mutations that are typically scattered throughout the entire coding region, making it difficult to detect all DNA mutations in a simple and cost-effective manner, DNA methylation changes always occur in the same location in the promoter region, thus facilitating the development of clinically useful assays.

IDENTIFICATION OF DNA METHYLATION MARKERS

DNA methylation markers for a specific type of cancer can either be identified through a candidate gene approach or through genome-wide profiling. Currently, there are more than 100 genes reported being methylated in various types of cancers (Esteller, 2007). Early studies have been focused on the utility of a few of these genes in cancer detection. More recently, genome-wide profiling of DNA methylation has been used to identify novel cancer specific methylation markers. Global DNA methylation changes can be detected by utilizing methylation-sensitive restriction enzymes, which cleave unmethylated CpG islands but leave methylated CpG islands intact. Differentially methylated CpG loci are then detected by various subtractive hybridization methods (Jones and Baylin, 2002), including methylation-sensitive arbitrarily primed PCR (MS-AP-PCR), restriction landmark genomic scanning (RLGS), methylated CpG island amplification-representational difference analysis (MCA-RDA), and differential methylation hybridization (DMH). Detailed mapping of individual CpGs can be achieved by combining sodium bisulfite conversion and methylation-specific oligonucleotide-based microarrays

(CpG island arrays). After bisulfite modification, DNA is amplified by PCR and then hybridized to an oligonucleotide array to allow discrimination of bisulfite-converted unmethylated (TpG) from methylated (CpG) at specific CpG sites. The disadvantage of these approaches is that the sequences surveyed are limited to those that contain specific restriction sites, and only ~ 10% of CpG islands are interrogated by these methods.

Methylation-silenced cancer associated genes can also be identified through the use of epigenetic modifying agents such as 5-aza-2'-deoxycytidine (DAC, a DNA methylase inhibitor) and trichostatin A (TSA, a histone deacetylase (HDAC) inhibitor), followed by expression microarray analysis of reactivated genes (Jones and Baylin, 2002). This approach has been successfully used to identify novel methylated genes in cervical, colorectal, pancreatic, prostate, and head and neck cancers. In cervical cancer, six genes (SPARC, TFPI2, RRAD, SFRP1, MT1G, and NMES1) were identified that are methylated in cancer tissues but not in normal tissues (Sova *et al*., 2006). One drawback of this approach is that not all genes methylated are reactivated by the DAC/TSA treatment; and not all genes reactivated by the treatment are methylated. Further, only abundant transcripts can be identified by the expression array, and genes expressed at a low level are missed by this approach.

Currently, the state of art approach for global methylation profiling uses either 5-methylcytosine antibody or methyl-binding proteins (MBDs) to preferentially enrich for methylated DNA. Subsequently, these DNA targets are identified through genomic tiling array hybridization. Using this approach, it has been demonstrated that promoter sequences and gene functions are major predictors for DNA methylation in normal cells (Weber *et al*., 2007), while tumor-specific methylated genes belong to distinct functional categories, have common sequence motifs in their promoters, and are found in clusters on chromosomes (Keshet *et al*., 2006). When applied to lung cancer cell lines, several homeodomain containing genes are identified being methylated in lung cancer (Rauch *et al*., 2006).

Lastly, because DNA methylation and chromatin states are interrelated, genes silenced by DNA methylation are usually associated with heterochromatins, and heterochromatins can be mapped using heterochromatin binding proteins and histone modifications associated with inactive genes. Global heterochromatin regions can be identified by chromatin immunoprecipitation with antibodies against histone deacetylase, or specific histone lysine methylation (H3-K9, H3-K27 and H4-K20) (Strunnikova *et al*., 2005). These genomic regions are candidate loci for DNA methylation.

CIRCULATING TUMOR DNA AS A DIAGNOSTIC TOOL FOR CANCER DETECTION

Cancer patients have been reported to contain a higher level of circulating DNA in their blood than normal healthy individuals, with 50–80% of such circulating DNA being derived from tumor cells (Goessl *et al*., 2002). Although the origin of circulating tumor DNA is still not clear, it is hypothesized that it is released from dying tumor cells via apoptosis or necrosis. Several studies have shown that breast cancer patients have a higher level of free

plasma DNA (mean 65–211 ng/ml) than healthy control patients (mean 13–21 ng/ml) (Huang *et al*., 2006). Further, cancer specific alterations, including point mutations in either genomic or mitochondrial DNA, genomic instability markers [measured by LOH and by microsatellite instability (MI)], and epigenetic changes such as aberrant DNA methylation can be detected in plasma DNA from cancer patients. Both p53 mutations and LOH/MI markers were detected in plasma DNA samples in breast cancer patients (Schwarzenbach *et al*., 2004). Methylation of p16 was also detected in plasma DNA of breast cancer patients (Silva *et al*., 1999a).

Either plasma or serum can be used to isolate circulating tumor DNA. In general, the concentration of circulating DNA is higher in serum samples than in plasma samples, probably due to contamination of lymphocyte lysis during serum preparation (Taback *et al*., 2004). One study reported that the amount of circulating DNA detected in plasma and serum samples did not correlate and plasma DNA levels better represent in vivo levels of circulating DNA (Thijssen *et al*., 2002). Fleischhacker *et al*. (2007) also detected different levels of methylation in plasma and serum samples.

METHODS FOR METHYLATION ANALYSIS

Early DNA methylation analysis methods relied on differential digestion by methylation-dependent (only cleaving methylated DNA) and methylation-sensitive (cleavage inhibited by methylation) restriction enzymes coupled with southern blot hybridization (Dahl and Guldberg, 2003). These methods are tedious, time-consuming, require large amounts of genomic DNA of high quality, and the data interpretation is hindered by incomplete enzymatic digestion. Therefore, these methods have limited utility in clinical samples. The current gold standard for DNA methylation analysis is based on sodium bisulfite treatment, where unmethylated cytosine is converted to uracil while methylated cytosine remains unchanged. Thus, bisulfite modification converts methylation differences into sequence differences, which are then detected using various detection methods. Combined with PCR or real-time PCR techniques, the methylation specific PCR (MSP) (Herman *et al*., 1996) and MethyLight assays (Eads *et al*., 2000a) have dramatically accelerated DNA methylation analysis on clinical samples.

BLOOD SAMPLE COLLECTION

Either plasma or serum samples can be used to isolate circulating tumor DNA. For plasma samples, ~ 6–10 ml blood is collected into a purple top tube with EDTA as the anti-coagulant. Within 4 h of collection, samples are centrifuged at 800 g for 10–15 min. The supernatant (plasma) are collected, and aliquoted (1 ml each) into 1.8 ml Nunc tubes (VWR, West Chester, PA). The plasma samples are stored in a −80°C freezer. For serum samples, ~ 6–10 ml blood is collected into a red top tube, without any anti-coagulant. The blood sample is allowed to clot for 4 h, and then centrifuged at 800 g for 10–15 min. The supernatant (serum) is collected and aliquoted (1 ml each) into 1.8 ml Nunc tubes, and stored in a −80°C freezer.

DNA ISOLATION FROM BLOOD

Cell-free DNA from plasma or serum samples can be efficiently isolated using the proteinase K phenol/chloroform extraction method. The DNA is finally dissolved in 30 µl of TE, and 3 µl of DNA solution is used for the measurement of DNA concentration using the Picogreen dsDNA assay (Invitrogen, Carlsbad, CA) or quantitative PCR on β-actin (ACTB) gene.

DNA isolation protocol with proteinase K phenol/chloroform extraction

Materials and Reagents

1. TE-9 buffer: 1 ml 1 M Tris-HCl, pH9.0; 0.5 ml 0.5 M EDTA, pH8.0; 1.25 ml 5 M NaCl; 97.25 ml dH2O
2. 20% proteinase K solution: 100 mg proteinase K, 5 ml TE-9 buffer
3. 10% SDS-PK solution: 100 mg proteinase K, 10 ml 10% SDS; aliquot and store at −20°C
4. 1% SDS-PK solution: 5 ml 10% SDS-PK, 45 ml TE-9 buffer
5. PC-8: phenol:chloroform (1:1)
6. Phase lock gel (heavy) (Brinkmann Instrument, cat.#955154151)
7. 7.5 M ammonium acetate: dissolve 38.5 g ammonium acetate in 100 ml dH2O
8. Glycogen (20 mg/ml) (Roche Applied Science, cat.#901-393)
9. 100% ethanol
10. 70% ethanol: mix 70 ml 100% ethanol with 30 ml dH2O
11. TE buffer: 10 mM Tris-HCl, 1 mM EDTA (pH 8.0)

Procedure

1. Digest 1 ml plasma or serum with 2 ml 1% SDS-PK in 48°C water bath for 48 h
2. Add 50 µl 20% PK three times a day
3. Add 3 ml of PC-8 into each tube
4. Vortex each sample for 1 min to thoroughly expose the sample to the PC-8
5. For each sample, aliquot 1 ml each to six 1.5 ml Phase Lock Gel Heavy; spin at 6,000 g for 5 min
6. Decant all supernatant (~ 3 ml total) into a 15 ml Falcon tube
7. Add 1 ml ammonium acetate 7.5 M solution, 2 µl glycogen (20 mg/ml) and 8 ml 100% ethanol solution to each sample
8. Gently invert the tube 3–4 times to thoroughly mix the sample with the ethanol
9. Allow the tubes to remain at −20°C overnight
10. Centrifuge the samples for 50 min at 5,200 g at 4°C
11. After centrifugation, carefully decant the supernatant, being very careful that the pellet remains in the tube
12. Wash the samples by adding 10 ml 70% ethanol to each tube
13. Centrifuge samples again at 5,200 g for 10 min
14. Decant the solution while retaining the pellet
15. Allow the tubes to sit, inverted, for ~ 15 min to remove all excess ethanol
16. Resuspend the DNA pellet in 30 µl TE, let it remain overnight at 4°C
17. Transfer resuspended DNA into 1.5 ml Eppendorf tubes

Alternatively, commercially available DNA isolation kits can be used to isolate circulating DNA. Either QIAamp UltraSens Virus kit (Cat. #53704, Qiagen, Valencia, CA) or QIAamp MinElute spin kit (Cat. #57704, Qiagen, Valencia, CA) can be used to extract circulating DNA from plasma or serum samples, following manufacture's protocols. DNA from large volumes of plasma or

serum samples should be isolated using the QIAamp UltraSens Virus kit. Briefly, using QIAamp UltraSens kit, circulating DNA from 1 ml plasma or serum samples are first concentrated using isopropanol precipitation. The pellet is then resuspended in a small volume of buffer and digested with proteinase K prior to loading onto a QIAamp column. By centrifugation, DNA selectively binds to the QIAamp membrane as contaminants pass through. The remaining contaminants and enzyme inhibitors are further removed by centrifugation in two wash steps, and the circulating nucleic acids are eluted in low-salt buffer. DNA from up to 200 µl plasma or serum samples can be isolated using the QIAamp MinElute spin kit. Briefly, plasma or serum samples are first lysed and digested with protease under highly denaturing conditions at elevated temperatures, then loaded onto a QIAamp MinElute column. DNA is absorbed onto the silica-gel membrane, washed and eluted in 20 µl low-salt buffer. DNA concentration is measured using quantitative PCR on β-actin (ACTB) gene. Although commercial kits offer quality control and consistency between batches, it should be noted that most columns do not efficiently bind small DNA molecules (up to 150 bp), thus, part of the DNA is lost during preparation.

BISULFITE CONVERSION

Sodium bisulfite treatment converts unmethylated cytosine to uracil but does not affect methylated cytosine, thus the methylation differences in the samples is converted into sequence differences, which can then be detected using various PCR-based methods. Optimization of bisulfite treatment is key to successful experiments, because incomplete bisulfite conversion can lead to false positive results, while prolonged treatment results in nonspecific DNA degradation.

Two published methods can be used for sodium bisulfite conversion (Herman *et al.*, 1996; Weisenberger *et al.*, 2005). In both methods, high quality genomic DNA is first denatured, then modified by 5 M sodium bisulfite, desulfonated with NaOH, then purified using different nucleic acid affinity resins and resuspended in 80 µl LoTris buffer. Unmethylated control DNA (human sperm DNA) and *in vitro* fully methylated DNA are converted along with clinical samples to ensure the specificity of bisulfite treatment.

Bisulfite conversion protocol 1 (Herman et al., 1996)

Materials and Reagents

1. 3 M NaOH: dissolve 120 mg NaOH in 1 ml dH2O, or mix 0.3 ml 10 M NaOH with 0.7 ml dH2O
2. 2 M NaOH: dissolve 80 mg NaOH in 1 ml dH2O, or mix 0.2 ml 10 M NaOH with 0.8 ml dH2O
3. 1 M hydroquinone (always prepare fresh): dissolve 0.22 g Hydroquinone (Sigma cat.# H-7148) into 2 ml dH2O.
4. Bisulfite-hydroquinone solution (enough for 16 samples; always prepare fresh): dissolve 3.8 g sodium-metabisulfite (Sigma cat# S-1516) in 5 ml dH2O, add 1.0 ml 1 M hydroquinone and 1.4 ml 2 M NaOH; heat to 80°C and vortex to dissolve; extrapolate if needed for more than 16 samples
5. Wizard DNA clean-up system (Promega cat # A7280)

6. 80% isopropanol: mix 40 ml 2-propanol with 10 ml dH2O
7. 5 M ammonium acetate: dissolve 38.5 g ammonium acetate into 100 ml dH2O
8. Glycogen (20 mg/ml)
9. 100% ethanol
10. 70% ethanol: mix 70 ml 100% ethanol with 30 ml dH2O
11. LoTris buffer: 5 mM Tris (pH8)

Procedure

DNA-denaturing

1. Dilute 1–2 µg DNA in 20 µl TE buffer or water
2. Add 1 µl salmon sperm DNA (10 mg/ml)
3. Add 2 µl 3 M NaOH
4. Mix and incubate at 50°C for 20 min

Bisulfite conversion

5. Add 500 µl of bisulfite-hydroquinone solution to each denatured DNA sample
6. Incubate at 70°C in dark for 3 h

DNA clean-up and recovery

7. Assemble syringe barrel, Wizard mini-column and vacuum manifold
8. Add 1 ml of Wizard DNA clean-up resin to the bisulfite converted DNA and mix
9. Load the whole mixture onto the mini-column
10. Apply vacuum to draw the solution through the minicolumn, break vacuum
11. Wash by applying 2 ml 80% isopropanol and apply vacuum
12. Vacuum additional 30 s after the isopropanol runs through
13. Place the minicolumn into a microcentrifuge and centrifuge at 14,000 rpm for 2 min
14. Place the minicolumn into a new tube and elute DNA with 45 µl dH2O heated to 80°C, wait for 1 min
15. Centrifuge at 14,000 rpm for 1 min
16. Remove minicolumn, add 5 µl 3 M NaOH to the filtrate, mix and incubate at room temperature for 10 min
17. Add 75 µl 5 M ammonium acetate and incubate at room temperature for 5 min
18. Add 1 µl glycogen and 325 µl 100% ethanol
19. Precipitate at −20°C overnight
20. Centrifuge at 14,000 rpm for 15 min
21. Decant the supernatant, wash the pellet with 500 µl 70% ethanol
22. Centrifuge at 14,000 rpm for 10 min
23. Decant the supernatant, speed vacuum 10 min to dry the pellet
24. Resuspend the pellet in 30 µl LoTris buffer

Bisulfite conversion protocol 2 (Weisenberger et al., 2005)

Materials and Reagents

1. 3 M NaOH: dissolve 120 mg NaOH into 1 ml dH2O, or mix 0.3 ml 10 M NaOH with 0.7 ml dH2O
2. 2 M NaOH: dissolve 80 mg NaOH into 1 ml dH2O, or mix 0.2 ml 10 M NaOH with 0.8 ml dH2O
3. 1 M hydroquinone (always prepare fresh): dissolve 0.11 g Hydroquinone (Sigma cat.# H-7148) into 1 ml dH2O
4. 5 M sodium bisulfite solution (always prepare fresh): dissolve 1.9 g sodium-metabisulfite (Sigma cat.# S-1516) in 2.5 ml dH2O, add 0.7 ml 2 M NaOH, heat to 50°C to dissolve bisulfite, add 0.5 ml 1 M hydroquinone
5. QIAamp Viral RNA mini kit (Qiagen cat.# 52904)

Procedure

DNA-denaturing

1. Dilute 1–2 µg DNA in 18 µl TE buffer or water
2. Denature at 100°C for 10 min, centrifuge briefly and chill on ice
3. Add 2 µl 3 M NaOH
4. Mix and incubate at 42°C for 20 min

Bisulfite conversion

5. Add 120 µl of bisulfite-hydroquinone solution to each denatured DNA sample
6. Incubate at 50°C in dark for 16 h

DNA clean-up and recovery

7. Add 560 µl AVL/carrier RNA buffer from QIAamp Viral RNA mini kit and incubate at room temperature for 10 min
8. Add 560 µl 100% ethanol, mix well
8. Load the whole mixture onto a QIAamp column in two steps, centrifuge at 14,000 rpm for 1 min
9. Save the filtrate and reload onto the same column, centrifuge at 14,000 rpm for 1 min
10. Wash with 500 µl buffer AW1, centrifuge at 14,000 rpm for 1 min
11. Wash with 500 µl buffer AW2, centrifuge at 14,000 rpm for 1 min
12. Elute the DNA with 40 µl buffer AVE, centrifuge at 9,000 rpm for 1 min
13. Repeat step 12
14. Combine both filtrates, add 50 µl 0.2 M NaOH, mix and incubate at room temperature for 15 min
14. Add 1 µl 1 M HCl
15. Repeat step 7 through step 12

Alternatively, several commercially available kits can be used for bisulfite conversion. These include EZ DNA Methylation Gold kit (Zymo Research Corporation, Orange, CA), EpiTect Bisulfite kit (Qiagen, Valencia, CA), MethylSEQr Bisulfite Conversion kit (ABI, Foster City, CA), MethylCode Bisulfite Conversion kit (Invitrogen, Carlsbad, CA), MethylEasy kit (Human Genetic Signatures, Sydney, Australia), and MethylDetector™ kit (Active Motif, Carlsbad, CA).

Although both home-brew methods and commercially available kits rely on the same principle of sodium bisulfite reaction, slight changes in reaction conditions might cause differences in conversion efficiency. Currently, very few studies have systematically compared different bisulfite conversion methods, and no standard protocol exists. Thus, discrepancies among different studies might derive from different conversion protocols. In general, a kit is more expensive, but it comes with quality control on reagents to ensure consistent bisulfite treatment results. Because of the variability of bisulfite treatment efficiency between runs, it is essential to include both unmethylated (U-DNA) and methylated (M-DNA) controls along with clinical samples for the treatment.

SIGNAL DETECTION

Currently the most sensitive detection methods following bisulfite conversion are all PCR-based. Methylation specific PCR is qualitative, while both MethyLight assay and quantitative multiplex MSP are semi-quantitative.

METHYLATION SPECIFIC PCR (MSP) ASSAY

Methylation specific PCR was first reported by Herman *et al.* (1996). In principle, two sets of primers are designed; the unmethylated

(U) primers will only amplify bisulfite converted unmethylated DNA, while the methylated (M) primers will amplify bisulfite converted methylated DNA. Because the differences between unmethylated and methylated DNA after bisulfite conversion are limited, primer design and PCR amplification conditions are keys for successful methylation analysis. The primers should contain a substantial number of non-CpG cytosines to ensure that only bisulfite converted DNA is amplified. The CpGs covered by the primers (~ 1–3 CpGs for each primer) should be located in the 3' region of the primers to maximize the discriminative power between methylated and unmethylated DNA and increase the specificity of the primer annealing. Alternatively, MSP primers can be designed using online available software, such as MethPrimer (www.urogene.org/methprimer). Because most CpG islands are GC-rich DNA sequences, it is recommended that β-mercaptoethanol is included in the PCR buffer to improve amplification efficiency. Both the annealing temperature for a specific primer set and number of amplification cycles should be determined empirically on positive and negative control samples. However, it is not known whether the amount of unmethylated DNA in a clinical sample affects the performance of MSP.

The MSP PCR product can be visualized on either nondenaturing 6–8% polyacrylamide gels or 2% agarose gels. The U-DNA and M-DNA treated along with clinical samples are ideal control samples for MSP. The assays are valid if the U-DNA is only amplified by the U-specific primers, but not by the M-specific primers, and M-DNA is only amplified by the M-specific primers, but not by the U-specific primers.

METHYLIGHT ASSAY

Because MSP can not quantify the amount of methylated alleles, the MethyLight assay was developed for quantitative methylation analysis by combining the MSP assay with quantitative TaqMan technology based real-time PCR. Two primers and a probe specific for bisulfite converted methylated DNA are used in each MethyLight assay (Eads *et al.*, 2000a). The primers are designed similarly to the M-primers in MSP assays. The probe should span 2–4 CpGs, preferably located in the middle of the probe. The probe is dually labeled with a reporter dye (6-FAM, VIC, TET, or NED) at the 5′ end and a quencher dye at the 3′ end (TAMRA, BHQ or MGB). The probe is not fluorescent when it is intact, due to the presence of the quencher dye. During PCR amplification, the 5′-3′ exonuclease activity of the Taq DNA polymerase cleaves the reporter dye from the quencher dye, resulting in an increase in the fluorescent signal which is quantitated in each PCR cycle.

A reference gene, usually β-actin (ACTB), is used to quantitate for input DNA. Other genes, including MYOD1 and COL2A1, have been used as reference genes (Eads *et al.*, 2000a; Ogino *et al.*, 2006). The primers and probe for the reference gene are designed outside the CpG island without encompassing any CpGs, so that samples will be amplified regardless of the methylation status of the reference gene. The reference gene primers and probe set is used concurrently with each gene specific primers and probe set. The Percentage Methylated Reference (PMR) for each locus is calculated by dividing the

GENE: reference ratio of a sample by the GENE: reference ratio of *in vitro* fully methylated DNA and multiplying by 100 (Eads *et al*., 2000b). Each run contains a no-DNA control to exclude PCR contamination, and a bisulfite converted unmethylated DNA (human sperm DNA) for PCR specificity.

The different PMRs reflect either the difference of methylation density in tumor DNA or the different amount of tumor DNA in clinical samples; thus, the assay is semi-quantitative. Further, the assay cannot distinguish between a small percentage of tumor cells with both alleles methylated and a large percentage of tumor cells with only one allele methylated.

The advantages of MethyLight assays are several fold. (1) The MethyLight assay is more sensitive than conventional MSP. It is reported that MethyLight has a sensitivity of 1:10,000 (one methylated allele in 10,000 unmethylated alleles) (Eads *et al*., 2000a), while the conventional MSP has a sensitivity of 1:1000 (Herman *et al*., 1996). (2) The MethyLight assay is more specific than MSP due to the incorporation of methylation specific probe. However, MethyLight can only detect fully methylated molecules, while MSP can also detect partially methylated molecules, thus the sensitivity might be reduced. One study indicated that conventional MSP was more sensitive to detect methylation changes in plasma or urine samples from patients with early stage diseases than real-time quantitative MSP (Jeronimo *et al*., 2002). (3) The MethyLight assay is more objective than conventional MSP, and PCR cross-contamination is reduced, as it does not require post-PCR analysis, such as gel electrophoresis.

NESTED POLYMERASE CHAIN REACTION METHOD

Because circulating tumor DNA is normally present in nanograms in body fluids, nested-MSP was developed to increase the sensitivity of methylation detection in clinical samples harboring low amounts of DNA (Palmisano *et al*., 2000). The first round of PCR is performed using primers that amplify bisulfite converted DNA, but do not distinguish unmethylated and methylated DNA, similar to bisulfite sequencing primers. The PCR product is subsequently diluted and used as the template for the second round of PCR using U and M specific primers. Compared to conventional MSP assays, the nested MSP assay has a sensitivity of 1:50,000. Because both unmethylated and methylated DNA are amplified in the first round of PCR, the percentage of methylated DNA can be quantitated in the second round of PCR as the amount of DNA amplified by M-specific primers divided by the sum of DNA amplified by both U and M-specific primers, as long as the amplification efficiencies of the U and M primers are the same. Recently, a quantitative multiplex nested MSP (QM-MSP) assay has been developed for this purpose (Fackler *et al*., 2004). Instead of performing nested PCR for each individual gene, the first round of PCR is multiplexed to amplify up to five gene loci. In the second round of PCR, methylation of each individual gene is quantitated using real time PCR. Similar to nested PCR, it is essential to ensure equal amplification of both unmethylated and methylated DNA in the first round PCR in order to obtain meaningful quantitative data. It is

estimated that as few as 1–10 methylated alleles among approximately 100,000 alleles of unmethylated DNA, or 40 pg of methylated genomic DNA in the presence of 1,500-fold excess unmethylated DNA can be detected (Fackler *et al.*, 2004).

POTENTIAL NOVEL METHODS

Although the MethyLight assay is the current gold standard for DNA methylation analysis, it lacks the sensitivity for use as a clinical blood assay, where tumor DNA is present in nanogram quantities or less. The main drawback is the non-specific DNA degradation associated with bisulfite conversion. It is estimated that even under the optimized bisulfite treatment condition, ~ 84–96% of the input DNA is degraded (Grunau *et al.*, 2001). Several non-bisulfite approaches have been developed for methylation detection, relying on methylcytosine affinity reagents (either the methyl-CpG-binding domain protein (MBD2) or 5-methyl cytosine antibody) to preferentially enrich for methylated DNA (Rauch and Pfeifer, 2005), then detected by downstream PCR amplification.

The methylated-CpG island recovery assay (MIRA) has been developed to address shortcomings of sodium bisulfite conversion (Rauch and Pfeifer, 2005). The assay relies on the ability of methyl-CpG binding protein 2 (MBD2/MBD3L1) to selectively pull down methylated DNA, followed by PCR amplification of the CpG island of interest. This assay has been successfully applied to DNA methylation analysis on cultured cell lines, and showed a comparable sensitivity and specificity to MSP (Rauch and Pfeifer, 2005). However, it is not clear whether similar sensitivity and specificity can be achieved on DNA isolated from clinical specimens. More recent studies have demonstrated the feasibility to perform global methylation profiling using an antibody against 5-methylcytosine to preferentially immunoprecipitate methylated DNA (Weber *et al.*, 2007), suggesting that the 5-methylcytosine antibody might be used to replace MBD2 protein in the MIRA assay.

DATA ANALYSIS

Methylation data can be treated as dichotomous or continuous data. Conventional MSP data are dichotomous (methylated or unmethylated). The chi square test or Fisher's exact test, when appropriate, can be used to assess the difference in proportions of methylated genes between cases and controls. On the other hand, both MethyLight and QM-MSP data can be dichotomous (methylated or unmethylated), or continuous, with the latter reflecting the amount of a particular methylation change for a specific gene. In MethyLight assay, the Percentage Methylated Reference (PMR) values described above generally fall between 0 and 100, however, they can rise above 100 due to fluctuations in real-time PCR amplification measurements, or if the *in vitro* SssI treatment is incomplete. In most studies, the MethyLight data are treated as dichotomous data using a fixed cutoff value (PMR below cutoff value as unmethylated, and PMR equal or greater than the cutoff as methylated). If PMR zero is used as the cutoff value, any methylation including very low level of methylation is

considered positive for methylation. One study suggests that a 4% cutoff best discriminated normal and premalignant/malignant tissues (Eads *et al*., 2000b). This was further supported by the detailed analysis correlating methylation data with protein expression analysis (Ogino *et al*., 2006). Because the circulating tumor DNA in blood is low, investigators have used training sample set to determine a cutoff value that best distinguishes case and control samples, then test them on test sample set (Hoque *et al*., 2006). However, little is known whether these experimentally determined cutoff values can be utilized in other independent sample sets, as usually different cutoff values are reported in different studies. Alternatively, the quantity of methylation will be modeled based upon PMR values that have been divided into four levels: zero (unmethylated), "low", "medium", and "high" levels of methylation. The latter three are defined by tertiles among positive values. The difference between levels of methylation in cases and controls is tested using the chi square test or Fisher's exact test (Siegmund and Laird, 2002).

EXAMPLES OF APPLICATIONS

Many genes in all cellular pathways have been reported to be aberrantly methylated at a high frequency in breast tumors but not in normal breast tissues, including p16, GSTP1, CDH1, CCND2, RASSF1A, RARB, HIN1, TWIST, and APC (Campan *et al*., 2006; Esteller *et al*., 2001; Evron *et al*., 2001; Fackler *et al*.; 2003, Jin *et al*., 2001). Several studies have shown that these aberrant methylated genes can also be detected in blood samples (serum or plasma) from breast cancer patients. Earlier studies relied on methylation-sensitive restriction enzyme and PCR amplification to detect methylation changes. For example, p16 methylation was detected in 14% of plasma samples from 35 breast cancer patients (Silva *et al*., 1999b). The presence of p16 methylation 1 year after mastectomy in some patients suggests that methylation assay can detect clinically undetectable micrometastatic disease (Silva *et al*., 2002). Using conventional MSP assays, Hu *et al*. (2003) showed that methylation of p16 or CDH1 was present in 25% of plasma samples from 36 breast cancer patients. Dulaimi *et al*. (2004) showed that methylation of a panel of three genes (RASSF1A, APC, and DAPK1) was present in 76% of sera from 34 breast cancer patients, including 15 precancerous lesions and early stage cancers, but was not present in 28 controls. Rykova *et al*. (2004) detected methylation of APC, RASSF1A, RARB, CDH1 and CDH13 at high frequencies (33–47%) in serum samples from breast cancer patients, though methylation of CDH1 and CDH13 was also detected in serum samples (17%) from patients with benign diseases. Jing *et al*. (2007) showed that methylation of BRCA1, p16 and 14-3-3sigma was present in 27 of 38 (71%) breast cancer patient serum samples, but not in serum samples from 20 patients with fibroadenoma or 20 healthy controls (Jing *et al*., 2007). Sharma *et al*. (2007) analyzed methylation of four genes (p16, p14, Cyclin D2, and Slit2) in 36 breast cancer patients, and 30 (83%) serum samples were positive for methylation of at least one of these four genes. Skvortsova *et al*. (2006) showed that circulating tumor DNA is present as both cell-free and cell-bound fractions

in blood, and methylation of RARB and RASSF1 was present in 95% of breast cancer patients' blood, in 60% of patients with benign lesions and absent in healthy women. Using quantitative MethyLight assays, Taback *et al*. (2006) showed that methylation of RARB, MGMT, RASSF1 and APC was detected in 9 (27%) of 33 early-stage breast cancer patients' serum samples. In a study of 93 African women with advanced breast cancer using quantitative MSP assays, methylation of a panel of four genes (APC, GSTP1, RASSF1 and RARB) had a sensitivity and specificity of 62% and 87%, respectively to detect breast cancer in plasma (Hoque *et al*., 2006). Several studies also correlated the presence of tumor specific DNA methylation changes in breast cancer patient blood with survival and response to treatment. The presence of methylation of RASSF1A and/or APC in serum is associated with poor survival (Muller *et al*., 2003, 2004a), and the detection of RASSF1A methylation correlated the response to adjuvant systemic treatment (Fiegl *et al*., 2005).

PERSPECTIVES AND LIMITATIONS

Recent studies have suggested that detection of circulating tumor DNA using methylation markers has great potential for clinical usage for breast cancer management. Similar to genetic alterations, DNA methylation changes are ubiquitous during tumor development. It can be used not only for early cancer detection, but also for risk assessment, individualization of therapy, prognosis and monitoring effectiveness of treatment.

Caution should be taken when interpreting methylation data, as many factors can affect the sensitivity and specificity of DNA methylation markers for cancer detection. Technically, the low number of starting molecules may result in stochastic amplification, leading to the generation of a PCR product that does not accurately reflect the distribution of methylated and unmethylated molecules in the original sample (Dahl and Guldberg, 2003). Further, a number of potential artifacts and pitfalls should be carefully considered, including PCR bias, incomplete conversion of cytosine, and unintended conversion of 5-methylcytosine. Finally, current assays can only detect fully methylated sequence regions covered by primers and probes, but are unable to detect partially methylated sequences. Biologically, most methylation markers are tumor-associated but not tumor-specific. Many of these tumor specific methylation changes are present at low frequencies in healthy controls and can become prevalent under specific normal physiological conditions. For example, methylation of APC, RASSF1A and TIMP3 was rarely detected in sera of healthy women, but was detected in sera of women in early pregnancy at similar frequencies as in advanced breast cancer patients (Muller *et al*., 2004b). Methylation of certain genes, such as MYOD1, IGF2 and N33, accumulates with age (Fraga *et al*., 2007; Issa, 2003). Although the lack of complete concordance between methylation detected in tumor and those detected in blood can be explained by tumor heterogeneity, it is also possible that some methylation changes detected in blood are not derived from tumor cells. Only when we fully understand the biology behind the origin of DNA methylation, will we

be able to fully harness the power of this epigenetic change in clinical settings.

Several studies have shown that DNA methylation detection in blood has potential in clinical breast cancer management, including diagnosis, prognosis and response to treatment. Larger well-controlled studies are necessary to validate the usefulness of current methylation markers. Novel markers are needed to increase the sensitivity and specificity of current markers. New technologies such as bisulfite-independent methylation detection, microfluidity and PCR on chip will no doubt be needed for the transition of epigenetic markers from the lab to the bedside.

REFERENCES

Campan, M., Weisenberger, D.J., and Laird, P.W. 2006. DNA methylation profiles of female steroid hormone-driven human malignancies. *Curr. Top. Microbiol. Immunol. 310*: 141–178.

D'Alessio, A.C., and Szyf, M. 2006. Epigenetic tete-a-tete: the bilateral relationship between chromatin modifications and DNA methylation. *Biochem. Cell Biol. 84*: 463–476.

Dahl, C., and Guldberg, P. 2003. DNA methylation analysis techniques. *Biogerontology 4*: 233–250.

Eads, C.A., Danenberg, K.D., Kawakami, K., Saltz, L.B., Blake, C., Shibata, D., Danenberg, P.V., and Laird, P.W. 2000a. MethyLight: a high-throughput assay to measure DNA methylation. *Nucleic Acids Res. 28*: E32.

Eads, C.A., Lord, R.V., Kurumboor, S.K., Wickramasinghe, K., Skinner, M.L., Long, T.I., Peters, J.H., DeMeester, T.R., Danenberg, K.D., Danenberg, P.V., Laird, P.W., and Skinner, K.A. 2000b. Fields of aberrant CpG island hypermethylation in Barrett's esophagus and associated adenocarcinoma. *Cancer Res. 60*: 5021–5026.

Esteller, M. 2007. Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat. Rev. Genet. 8*: 286–298.

Esteller, M., Sparks, A., Toyota, M., Sanchez-Cespedes, M., Capella, G., Peinado, M.A., Gonzalez, S., Tarafa, G., Sidransky, D., Meltzer, S.J., Baylin, S.B., and Herman, J.G. 2000. Analysis of adenomatous polyposis coli promoter hypermethylation in human cancer. *Cancer Res. 60*: 4366–4371.

Esteller, M., Corn, P.G., Baylin, S.B., and Herman, J.G. 2001. A gene hypermethylation profile of human cancer. *Cancer Res. 61*: 3225–3229.

Evron, E., Umbricht, C.B., Korz, D., Raman, V., Loeb, D.M., Niranjan, B., Buluwela, L., Weitzman, S.A., Marks, J., and Sukumar, S. 2001. Loss of cyclin D2 expression in the majority of breast cancers is associated with promoter hypermethylation. *Cancer Res. 61*: 2782–2787.

Fackler, M.J., McVeigh, M., Evron, E., Garrett, E., Mehrotra, J., Polyak, K., Sukumar, S., and Argani, P., 2003. DNA methylation of RASSF1A, HIN-1, RAR-beta, Cyclin D2 and Twist in in situ and invasive lobular breast carcinoma. *Int. J. Cancer 107*: 970–975.

Fackler, M.J., McVeigh, M., Mehrotra, J., Blum, M.A., Lange, J., Lapides, A., Garrett, E., Argani, P., and Sukumar, S. 2004. Quantitative multiplex methylation-specific PCR assay for the detection of promoter hypermethylation in multiple genes in breast cancer. *Cancer Res. 64*: 4442–4452.

Fiegl, H., Millinger, S., Mueller-Holzner, E., Marth, C., Ensinger, C., Berger, A., Klocker, H., Goebel, G., and Widschwendter, M. 2005. Circulating tumor-specific DNA: a marker for monitoring efficacy of adjuvant therapy in cancer patients. *Cancer Res. 65*: 1141–1145.

Fraga, M.F., Agrelo, R., and Esteller, M. 2007. Cross-talk between aging and cancer: the epigenetic language. *Ann. N. Y. Acad. Sci. 1100*: 60–74.

Goessl, C., Muller, M., Straub, B., and Miller, K. 2002. DNA alterations in body fluids as molecular tumor markers for urological malignancies. *Eur. Urol. 41*: 668–676.

Grunau, C., Clark, S.J. and Rosenthal, A. 2001. Bisulfite genomic sequencing: systematic investigation of critical experimental parameters. *Nucleic Acids Res. 29*: e65.

Herman, J.G., Graff, J.R., Myohanen, S., Nelkin, B.D. and Baylin, S.B. 1996. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc. Natl. Acad. Sci. U. S. A. 93*: 9821–9826.

Hoque, M.O., Feng, Q., Toure, P., Dem, A., Critchlow, C.W., Hawes, S.E., Wood, T., Jeronimo, C., Rosenbaum, E., Stern, J., Yu, M., Trink, B.,

Kiviat, N.B. and Sidransky, D. 2006. Detection of aberrant methylation of four genes in plasma DNA for the detection of breast cancer. *J. Clin. Oncol. 24*: 4262–4269.

Huang, Z.H., Li, L.H. and Hua, D. 2006. Quantitative analysis of plasma circulating DNA at diagnosis and during follow-up of breast cancer patients. *Cancer Lett. 243*: 64–70.

Issa, J.P. 2003. Age-related epigenetic changes and the immune system. *Clin. Immunol. 109*: 103–108.

Jeronimo, C., Usadel, H., Henrique, R., Silva, C., Oliveira, J., Lopes, C. and Sidransky, D. 2002. Quantitative GSTP1 hypermethylation in bodily fluids of patients with prostate cancer. *Urology 60*: 1131–1135.

Jin, Z., Tamura, G., Tsuchiya, T., Sakata, K., Kashiwaba, M., Osakabe, M. and Motoyama, T. 2001. Adenomatous polyposis coli (APC) gene promoter hypermethylation in primary breast cancers. *Br. J. Cancer 85*: 69–73.

Jing, F., Zhang, J., Tao, J., Zhou, Y., Jun, L., Tang, X., Wang, Y. and Hai, H. 2007. Hypermethylation of tumor suppressor genes BRCA1, p16 and 14-3-3sigma in serum of sporadic breast cancer patients. *Onkologie 30*: 14–19.

Jones, P.A. and Baylin, S.B. 2002. The fundamental role of epigenetic events in cancer. *Nat. Rev. Genet. 3*: 415–428.

Jones, P.A. and Baylin, S.B. 2007. The epigenomics of cancer. *Cell 128*: 683–692.

Keshet, I., Schlesinger, Y., Farkash, S., Rand, E., Hecht, M., Segal, E., Pikarski, E., Young, R.A., Niveleau, A., Cedar, H. and Simon, I. 2006. Evidence for an instructive mechanism of de novo methylation in cancer cells. *Nat. Genet. 38*: 149–153.

Kouzarides, T. 2007. Chromatin modifications and their function. *Cell 128*: 693–705.

Muller, H.M., Widschwendter, A., Fiegl, H., Ivarsson, L., Goebel, G., Perkmann, E., Marth, C. and Widschwendter, M. 2003. DNA methylation in serum of breast cancer patients: an independent prognostic marker. *Cancer Res. 63*: 7641–7645.

Muller, H.M., Fiegl, H., Widschwendter, A. and Widschwendter, M. 2004a. Prognostic DNA Methylation marker in serum of cancer patients. *Ann. N. Y. Acad. Sci. 1022*: 44–49.

Muller, H.M., Ivarsson, L., Schrocksnadel, H., Fiegl, H., Widschwendter, A., Goebel, G., Kilga-Nogler, S., Philadelphy, H., Gutter, W., Marth, C. and Widschwendter, M. 2004b. DNA methylation changes in sera of women in early pregnancy are similar to those in advanced breast cancer patients. *Clin. Chem. 50*: 1065–1068.

Ogino, S., Kawasaki, T., Brahmandam, M., Cantor, M., Kirkner, G.J., Spiegelman, D., Makrigiorgos, G.M., Weisenberger, D.J., Laird, P.W., Loda, M. and Fuchs, C.S. 2006. Precision and performance characteristics of bisulfite conversion and real-time PCR (MethyLight) for quantitative DNA methylation analysis. *J. Mol. Diagn. 8*: 209–217.

Palmisano, W.A., Divine, K.K., Saccomanno, G., Gilliland, F.D., Baylin, S.B., Herman, J.G. and Belinsky, S.A. 2000. Predicting lung cancer by detecting aberrant promoter methylation in sputum. *Cancer Res. 60*: 5954–5958.

Rauch, T. and Pfeifer, G.P. 2005. Methylated-CpG island recovery assay: a new technique for the rapid detection of methylated-CpG islands in cancer. *Lab Invest. 85*: 1172–1180.

Rauch, T., Li, H., Wu, X. and Pfeifer, G.P. 2006. MIRA-assisted microarray analysis, a new technology for the determination of DNA methylation patterns, identifies frequent methylation of homeodomain-containing genes in lung cancer cells. *Cancer Res. 66*: 7939–7947.

Schwarzenbach, H., Muller, V., Stahmann, N. and Pantel, K. 2004. Detection and characterization of circulating microsatellite-DNA in blood of patients with breast cancer. *Ann. N. Y. Acad. Sci. 1022*: 25–32.

Siegmund, K.D. and Laird, P.W. 2002. Analysis of complex methylation data. *Methods 27*: 170–178.

Silva, J.M., Dominguez, G., Garcia, J.M., Gonzalez, R., Villanueva, M.J., Navarro, F., Provencio, M., San Martin, S., Espana, P. and Bonilla, F. 1999a. Presence of tumor DNA in plasma of breast cancer patients: clinicopathological correlations. *Cancer Res. 59*: 3251–3256.

Silva, J.M., Dominguez, G., Villanueva, M.J., Gonzalez, R., Garcia, J.M., Corbacho, C., Provencio, M., Espana, P. and Bonilla, F. 1999b. Aberrant DNA methylation of the p16INK4a gene in plasma DNA of breast cancer patients. *Br. J. Cancer 80*: 1262–1264.

Silva, J.M., Garcia, J.M., Dominguez, G., Silva, J., Miralles, C., Cantos, B., Coca, S., Provencio, M., Espana, P. and Bonilla, F. 2002. Persistence of tumor DNA in plasma of breast cancer patients after mastectomy. *Ann. Surg. Oncol. 9*: 71–76.

Sova, P., Feng, Q., Geiss, G., Wood, T., Strauss, R., Rudolf, V., Lieber, A. and Kiviat, N. 2006. Discovery of novel methylation biomarkers in cervical carcinoma by global demethylation and microarray analysis. *Cancer Epidemiol. Biomarkers Prev. 15*: 114–123.

Strunnikova, M., Schagdarsurengin, U., Kehlen, A., Garbe, J.C., Stampfer, M.R. and Dammann, R. 2005. Chromatin inactivation precedes de novo DNA methylation during the progressive epigenetic silencing of the RASSF1A promoter. *Mol. Cell Biol. 25*: 3923–3933.

Taback, B., O'Day, S.J. and Hoon, D.S. 2004. Quantification of circulating DNA in the plasma and serum of cancer patients. *Ann. N. Y. Acad. Sci. 1022*: 17–24.

Taback, B., Giuliano, A.E., Lai, R., Hansen, N., Singer, F.R., Pantel, K. and Hoon, D.S. 2006. Epigenetic analysis of body fluids and tumor tissues: application of a comprehensive molecular assessment for early-stage breast cancer patients. *Ann. N. Y. Acad. Sci. 1075*: 211–221.

Thijssen, M.A., Swinkels, D.W., Ruers, T.J. and de Kok, J.B. 2002. Difference between free circulating plasma and serum DNA in patients with colorectal liver metastases. *Anticancer Res. 22*: 421–425.

Weber, M., Hellmann, I., Stadler, M.B., Ramos, L., Paabo, S., Rebhan, M. and Schubeler, D. 2007. Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nat. Genet. 39*: 457–466.

Weisenberger, D.J., Campan, M., Long, T.I., Kim, M., Woods, C., Fiala, E., Ehrlich, M. and Laird, P.W. 2005. Analysis of repetitive element DNA methylation by MethyLight. *Nucleic Acids Res. 33*: 6823–6836.

12
Breast Cancer Patients: Detection of Circulating Cancer Cell-Related mRNA Markers with Membrane Array Method

Shiu-Ru Lin, Jaw-Yuan Wang, Ming-Yii Huang, and Chung-Chi Chen

INTRODUCTION

The diagnosis and the therapy of early-stage tumors have the potential to decrease morbidity and mortality of patients with malignancy. Although promising advances in imaging technology and other diagnostic modalities have been achieved recently, early diagnosis of patients with malignancy remains a challenge for physicians. It was reported by Weidner (1993) that active angiogenesis may occur in cancer tissues growing to 2 mm in diameter. Evidence is accumulating that primary cancers begin shedding neoplastic cells into the circulation at an early stage (Smirnov *et al*., 2005); ~ 10^6 cells are shed daily per gram of tumor (Chang *et al*., 2000). Thus, circulating tumor cells (CTCs) is a potential source for the noninvasive and early diagnosis for cancer patients. O'Sullivan *et al*. (1997) indicated that preoperative detection of micrometastases may reflect either transient shedding of tumor cells, metastatic potential, or residual disease, but postoperative micrometastases are likely to indicate minimal residual disease. Such neoplastic cells may be present in the bloodstream in very low numbers and would hardly be detected by conventional methods. Consequently, a sensitive and powerful method for detection of CTCs is essential not only to increase the accuracy, but also to aid in the development of a novel non-invasive diagnostic strategy.

LIMITATION OF TRADITIONAL STANDARD METHOD FOR DETECTING THE CIRCULATING CANCER CELLS

In recent years, several methods, including flow cytometry and immunohistochemistry (Giuliani *et al*., 2005) have been developed to detect CTCs in the blood of patients with different types of malignancies. However, to date, a high-sensitivity and high-throughput method for the detection of CTCs is lacking. In colorectal cancer (CRC) many researchers have demonstrated that diagnosis and therapy of early-stage tumors have the potential to decrease the mor-

bidity and mortality of CRC patients (Hermanek, 1995). However, many traditional clinical diagnostic methods, such as sigmoidoscopies, colonoscopies, and double-contrast barium enemas, are unsuitable for broad screening programs mainly because of their low general acceptance and highly-invasive nature (Frazier *et al.*, 2000). Tests for fecal occult blood are noninvasive and useful, especially as an adjunct to sigmoidoscopies. However, the relatively high false-positive rates and other problems have led to a search for more specific noninvasive tests (Ahlquist and Shuber, 2002). Because early detection is one of the most effective means of reducing cancer mortality, the development of a sensitive, specific, and convenient diagnostic method for detection of CTCs at a very early stage is an issue of utmost importance (Wu *et al.*, 2006).

The analysis of genetic anomalies has led to fundamental progress and clinical advances. The following techniques study genetic anomalies: Flowcytometry evaluates the quantity of DNA in the nucleus during the cell cycle. Cytogenetics is the study of karyotype anomalies by loss or gain of chromosome material and structural changes. Molecular biology gives a means of recognizing chromosome losses and in particular allows the study of oncogenic or antioncogenic mutations. These anomalies correspond to alterations found in tumors. Studying these alterations will allow better prediction of high-risk subjects in high-risk cancer families (Salmon *et al.*, 1994). Despite having been proven to be powerful tools, these techniques are limited due to their ability to monitor only one or a few tumor-related molecules for each specimen in a single test.

DETECTION OF MRNA-RELATED MOLECULES BY REVERSE-TRANSCRIPTASE PCR (RT-PCR)

Hematogenous tumor-cell dissemination during diagnostic and therapeutic procedures in patients with CRC has been demonstrated. Approximately 20–45% of CRC patients ultimately develop local recurrence or metastasis following curative surgical resection. The problem is caused by tumor cells being shed from the primary carcinoma prior to or during operation, which is currently undetected by standard clinical staging. Fortunately, the presence of tumor cells in peripheral blood can be detected by molecular methods and is being regarded increasingly as a clinically relevant prognostic factor.

Metastasis is a multistep process which requires highly adapted interactions of tumor cells with host target organs. Cancer cells detach from the site of primary tumor and are distributed to hematopoietic of lymphatic tissue. This can lead to the appearance of gene transcripts that are not normally expressed in these host tissues. Tumor-specific abnormalities present in the DNA or mRNA of malignant cells can be detected for the prediction of disease progression (Ghossein *et al.*, 1999). In the last decade, numerous groups have attempted to detect occult tumor cells in solid malignancies using assays that were, in the vast majority of cases, directed against tissue-specific markers. Tissue-

specific transcripts (mRNA) were detected with high specificity in the blood of patients with malignant solid tumors. The molecular detection and characterization of occult tumor cells offer an opportunity for better stratifying patients with solid tumors and for developing new prognostic markers and targeted therapies (Ghossein and Bhattacharya, 2000).

Using RT-PCR to select some of the most suitable mRNAs that were assayed in blood samples from normal subjects and patients with CRC as possible markers for the presence of epithelial cells in the blood can be performed. In our previous study, we simultaneously examined human telomerase reverse transcriptase (hTERT), cytokeratin-19 (CK-19), cytokeratin-20 (CK-20), and carcinoembryonic antigen (CEA) mRNA in the peripheral blood of 72 CRC patients and 30 healthy individuals for detecting the presence of CTCs and evaluate their relationship to postoperative metastatic relapse. Tumor-related mRNAs were amplified using a RT-PCR; in addition, analysis was carried out to find the correlation with patients' clinicopathologic features as well as the occurrence of postoperative metastasis. The results show that CRC patients expressing positive CEA mRNA in peripheral blood have a significantly higher risk of postoperative metastasis (Wang *et al*., 2006a).

It becomes obvious that it is feasible to use RT-PCR for the detection of CEA mRNA, and this may be a promising tool for early detection of micrometastatic CTCs in CRC patients. Nevertheless, confirmation of CEA mRNA as a prognostic predictive factor requires the continuation of patient follow-up (Wang *et al*., 2006a).

HISTOLOGICAL REVIEW OF DEVELOPING A POWER TOOL FOR DETECTING CIRCULATING CANCER CELLS WITH THE MEMBRANE ARRAY

Reverse transcriptase-polymerase chain reaction (RT-PCR) is limited to monitoring only a few markers for each specimen in a single test. Examination containing quantities of samples or molecular markers will consume much time and effort. In addition, most tumors have the characteristics of heterogeneity, and the degrees of sensitivity achieved by the mRNA-based assays reported in the studies detecting single mRNA markers remain too low for clinical applications. In contrast, use of multiple markers in combination has been demonstrated to be capable of significantly raising the sensitivity of molecular detection for cancers (Hoon *et al*., 1995; Taback *et al*., 2001).

Rapidly developed in recent years, gene chips are one effective tool to detect biological molecules. A large amount of biologically-associated examination can be done on a small area of material. The advantages are: (1) Simultaneous fast and accurate analysis of multiple genes. Its efficiency is thousand times higher than that of traditional biochemical examinations, which is a significant improvement over previous methods. In addition, most biological responses or diseases involve the interaction and regulation of multiple genes, so gene chips can provide researchers with wider and more comprehensive gene information. It also changes the traditional method of gene study where one researcher concentrates on only one

gene for their whole life. (2) The advantages of miniaturization are: Gene chips need only a small sample, so test results can be obtained even if the sample is minute. Furthermore, parallel, simultaneous analysis can decrease the consumption of reagents and materials, so the cost and required amount of sample can be reduced. (3) Automatic analysis: There are tens of thousands of closely arranged molecular microarrays on the gene chip. Through automatic detecting software, a large amount of biological molecules can be analyzed in a short duration, and two of its major functions (gene quantitation and research for different genes) can be utilized for fast and accurate analysis. The development of the gene chip resolves the problem of costs and artifacts in traditional detecting methods, and thus becomes an important milestone in the field of clinical diagnosis. However, gene chips and fluorescence scanners for reading test results are still expensive; therefore, their popularity is still limited. At present, gene chips are used mainly for research purposes in laboratories and are not applied clinically for cancer diagnosis.

For this reason, it is believed that creating a low-cost, easily operative and highly effective system to detect cancer molecular markers will be a great improvement in cancer diagnosis. We have developed the membrane-array method, which uses the chemical colorimetric method, that replaces glass chips with the fluorescence of gene chips and nylon membranes. The cost and technology threshold is thus reduced significantly and the original advantages of the gene chip are also preserved. The flowchart of this technique is as follows (Figure 12.1).

Membrane Array Preparation

We used software to design oligonucleotide probe sequences for target genes, and housekeeping genes served as an internal control. The newly synthesized oligonucleotide fragments were dissolved in DI-water to a concentration of 20 mM and then applied to a BioJet Plus 3,000 nl dispense system, which blotted the oligonucleotides (50 nl per spot and 1.5 mm between spots) on SuPerCharge nylon membrane sequentially, in triplicate. DMSO

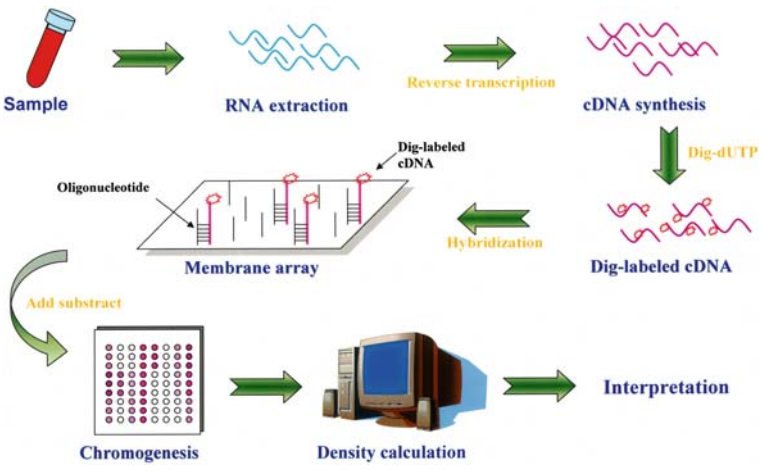

FIGURE 12.1. The platform of colorimetric membrane array method. The flowchart of detecting mRNAs from peripheral blood by colorimetric membrane array method

was also dispensed onto the membrane as a blank control. After rapid drying and cross-linking procedures, the preparation of the diagnostic membrane array was accomplished.

Preparation of Digoxigenin-Labeled cDNA Targets and Hybridization

First-strand cDNA targets for hybridization were produced by using SuperScript II reverse transcriptase (Gibco-BRL) in the presence of digoxigenin (DIG)-labeled UTP (Roche Diagnostics GmbH, Penzberg, Germany). After procedures of prehybridization and blocking, the chips were subjected to hybridization. The lifts were covered with the Express Hyb Hybridization Solution (BD biosciences, Palo Alto, CA, USA) containing DIG-11-UTP-labeled cDNA probes, and then incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Diagnostics). For hybridization, the arrays were incubated at 42°C for 12 h in a humidity chamber. After washing, the arrays were exposed to light. For signal detection, the gene chips were incubated in chromogen solution containing nitroblue-tetrazolium and 5-bromo-4-chloro3-indoyl-phosphate (NBT/BCIP) for 15 min. Subsequent quantification analysis of each spot's intensity was carried out by using AlphaEase® FC software (Alpha Innotech Corp., San Leandro, CA, USA). Spots consistently carrying by a factor of two or more were taken as differentially expressed.

This technique was first applied in sensing the activation of the K-ras oncogene in the peripheral blood of cancer patients. The objective of this study was to develop a diagnostic membrane array using activated K-ras oncogene-associated molecules as detection targets. In our previous study, cDNA microarray analysis showed that there were 94 genes differentially expressed in K-ras mutant stably transfected adrenocortical cells. In the present study, we obtained 22 up-regulated genes with the closest relation to the K-ras oncogene through bioinformatic analysis. Sensitivity and specificity along with various numbers of differentially expressed genes on the diagnostic membrane were calculated and ROC curves were constructed. According to the analysis of the ROC curves, the optimal cut-off point for the number of differentially expressed genes was 11, i.e., a diagnostic membrane on which 11 (or > 11) of 22 genes expressing twofold higher than normal levels was considered to be positive and vice versa (Chen *et al*., 2005).

The results revealed that the sensitivity, specificity, and accuracy of the diagnostic membrane arrays were 83.7%, 90.9%, and 86.8%, respectively. In order to explore the feasibility and detecting sensitivity of membrane arrays, we introduced *in vitro* cultures of K-ras mutant stably transfected adrenocortical cells into normal blood specimens to evaluate our diagnostic membrane analysis. We found that it could positively detect the activation of the K-ras gene in 5 ml blood specimens containing as few as 25 transfected cells. The results demonstrated that our diagnostic method was highly feasible. This technique can, therefore, be applied in clinical diagnosis and is a test platform with high sensitivity (Chen *et al*., 2005).

Although this technique can significantly reduce the difficulty and cost of experiments

and help to increase its popularity and application in the medical marketplace, the operation and reading of colorimetric methods are easily affected by artifacts. Therefore, we are starting to develop another chemiluminescent gene chip to detect CTCs in peripheral blood (Figure 12.2). The chemiluminescent method can provide excellent sensitivity and stability, and has a low background level and an extended range of detection. By real-time luminometric approach, quantitative data can be obtained to decrease the artifacts in the colorimetric method. Results from initial tests reveal that the sensitivity and specificity of luminescence gene chips were 92% and 93%, respectively, in CTCs in peripheral blood of breast cancer patients (Figure 12.3). Furthermore, luminescence gene chips can detect as few as two cancer cells per milliliter of blood (Figure 12.4). The initial results show that luminescence gene chips can increase the accuracy of diagnosis as a whole; however, a large amount of clinical samples from different cancers are still required for further experiments.

Comparison of Membrane Array Method with Real-Time PCR

Because real-time PCR is highly accurate and easily reproductive, it is considered the pinnacle of tumor detection. Therefore, we further compared the clinical diagnostic value of real-time PCR and colorimetric membrane array. In the study of breast cancer, we collected peripheral blood from 80 healthy females and 102 patients. Real-time PCR and membrane array were used to detect the expression of cytokeratin 19 (CK-19), carcinoembryonic antigen (CEA), c-Met, Her2/*neu*, and mammaglobin (hMAM) in the blood. Then we compared the correlation between data from real-time PCR and membrane array by linear regression and Pearson correlation, and the results revealed a high correlation ($r = 0.979$, $P < 0.0001$) (Chen *et al*., 2006b). In previous study, we collected samples from 88 patients of colorectal cancer and 50 healthy individuals. Real-time PCR and membrane array were used to detect the expression of 18 genes associated with colorectal cancer.

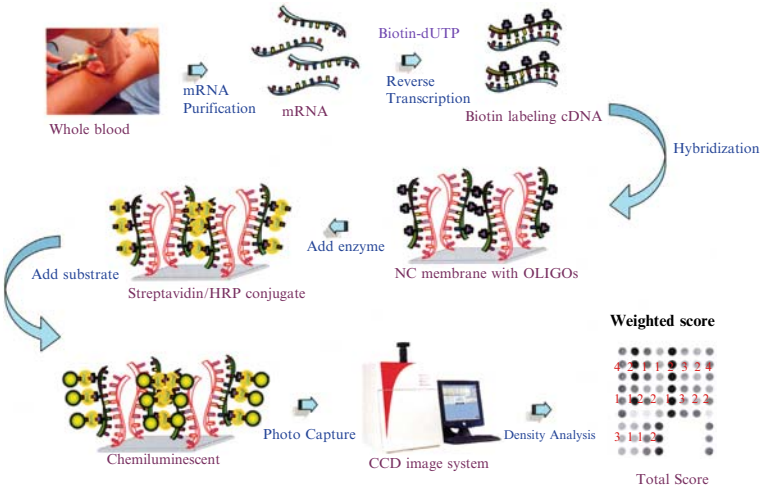

FIGURE 12.2. The platform of chemiluminescent membrane array method. The flowchart of detecting mRNAs from peripheral blood by chemiluminescent membrane array method

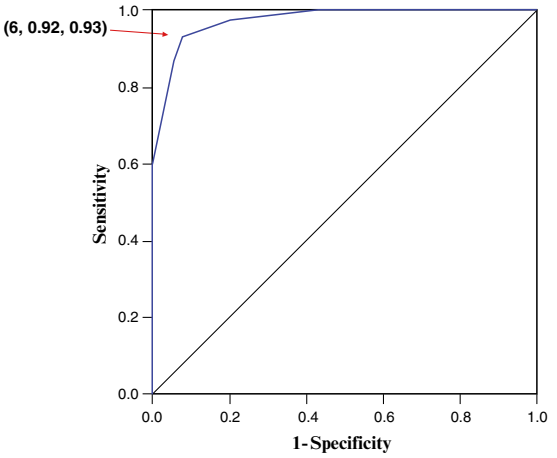

FIGURE 12.3. ROC curve of chemiluminescent membrane array. An ROC curve is drawn according to the analysis of our experimental results by Statistical Package for the Social Sciences Version 10.0. An optimal cut-off point is also obtained (6, 0.92, 0.93)

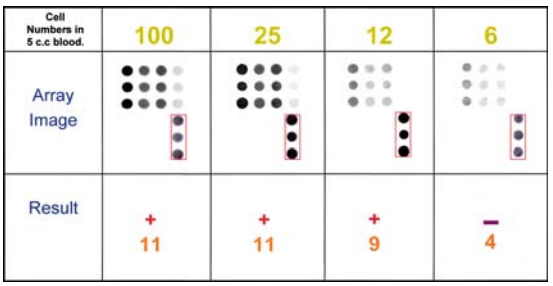

FIGURE 12.4. Detective limitation of chemiluminescent membrane array. Cell dilution experiments with the breast cancer cell line MCF-7 for the detection of the sensitivity of the membrane array. Five milliliters of normal blood samples were mixed with a known number of MCF-7 cells (100, 25, 12 and 6 cells) and then subjected to membrane array. After computer analysis, each 5-ml sample blood containing at least 12 cancer cells was positively detected.

High correlation was also demonstrated between these two methods ($r = 0.979$, $P < 0.001$) (Wang *et al*., 2006b). Compared to real-time PCR, colorimetric membrane array can detect multiple gene targets simultaneously and therefore saves time and cost. For this reason, applying the colorimetric membrane array technique to detect CTCs in a patient's peripheral blood shows promise for future applications.

Cancer is a leading cause of death worldwide. From a total of 58 million deaths worldwide in 2005, cancer accounted for 7.6 million (13%) of all deaths (World Health Organization, 2005). Early detection of cancer is one of the goals of cancer treatment; researchers have demonstrated that early detection is one of the most effective methods to decrease the incidence and mortality of breast cancer. Early diagnosis can increase not only the options of treatment, but also the success rate. Accordingly, if an accurate diagnostic technology can be developed to be an adjunctive diagnostic tool for cancer, this malignancy can be more effectively diagnosed. Our technique was developed with this goal; relevant research has proved that this technique can provide patients with a simple and comfortable examination, increase the general sensitivity and accuracy of examination (Chen *et al*., 2005, 2006a, b; Chong *et al*., 2006; Wang *et al*., 2006 a, b; Yeh *et al*., 2006), and also decrease artifacts. Providing fast and effective diagnosis, it has the potential to become a screening tool of the future and help patients to obtain timely treatment. We believe that this technique will become an invaluable tool in the fight against cancer.

Potential Clinical Application of Membrane Array Method

Peripheral blood sampling is relatively easy as a source of tumor markers. In consideration of maximizing the potential of these markers in clinical implication, blood is regarded as an alternative option when compared to tissue specimens on the

basis of our recent studies (Wang *et al*., 2004a, b; Yeh *et al*., 2006) and by other investigators (Ghossein *et al*., 1999; Mori *et al*., 1998) demonstrating either a significant overexpression of these markers or a prominent correlation between the expression of these markers in tumor tissue and that in peripheral blood of gastric or colorectal cancer patients. For clinical practice, the mRNA markers from blood samples have the advantage of being potential markers in the postoperative surveillance for cancer patients, whereas those from tissue samples could not be used as tumor markers. Therefore, CTCs in peripheral blood seem to be more feasible as a target for early detection of cancer patients.

In an attempt to increase the sensitivity of detection of these CTCs, several approaches have been extensively exploited recently. With recent developments in molecular technology, the use of PCR, RT-PCR, or real-time PCR assays now permit sensitive detection of CTCs in peripheral blood. In fact, accumulated reports describing the detection of CTCs in the peripheral blood of cancer patients have important prognostic and therapeutic implications (Bepler *et al*., 2004; Burchill *et al*., 1999; Ghossein *et al*., 1999). Using RT-PCR or real-time PCR, our previous investigations have also demonstrated the clinical significance of CTCs-related mRNA markers as prognostic markers in various human cancers (Uen *et al*., 2006; Wu *et al*., 2006). Although these methods provide useful data, one of the limitations is that the methodology can analyze only one molecular target in one experiment. Consequently, for multiple gene detection, RT-PCR or real-time PCR is too time-consuming and laborious to apply in clinical diagnosis. Additionally, due to the heterogeneity of tumor-related genes, a multi-marker assay is regarded as more reliable and sensitive than a single marker assay (Conzelmann *et al*., 2005; Hoon *et al*., 1995; Racila *et al*., 1998). Likewise, in our RT-PCR analysis of peripheral blood for colorectal cancer patients, the positive rates of hTERT, CK-19, CK-20, and CEA mRNA for the detection of CTCs were 69.4%, 66.7%, 52.8%, and, 72.2%, respectively. However, the detection rate of CTCs increased to 87.5% using these four mRNAs concurrently (Wang *et al*., 2006). Consequently, a group of candidate genes related to carcinogenesis would likely overcome inter-tumoral variations, and increase the detection rate for CTCs.

With respect to the detection sensitivity of each mRNA marker in gastric cancer patients, most investigators indicated about one to two cancer cells in 1 ml blood samples, or ~ one cancer cell per 1×10^6 to 2×10^6 lymphocytes (Miyazono *et al*., 2001). Our detection sensitivity of colorimetric membrane-array method was five cancer cells in 1 ml blood using *in vitro* cell-dilution test, which was apparently inferior to previous investigations due to multi-marker analysis. Nevertheless, for the detection of each mRNA marker, our colorimetric membrane-array method was comparable to that by RT-PCR with a similar detection limit. Furthermore, the result of the cell dilution test seemed to consistently reflect the tumor cell number in blood samples.

Using colorimetric membrane-array method, we observed that the detection rates of using single markers for CTCs in gastric cancer patients ranged from 78.1% to 82.8%, apparently higher than the results with RT-PCR (61.9–78.6%) in our previous observation (Wu *et al*., 2006) or those in another investigation from (35.5–51.6%)

(Huang *et al*., 2003). Similarly, our recent works showed that detection sensitivity (77.1–82.8%, Table 12.1) of the colorimetric membrane-array method is considerably higher than that of our previous RT-PCR assay (52.8–72.2%) for the detection of CTCs in each corresponding molecular marker for colorectal cancer patients (Wang *et al*., 2006a, b).

This colorimetric membrane-array assay is more accurate in discriminating cancer patients from normal subjects, with the advantages being more time-saving and cost-effectiveness than PCR-based techniques (Chen *et al*., 2006; Chong *et al*., 2006; Wang *et al*., 2006a, b; Wu *et al*., 2006). Presently, real-time PCR has been widely accepted as a sensitive method in specifically detecting the expression of mRNA markers (Mocellin *et al*., 2003). Our study also demonstrates that membrane-arrays are highly significantly correlated with real-time PCR for the detection of each target gene expressed in CTCs. Meanwhile, the sensitivity, specificity, and accuracy of each gene by membrane-arrays are closely compatible with real-time PCR (Chen *et al*., 2006; Chong *et al*., 2006; Wu *et al*., 2006). To detect the gene expression for five markers in peripheral blood of 102 patients with breast cancer by real-time PCR, showed the correlation of the genes expression ratio analyzed by real-time PCR and membrane-arrays. There was a highly significant correlation between these two methods ($P < 0.001$, r = 0.979) (Chen *et al*., 2006).

In addition, membrane-arrays outstanding accuracy and striking correlation with the clinical stage enables its potential application in the early detection and post-operative surveillance of gastric, colorectal, breast, and lung cancer patients. Furthermore, the detection of CTCs by membrane-arrays was observed with a tendency of higher detection rate in cancer patients with depth of tumor invasion, lymph node metastasis, or advanced stage (Wang *et al*., 2006a).

Similarly, Mori *et al*. (1998) indicated that the positive detection rate for tumor-specific mRNA in peripheral blood samples increased with the advanced stages of gastrointestinal malignancies. As analyzed in combination, expression of more mRNA markers was more significantly correlated with the clinicopathologi-

Table 12.1. Sensitivity, specificity, positive predictive value, negative predictive value and accuracy of each mRMA marker and the combination between colorectal cancer patients and healthy individuals.

	Sensitivity (%)	Specificity (%)	Positive predictive value(%)	Negative predictive value (%)	Accuracy (%)
	(95% confidence interval)				
hTERT mRNA	80.3 (66.7–93.8)	80.0 (66.4–93.6)	88.7 (77.9–99.5)	67.4 (51.4–83.4)	80.2
CK-19 mRNA	79.6 (64.0–92.2)	78.8 (64.8–92.7)	88.0 (77.0–99.1)	66.3 (50.2–82.4)	79.3
CK-20 mRNA	77.1 (62.7–91.4)	77.5 (63.3–91.7)	87.1 (75.6–98.5)	63.3 (46.8–79.7)	77.2
CEA mRNA	82.8 (69.9–95.7)	81.3 (67.9–94.6)	89.7 (79.3–100.0)	70.7(55.1–86.2)	82.3
Any one mRNA	92.4 (83.3–101.4)	67.5 (51.5–83.5)	84.8 (72.5–97.0)	81.8 (68.7–95.0)	84.0
All four mRNA	57.3 (40.4–74.2)	100.0	100.0	54.4 (37.4–71.4)	71.7

Source: adapted from wang et al; World J Surg. 30: 1007–1013, 2006

cal characteristics than a single marker. Concomitant molecular analysis for CTCs with a multi-marker panel is justifiable in supplementary approaches to the current pathological staging system, which may help physicians make judgments that are more accurate on clinical treatments and predicting prognosis for cancer patients. In fact, the membrane-array method could identify patients at risk of postoperative relapse, even colorectal cancer patients with normal perioperative serum CEA levels. Concurrently, cancer patients expressing all mRNA markers are found to have a poorer survival rate (Wang *et al*., 2006a, b; Wu *et al*., 2006). Therefore, the identification of CTCs in cancer patients could lead to novel staging approaches or prognostic values, or even new treatment modalities.

A high false-positive prediction rate of postoperative relapse by membrane-array methods occurred in our previous investigations (Wu *et al*., 2006); hence, it indicated that there is room for the improvement of this method. First, the high false-positive rate of membrane array in the prediction of postoperative relapse may be attributable to the artifactual expression of mRNA, notably by macrophages, leukocytes, and hematopoietic cells (Pantel *et al*., 1999). Another cause of a high false-positive rate, at least in part, might result from the timing of blood sampling during surgical procedures (Miyazono *et al*., 2001), which probably enhances the relief of CTCs. A blood sampling procedure carried out after operation can be one way of overcoming such a problem. Alternatively, this may be quite reasonable because few carcinoma cells shed into the bloodstream succeed in establishing metastatic disease (O'Sullivan *et al*., 1997). In addition, extending the follow-up period may really identify patients who ultimately develop postoperative relapse. In fact, using microarray technology and gene-expression profiling to identify more specific markers of risk of relapse in cancer patients might be another way to improve the accuracy of molecular detection methods. Finally, the concept of serial measurement of mRNA markers might be probably formed based on the clinical experience of serum tumor markers for the follow-up of cancer patients. However, large scale and long-term clinical study follow-up is warranted to confirm these hypotheses.

Incidentally, the false-negative rate of the membrane-array method in predicting postoperative relapse, at least in part, might result from CTCs intermittently flowing into the bloodstream of the bowel or the heterogeneous character of the tumor itself (Mori *et al*., 1998; Sher *et al*., 2005; Wang *et al*., 2006a, b). Using multiple blood sampling or a refined normalization procedure might improve the sensitivity of the membrane-array method (Wharton *et al*., 1999). On the other hand, a deficient expression of the marker genes in micro-metastatic tumor cells or insufficient numbers of tumor cells in blood samples may subsequently lead to false-negatives (Pantel *et al*., 1999). Thus, it will be important to define the critical variables in the methods and to introduce at least some level of standardization to allow for more reliable and reproducible results.

Conversely, some recently published studies have reported conflicting results regarding the prognostic value of CTCs (Bessa *et al*., 2003; Bosch *et al*., 2003; Wyld *et al*., 1998). A major problem of most of the published studies is that only small, inhomogeneous patient groups with

short follow-up periods were evaluated. Another important issue concerns the most adequate time of blood sampling because some of the studies obtained blood samples before, during, or immediately after operation. In fact, blood samples should be obtained 1 week after operation to accurately represent the real residual CTCs in peripheral blood. Moreover, the methods used for CTCs detection also need to be taken into account, as sensitivity and specificity are of major importance and may differ significantly (Koch *et al*., 2006). It is conceivable that the biologic significance of CTCs would depend on the amount of such cells entering the bloodstream, and thereafter, quantitative instead of qualitative analysis would be more adequate to assess the prognostic significance of cancer patients.

For postoperative follow-up of cancer patients, serum tumor protein markers are important diagnostic tools in clinical practice. For colorectal cancer patients, CEA has been the most extensively investigated tumor marker. In fact, serum CEA protein is currently the most widely used marker in the surveillance or follow-up of colorectal cancer. The monitoring of recurrence or metastasis in asymptomatic patients without accompanying serum CEA elevation after curative resection has rarely been addressed. In the recent investigation of 157 stage I–III CRC patients with normal peri-operative serum CEA levels undergoing radical resection, we demonstrated that our constructed colorimetric membrane-array could detect CTCs in peripheral blood of CRC patients with normal peri-operative serum CEA levels at an earlier stage, with a median time of 6 months earlier than the elevation of serum CEA levels. Indeed, 6 months is a good lead-time for the introduction of new therapeutic strategies to possibly cure these patients. Accordingly, concomitant molecular detection of CTCs with a multimarker panel is a justifiable supplementary approach to the current pathology staging system, which may help physicians make accurate judgment on clinical treatment and in predicting prognosis. A more intensive follow-up plan may be recommended for these patients despite normal perioperative serum CEA levels after radical resection.

FUTURE PERSPECTIVE

Identification of early-stage cancer patients at risk of relapse post-resection remains a challenge for physicians. For example, patients diagnosed with early-stage (UICC stage II) colorectal cancer undergo curative resection, yet up to 30% of these patients develop postoperative relapse within 5 years of surgery. For stage III colorectal cancer, surgery and adjuvant chemotherapy are standard treatment; however, adjuvant chemotherapy is not routinely recommended for stage II colorectal cancer. Increasing attention is being focused on the identification of unfavorable prognostic factors that could aid in making the decision for or against treatment on the basis of the relative benefits and risks for stage II colorectal cancer patients. Indeed, the clinical outcome of patients with high-risk stage II disease is similar to that of patients with stage III disease. With regard to stage II colorectal cancer, the most important factors for predicting the risk of recurrence are emergency presentation (bowel perforation or obstruction), poorly differentiated tumor (histological grade),

depth of tumor invasion, number of examined lymph nodes, adjacent organ involvement (T4), extramural venous invasion, and peritoneal involvement.

More recently, there has been an attempt to identify novel panels of molecular and biochemical markers that may be used to more precisely define prognosis and predict benefit of adjuvant treatment in colorectal cancer. Several retrospective studies have suggested that a number of molecular markers may now define patients with a higher risk of relapse with both stage II and stage III disease (Allegra *et al*., 2002; Ribic *et al*., 2003; Watanabe *et al*., 2001). It is, therefore, of high importance to define reliable prognostic factors for this patient group to help identify high-risk patients (for tumor relapse) who might benefit from adjuvant therapeutic regimes. Clinical trials in stage II colorectal cancer patients could incorporate better risk stratification using molecular markers. Our constructed membrane-array method could detect CTCs in 80% of these stage II colorectal cancer patients with postoperative relapse, with a median lead-time (the time between the presence of molecular markers and the onset of clinically detectable recurrence) of 7 months (data not published). Incidentally, the lead-time advantage of routine serum CEA measurement for surveillance of CRC patients is only 4 months (Northover, 1986). Therefore, it is an approximate 3-month benefit for the earlier prediction of postoperative relapse when comparing our membrane-array method and serum CEA measurement. Consequently, to determinate whether the introduction of adjuvant chemotherapy for stage II patients with positive CTCs is advantageous and efficacious would be an imperative issue for future investigation. Integrating molecular markers into therapeutic strategies might be a crucial step for cancer treatment planning. Lately, the argument regarding using CTCs as a promising tool for choosing and monitoring adjuvant therapy in patients with early breast cancer has been proposed (Dirix *et al*., 2005). Ultimately, the presence of CTCs will probably redefine the most appropriate treatment and affect future patient outcome for malignancies. Nevertheless, large prospective clinical studies regarding the role of presence of CTCs in early stage cancer patients in determining those patients who will benefit from adjuvant chemotherapy should be conducted.

The concept of cancer screening using molecular markers, by either DNA- or RNA-based tests, is gradually being accepted. For instance, the DNA-based stool test for the early detection of colorectal cancer is now available for use by physicians to screen their patients for colorectal cancer. When a cancer grows in the colon, the tumor sheds cells into the stool. PreGen Plus tests a stool sample for 23 DNA markers that are associated with colorectal cancer and precancerous polyps (http://genesanddrugs.dnadirect.com/patients/tests/colon_cancer/what.jsp). This simple, safe, and convenient test could possibly replace the conventional stool occult blood test as a promising noninvasive screening tool. Meanwhile, some investigators have demonstrated the feasibility of detecting early prostate cancer, with high sensitivity and specificity, in body fluids (serum, plasma, urine, and ejaculates) and tissue samples by using a set of prostate cancer-related genes (Costa *et al*., 2007). Although large, prospective trials are needed to validate these findings, the clinical use of these markers

might be envisaged for the near future. Correspondingly, our constructed membrane-array method can be potentially useful for early screening of CTCs in the healthy individuals. The specialized membrane-array would be probably designed for the early detection of CTCs in each human cancer, or even multiple human cancers in one membrane-array with distinct sections. This is the challenge and confrontation in years ahead.

REFERENCES

Ahlquist, D.A., and Shuber, A.P. 2002. Stool screening for colorectal cancer: evolution from occult blood to molecular markers. *Clin. Chim. Acta 315*: 157–168.

Allegra, C.J., Parr, A.L., Wold, L.E., Mahoney, M.R., Sargent, D.J., Johnston, P., Klein, P., Behan, K., O'Connell, M.J., Levitt, R., Kugler, J.W., Tria Tirona, M., and Goldberg, R.M. 2002. Investigation of the prognostic and predictive value of thymidylate synthase, p53, and Ki-67 in patients with locally advanced colon cancer. *J. Clin. Oncol. 20*: 1735–1743.

Bepler, G., Sharma, S., Cantor, A., Gautam, A., Haura, E., Simon, G., Sharma, A., Sommers, E., and Robinson, L. 2004. RRM1 and PTEN as prognostic parameters for overall and disease-free survival in patients with non-small-cell lung cancer. *J. Clin. Oncol. 22*: 1878–1885.

Bessa, X., Pinol, V., Castellvi-Bel, S., Piazuelo, E., Lacy, A.M., Elizalde, J.I., Pique, J.M., and Castells, A. 2003. Prognostic value of postoperative detection of blood circulating tumor cells in patients with colorectal cancer operated on for cure. *Ann. Surg. 237*: 368–375.

Bosch, B., Guller, U., Schnider, A., Maurer, R., Harder, F., Metzger, U., and Marti, W.R. 2003. Perioperative detection of disseminated tumour cells is an independent prognostic factor in patients with colorectal cancer. *Br. J. Surg. 90*: 882–888.

Burchill, S.A., Lewis, I.J., and Selby, P. 1999. Improved methods using the reverse transcriptase polymerase chain reaction to detect tumour cells. *Br. J. Cancer 79*: 971–977.

Chang, Y.S., di Tomaso, E., McDonald, D.M., Jones, R., Jain, R.K., and Munn, L.L. 2000. Mosaic blood vessels in tumors: frequency of cancer cells in contact with flowing blood. *Proc. Natl. Acad. Sci. 97*: 14608–14613.

Chen, C.C., Chang, T.W., Chen, F.M., Hou, M.F., Hung, S.Y., Chong, I.W., Lee, S.C., Zhou, T.H., and Lin, S.R. 2006a. Combination of multiple mRNA markers (PTTG1, Survivin, UbcH10 and TK1) in the diagnosis of Taiwanese patients with breast cancer by membrane array. *Oncology 70*: 438–446.

Chen, C.C., Hou, M.F., Wang, J.Y., Chang, T.W., Lai, D.Y., Chen, Y.F., Hung, S.Y., and Lin, S.R. 2006b. Simultaneous detection of multiple mRNA markers CK19, CEA, c-Met, Her2/neu and hMAM with membrane array, an innovative technique with a great potential for breast cancer diagnosis. *Cancer Lett. 240*: 279–288.

Chen, Y.F., Wang, J.Y., Wu, C.H., Chen, F.M., Cheng, T.L., and Lin, S.R. 2005. Detection of circulating cancer cells with K-ras oncogene using membrane array. *Cancer Lett. 229*: 115–122.

Chong, I.W., Chang, M.Y., Chang, H.C., Yu, Y.P., Sheu, C.C., Tsai, J.R., Hung, J.Y., Chou, S.H., Tsai, M.S., Hwang, J.J., and Lin, S.R. 2006. Great potential of a panel of multiple hMTH1, SPD, ITGA11 and COL11A1 markers for diagnosis of patients with non-small cell lung cancer. *Oncol. Rep. 16*: 981–988.

Conzelmann, M., Linnemann, U., and Berger, M.R. 2005. Molecular detection of clinical colorectal cancer metastasis: how should multiple markers be put to use? *Int. J. Colorect. Dis. 20*: 137–146.

Costa, V.L., Henrique, R., and Jeronimo, C. 2007. Epigenetic markers for molecular detection of prostate cancer. *Dis. Markers 23*: 31–41.

Dirix, L., Van Dam, P., and Vermeulen, P. 2005. Genomics and circulating tumor cells: promising tools for choosing and monitoring adjuvant therapy in patients with early breast cancer? *Curr. Opin. Oncol. 17*: 551–558.

Frazier, A.L., Colditz, G.A., Fuchs, C.S., and Kuntz, K.M. 2000. Cost-effectiveness of screening for colorectal cancer in the general population. *Jama. 284*: 1954–1961.

Ghossein, R.A., and Bhattacharya, S. 2000. Molecular detection and characterisation of circulating tumour cells and micrometastases in solid tumours. *Eur. J. Cancer 36*: 1681–1694.

Ghossein, R.A., Bhattacharya, S., and Rosai, J. 1999. Molecular detection of micrometastases and circulating tumor cells in solid tumors. *Clin. Cancer Res. 5*: 1950–1960.

Giuliani, L., Ciotti, M., Stoppacciaro, A., Pasquini, A., Silvestri, I., De Matteis, A., Frati, L., and Agliano, A.M. 2005. UDP-glucuronosyltransferases 1A expression in human urinary bladder and colon cancer by immunohistochemistry. *Oncol. Rep. 13*: 185–191.

Hermanek, P. 1995. pTNM and residual tumor classifications: problems of assessment and prognostic significance. *World J. Surg. 19*: 184–190.

Hoon, D.S., Wang, Y., Dale, P.S., Conrad, A.J., Schmid, P., Garrison, D., Kuo, C., Foshag, L.J., Nizze, A.J., and Morton, D.L. 1995. Detection of occult melanoma cells in blood with a multiple-marker polymerase chain reaction assay. *J. Clin. Oncol. 13*: 2109–2116.

Huang, P., Wang, J., Guo, Y., and Xie, W. 2003. Molecular detection of disseminated tumor cells in the peripheral blood in patients with gastrointestinal cancer. *J. Cancer Res. Clin. Oncol. 129*: 192–198.

Koch, M., Kienle, P., Kastrati, D., Antolovic, D., Schmidt, J., Herfarth, C., von Knebel Doeberitz, M., and Weitz, J. 2006. Prognostic impact of hematogenous tumor cell dissemination in patients with stage II colorectal cancer. *Int. J. Cancer 118*: 3072–3077.

Miyazono, F., Natsugoe, S., Takao, S., Tokuda, K., Kijima, F., Aridome, K., Hokita, S., Baba, M., Eizuru, Y., and Aikou, T. 2001. Surgical maneuvers enhance molecular detection of circulating tumor cells during gastric cancer surgery. *Ann. Surg. 233*: 189–194.

Mocellin, S., Provenzano, M., Rossi, C.R., Pilati, P., Nitti, D., and Lise, M. 2003. Use of quantitative real-time PCR to determine immune cell density and cytokine gene profile in the tumor microenvironment. *J. Immunol. Methods 280*: 1–11.

Mori, M., Mimori, K., Ueo, H., Tsuji, K., Shiraishi, T., Barnard, G.F., Sugimachi, K., and Akiyoshi, T. 1998. Clinical significance of molecular detection of carcinoma cells in lymph nodes and peripheral blood by reverse transcription-polymerase chain reaction in patients with gastrointestinal or breast carcinomas. *J. Clin. Oncol. 16*: 128–132.

Northover, J. 1986. Carcinoembryonic antigen and recurrent colorectal cancer. *Gut 27*: 117–122.

O'Sullivan, G.C., Collins, J.K., Kelly, J., Morgan, J., Madden, M., and Shanahan, F. 1997. Micrometastases: marker of metastatic potential or evidence of residual disease? *Gut 40*: 512–515.

Pantel, K., Cote, R.J., and Fodstad, O. 1999. Detection and clinical importance of micrometastatic disease. *J. Natl. Cancer Inst. 91*: 1113–1124.

Racila, E., Euhus, D., Weiss, A.J., Rao, C., McConnell, J., Terstappen, L.W., and Uhr, J.W. 1998. Detection and characterization of carcinoma cells in the blood. *Proc. Natl. Acad. Sci. 95*: 4589–4594.

Ribic, C.M., Sargent, D.J., Moore, M.J., Thibodeau, S.N., French, A.J., Goldberg, R.M., Hamilton, S.R., Laurent-Puig, P., Gryfe, R., Shepherd, L.E., Tu, D., Redston, M., and Gallinger, S. 2003. Tumor microsatellite-instability status as a predictor of benefit from fluorouracil-based adjuvant chemotherapy for colon cancer. *N. Engl. J. Med. 349*: 247–257.

Salmon, R.J., Girodet, J., Remvikos, Y., Muleris, M., Olschwang, S., and Thomas, G. 1994. Genetic anomalies of colorectal cancers. *J. Chir* (Paris). *131*: 511–516.

Sher, Y.P., Shih, J.Y., Yang, P.C., Roffler, S.R., Chu, Y.W., Wu, C.W., Yu, C.L., and Peck, K. 2005. Prognosis of non-small cell lung cancer patients by detecting circulating cancer cells in the peripheral blood with multiple marker genes. *Clin. Cancer Res. 11*: 173–179.

Smirnov, D.A., Zweitzig, D.R., Foulk, B.W., Miller, M.C., Doyle, G.V., Pienta, K.J., Meropol, N.J., Weiner, L.M., Cohen, S.J., Moreno, J.G., Connelly, M.C., Terstappen, L.W., and O'Hara, S.M. 2005. Global gene expression profiling of circulating tumor cells. *Cancer Res. 65*: 4993–4997.

Taback, B., Chan, A.D., Kuo, C.T., Bostick, P.J., Wang, H.J., Giuliano, A.E., and Hoon, D.S. 2001. Detection of occult metastatic breast cancer cells in blood by a multimolecular marker assay: correlation with clinical stage of disease. *Cancer Res. 61*: 8845–8850.

Uen, Y.H., Lin, S.R., Wu, C.H., Hsieh, J.S., Lu, C.Y., Yu, F.J., Huang, T.J., and Wang, J.Y.

2006. Clinical significance of MUC1 and c-Met RT-PCR detection of circulating tumor cells in patients with gastric carcinoma. *Clin. Chim. Acta 367*: 55–61.

Wang, J.Y., Hsieh, J.S., Chang, M.Y., Huang, T.J., Chen, F.M., Cheng, T.L., Alexandersen, K., Huang, Y.S., Tzou, W.S., and Lin, S.R. 2004a. Molecular detection of APC, K- ras, and p53 mutations in the serum of colorectal cancer patients as circulating biomarkers. *World J. Surg*. 28: 721–726.

Wang, J.Y., Hsieh, J.S., Chen, C.C., Tzou, W.S., Cheng, T.L., Chen, F.M., Huang, T.J., Huang, Y.S., Huang, S.Y., Yang, T., and Lin, S.R. 2004b. Alterations of APC, c-met, and p53 genes in tumor tissue and serum of patients with gastric cancers. *J. Surg. Res. 120*: 242–248.

Wang, J.Y., Wu, C.H., Lu, C.Y., Hsieh, J.S., Wu, D.C., Huang, S.Y., and Lin, S.R. 2006a. Molecular detection of circulating tumor cells in the peripheral blood of patients with colorectal cancer using RT-PCR: significance of the prediction of postoperative metastasis. *World J. Surg. 30*: 1007–1013.

Wang, J.Y., Yeh, C.S., Chen, Y.F., Wu, C.H., Hsieh, J.S., Huang, T.J., Huang, S.Y., and Lin, S.R. 2006b. Development and evaluation of a colorimetric membrane-array method for the detection of circulating tumor cells in the peripheral blood of Taiwanese patients with colorectal cancer. *Int. J. Mol. Med. 17*: 737–747.

Watanabe, T., Wu, T.T., Catalano, P.J., Ueki, T., Satriano, R., Haller, D.G., Benson, A.B., III, and Hamilton, S.R. 2001. Molecular predictors of survival after adjuvant chemotherapy for colon cancer. *N. Engl. J. Med. 344*: 1196–1206.

Weidner, N. 1993. Tumor angiogenesis: review of current applications in tumor prognostication. *Semin. Diagn. Pathol. 10*: 302–313.

Wharton, R.Q., Jonas, S.K., Glover, C., Khan, Z.A., Klokouzas, A., Quinn, H., Henry, M., and Allen-Mersh, T.G. 1999. Increased detection of circulating tumor cells in the blood of colorectal carcinoma patients using two reverse transcription-PCR assays and multiple blood samples. *Clin. Cancer Res. 5*: 4158–4163.

World Health Organization. 2005. http://www.who.int/mediacentre/factsheets/fs297/en/.

Wu, C.H., Lin, S.R., Yu, F.J., Wu, D.C., Pan, Y.S., Hsieh, J.S., Huang, S.Y., and Wang, J.Y. 2006. Development of a high-throughput membrane-array method for molecular diagnosis of circulating tumor cells in patients with gastric cancers. *Int. J. Cancer 119*: 373–379.

Wyld, D.K., Selby, P., Perren, T.J., Jonas, S.K., Allen-Mersh, T.G., Wheeldon, J., and Burchill, S.A. 1998. Detection of colorectal cancer cells in peripheral blood by reverse-transcriptase polymerase chain reaction for cytokeratin 20. *Int. J. Cancer 79*: 288–293.

Yeh, C.S., Wang, J.Y., Wu, C.H., Chong, I.W., Chung, F.Y., Wang, Y.H., Yu, Y.P., and Lin, S.R. 2006. Molecular detection of circulating cancer cells in the peripheral blood of patients with colorectal cancer by using membrane array with a multiple mRNA marker panel. *Int. J. Oncol. 28*: 411–420.

13
Prediction of Metastasis and Recurrence of Breast Carcinoma: Detection of Survivin-Expressing Circulating Cancer Cells

Shang-mian Yie

INTRODUCTION

Breast cancer is the most common type of cancer for women in the world. According to Bray *et al*. (2004), it is responsible for over one million of the estimated ten million neoplasms diagnosed worldwide each year. Globally, it is also the leading cause of cancer deaths among women, responsible for 375,000 deaths in the year 2000 as documented by Ferlay *et al*. (2001). Major causes for these breast cancer-related deaths, as indicated by Zieglschmid *et al*. (2005) have been metastases and recurrences. As a result, the ability to predict an individual patient's risk of metastasis and recurrence after surgical resection of the primary tumor is of great importance. This ability would also allow for the planning of optimized adjuvant therapies as well as monitoring the efficacy of treatments.

It was explained by Cianfrocca and Goldstein (2004) that for decades the presence of metastatic breast cancer cells in the regional lymph nodes (that is cancer cells disseminated in the lymph nodes but before having spread to the peripheral blood or other distant locations such as the bone marrow) was considered to be the most valuable prognostic indicator for breast carcinoma. One method of detecting the cancer cells is through lymph node evaluation. However, with this method, the evaluation of tissues can only be undertaken at the time of initial diagnosis or surgery, and the procedures employed are often inaccurate, time consuming, and cannot be easily used in routine screening for the purpose of predicting disease recurrence. Another widely used method involves analyzing antibody-based assays for currently available tumor markers. The problem with this is that the markers are not tumor-specific and are not easily detectable in the early stage, which renders the method ineffective in monitoring whether or not a patient is responding well to treatments or whether there is a recurrence (Lacroix, 2006).

In works by Fisher *et al*. (2002), Gilbey *et al*. (2004) and Pantel and Brakenhoff (2004), it has been demonstrated that metastatic spreading occurs in ~ 50% of cases with apparently localized breast

cancer, and that up to 30% of patients with lymph node-negative disease will eventually develop distant metastases within 5 years. This suggests that: (1) breast cancer cells seem occasionally able to shed from the primary lesion very early during the natural history of the tumors, and subsequent recurrence is probably due to the establishment of micrometastases before primary locoregional treatment; (2) hematogenous spreading of tumor cells from the primary tumor can be considered as a crucial step in the metastasis cascade which eventually leads to the formation of clinically manifested metastases.

Histologically confirmed vessel infiltration is a well-known prognostic/predictive factor in nodal-negative breast carcinoma as summarized by Mirza *et al*. (2002). However, Denley *et al*. (2002) have pointed out that vessel infiltration is not very sensitive when evaluated with either a fine-needle aspiration cytology sample or a needle core biopsy specimen. Identification of disseminated cancer cells in the bone marrow, as stated by Muller and Pantel (2004), is another independent prognostic/predictive factor that is not only useful in the prediction of bone metastasis development, but also in the development of metastasis in other organs such as lung and liver. Nonetheless, as noted by Gilbey *et al*. (2004), the sampling of bone marrow aspiration for subsequent analysis is both a painful and costly procedure, and one that cannot be easily used in routine screenings.

Consequently, there have been numerous recent studies, which indicate that the detection of disseminated tumor cells in the peripheral blood might be of clinical use in terms of evaluating patient prognosis, predicating metastasis and recurrence, and monitoring therapy. Moreover, technically speaking, peripheral blood analysis appears to be an ideal source for monitoring disseminated tumor cells because the sampling is relatively painless and can be performed at frequent intervals. As such, the method offers an appealing approach for detecting occult metastasis in patients with early stages, for monitoring tumor progression, and for assessing the patients' response to cancer treatment. Nevertheless, some published data have also revealed a few problems such as unreliable assay methods and tumor markers that are non-malignant specific. Hence, the predictive effect of such a procedure remains to be fully substantiated. For this reason, there is still a great need to seek out specific tumor markers as well as to develop reliable and reproducible assay methods that can be used to unambiguously identify disseminated tumor cells in the peripheral blood.

DETECTION OF CIRCULATING BREAST CANCER CELLS

Methods Used to Detect Circulating Cancer Cells

At the present, various techniques have been deployed to identify circulating cancer cells. The most important of these can be broadly divided into two categories: cytometric methods and molecular methods. For molecular methods, the reverse transcription-polymerase chain reaction (RT-PCR) technique is most commonly used.

Cytometric Methods

Cytometric methods are used to isolate and enumerate individual cells. The advantage is that they allow for a deeper characterization

of the cells at the molecular level in terms of expressing key biological markers and conducting morphological cell analysis. Because only a small amount of epithelial cells can be found in the blood, even in patients with metastatic cancer, enrichment procedures may be performed by using immunomagnetic methods. However, despite enrichments that can result in the order of 10,000 times more epithelial cells, they are still greatly outnumbered by the residual white blood cells. Thus, immunocytochemical identification of the epithelial cells within this population is often required.

According to Ring *et al.* (2004), immunocytochemical methods based on monoclonal antibodies against various epithelium-specific antigens have been studied, for which cytokeratin is the most popular antigen. However, antibody-based techniques also have their limitations. For example, Lacroix (2006) mentions that many of the antibodies directed at epithelial and breast cancer cells are also known to stain hematopoietic cells including cytokeratins. Nonspecific staining of plasma cells can also occur due to alkaline phosphatase reactions against the *k* and *l* light chains on the cell surface. A review by Zieglschmid *et al.* (2005) further stresses that depending on the type of antibodies used, a false-positive detection rate of 1–3% can be expected. Moreover, the screening of large volumes of materials by using immunocytochemical techniques can be very time consuming. Thus, automated image-analysis systems or semi-automated alternatives should be used for clinical application purposes as suggested by Gross *et al.* (1995) and Pachmann *et al.* (2001).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Technique

The RT-PCR technique first involves the isolation and reverse-transcription of tumor-associated messenger RNA (mRNA) to complementary DNA (cDNA). Next, it requires a PCR-based amplification of the cDNA template with specific primers. This results in a several thousand-fold amplification of the signal which theoretically makes the method very sensitive. Comparative studies carried out by Lambrechts *et al.* (1999) and Smith *et al.* (2000) have shown that the RT-PCR technique has higher rates of positivity than cytometric methods.

A shortcoming of the RT-PCR technique is its inability to employ morphologic criteria to confirm the presence of metastatic cells. However, Zieglschmid *et al.* (2005) indicated that RT-PCR is the most successful technique for detecting tumor-associated mRNA, which could indicate the presence of circulating cancer cells in the blood of patients with most common types of carcinomas. In breast cancer studies, Gilbey *et al.* (2004), Ring *et al.* (2004), Zieglschmid *et al.* (2005) and Lacroix (2006) have described the extensive use of tumor-associated mRNA including various tissue-specific differentiation markers such as cytokeratin 19 (CK19), cytokeratin 20 (CK20), maspin, epidermal growth factor receptor (EGFR), epidermal growth factor receptor 2 (HER2/*neu*), mammaglobin, membrane-associated mucin1 (MUC1), or oncofetal antigens such as β-human chorionic gonadotropin (β-HCG), and carcinoembryonic antigen (CEA). However, the same studies revealed that most of the currently available tumor markers are not specific for malignancy as low expression levels

of CK19, CK20, EGFR, CEA, MUC1 and HER2 have been detected in normal patients, in patients with malignant hematological disorders, and in normal blood components. Furthermore, studies by Jung *et al.* (1998) and Goeminne *et al.* (1999) have detailed that the expression of CK19 and CEA can be induced in peripheral blood mononuclear cells by cytokines and growth factors that circulate at higher concentrations under inflammatory conditions and neutronpenia. As a consequence, false-positive results are more likely to occur under these circumstances.

With standard techniques, RT-PCR products are separated using a process of agarose gel electrophoresis and stained with ethidium bromide for detection. Samples are described as either positive or negative depending on the presence or absence of a PCR product and further quantification is not possible. Even so, the standard RT-PCR technique may not be sensitive enough to detect small quantities of PCR-amplified products by using the ethidium bromide staining. In such cases, a nested PCR is usually applied to increase the assay sensitivity as mentioned by Gilbey *et al.* (2004). However, by utilizing the highly sensitive nested RT-PCR, even low levels of illegitimate transcription in the blood can cause alternative false-positive results. Moreover, performing a nested RT-PCR is more time consuming, as there is a need to observe stringent procedures in order to minimize the risk of false-positives arising from possible cross-contamination of PCR products. Therefore, it is more desirable to carry out the RT-PCR in a single round so that potential contamination problems can be avoided. A further concern outlined by Gilbey *et al.* (2004) is that if the primers are not carefully designed, pseudogenes (DNA segments showing sequence homology with the target mRNA sequences) could be amplified leading to PCR products that are indistinguishable from the target mRNA.

Potential Clinical Significance

The development of RT-PCR tests to detect circulating cancer cells in the blood has been shown to be feasible in a number of studies. For instance, in studies concerning early stage of breast carcinoma, Stathopoulou *et al.* (2002) and Zach *et al.* (2002) by using CK19 mRNA and mammaglobin mRNA respectively, showed that early stage breast cancer patients with no detectable circulating tumor cells may experience a low risk of relapse or death regardless of what other prognostic/predictive factors may reveal. Adjuvant therapy may therefore be withheld. At the same time, patients with circulating tumor cells expressing either CK19 or mammaglobin may be judged to be at a higher risk of relapse, and therefore should be offered adjuvant systemic therapy irrespective of the standings of other prognostic/predictive factors.

In metastatic settings, reports by Manhani *et al.* (2001) and Smith *et al.* (2000) indicate that changes in the number of circulating cancer cells can be seen as a response to systemic therapy signifying that measurements on the circulating cancer cells, especially if accompanied by an assessment of the residual cell phenotypes, might be preferable to measurements on the primary lesion. This is so because the interval between the initial excision and the development of metastatic disease might enable a phenotypic drift to occur. All of this is useful not only in selecting

patients and monitoring their responses to the highly sophisticated tailoring of therapy, but also in assessing markers of biological response for the development of novel drugs.

Current clinical studies have also revealed a number of problems. For example, a review by Ring *et al*. (2004) illustrated that in studies using cytometric assays, cells with the characteristics of tumor cells have been shown to be in the blood of 0–100% of patients with operable (stages I–IIIa) breast cancer, and in 3–100% of patients with metastatic disease. Also presented in the same review is that in studies using RT-PCR techniques, cells with the characteristics of tumor cells have been shown to be in the blood of 0–88% of patients with operable breast cancer, and in 0–100% of patients with metastatic disease. In accordance with a number of recent related reviews, namely Gilbey *et al*. (2004), Ring *et al*. (2004), Zieglschmid *et al*. (2005) and Lacroix (2006), the observed variability may be due to: (1) sources from which the samples were taken given that most population bases are heterogeneous in nature whose members have different stages of disease which are likely to have a substantial effect on the presence of circulating tumor cells; (2) times at which the samples were taken given that samples taken before or after surgery, or before undergoing therapy, can all have considerable influence on the likelihood of detecting circulating tumor cells; (3) circumstances in which the samples were handled and prepared such as delays between collection and analysis, conditions of sample storage, and contamination of skin epithelial cells during sample collection; (4) guidelines in which the samples were to be evaluated, given that there is a lack of criteria for positivity and any sample containing evidence of epithelial cancer cells, has been labeled as positive in some studies. A more appropriate approach would be to describe samples as being positive only if the number of cancer cells in them exceeds those found in normal controls.

Taking into consideration the abovementioned points, it is therefore necessary to develop novel techniques that are reliable and reproducible with increased sensitivity and specificity. In addition, the use of multi-marker assays and "search-and-select" type of markers specific to certain malignancies should be further explored. Accordingly, the following sections will elaborate on a specific, sensitive, and reliable molecular technique to quantitatively determine circulating breast cancer cells that express *survivin* mRNA, a highly specific marker for most common types of malignancies.

DETECTION OF SURVIVIN-EXPRESSING CIRCULATING BREAST CANCER CELLS

Reverse Transcription-Polymerase Chain Reaction-Enzyme-Linked Immunosorbant Assay (RT-PCR-ELISA)

From the discussions in the previous section, it is evident that certain technical problems exist when using RT-PCR techniques to detect circulating breast cancer cells. To overcome these problems, Lacroix (2006) has referred to a number of studies that have utilized modern real-time RT-PCR techniques. In a study by Aerts *et al*. (2001), these techniques were used to determine the number of

circulating cancer cells in clinical samples by comparison with standard curves. The real-time PCR can also be used to establish a normal range if the assay is performed for some control samples. A cutoff point for positivity can then be identified, thereby improving the specificity of the test. However, as noted by Valasek and Repa (2005), the real-time PCR method can be very costly and certain limitations are present as with all PCR- or RT-PCR-based techniques.

In the study by Yie *et al*. (2006), a specific, sensitive, reproducible and yet simple procedure for measuring cancer-associated mRNA in peripheral blood cells was developed. This technique called the RT-PCR-ELISA was based on a hybridization-ELISA detection system for RT-PCR products. It combines the sensitivity of nucleic acid amplification and the high specificity of hybridization protocols with a non-isotopic quantification method that is in itself highly sensitive and specific. Consequently, it offers several advantages over conventional RT-PCR and nested PCR methods.

The first advantage is that the method dramatically increases the sensitivity of marker detection when compared to regular RT-PCR procedures. In a regular RT-PCR procedure, the most commonly used method to visualize PCR product on an agarose gel is ethidium bromide staining which has a relatively low detection limit. By comparison, the RT-PCR-ELISA procedure as demonstrated by Yie *et al*. (2006) was able to increase the sensitivity to detect PCR products by 8–16 times, and was capable of detecting one cancer cell in a 2 ml blood sample spiked with breast cancer cell lines. The second advantage is that the hybridization-ELISA detection system can also increase the specificity, thereby reducing the effects caused by contaminants that might otherwise be introduced during the RT-PCR process. The third advantage is that the PCR products can be directly quantified by interpolation from ELISA standard curves akin to real-time PCR techniques. As such, this provides a measure of the amount of PCR products that can be compared among different batches in the assay. The fourth advantage is that the method permits a rapid and simple quantification of mRNA within one working day. Also, it uses widely available commercial reagents and employs relatively inexpensive equipments such as a thermocycler and ELISA reader.

Selection of Breast Cancer-Associated Marker

As stated before, most of the currently available tumor markers used in the detection of circulating breast cancer cells are not specific for malignancy. Existing studies as mentioned by Gilbey *et al*. (2004) do not draw firm conclusions on whether one type of marker is more sensitive than another. However, published data have revealed that assay sensitivity can vary from one study to another even when the same marker is used. To improve upon this situation, Bosma *et al*. (2002) and Taback *et al*. (2001) have investigated the use of multiple marker assays. Because breast cancer is composed of a heterogeneous collection of cells with differing degrees of tumor marker expression, circulating tumor cells within a particular breast cancer patient may not express the particular tumor marker being assayed. Therefore, a multiple marker assay, taking into account tumor heterogeneity and mRNA expression

variability, would enhance the detection of circulating tumor cells when comparing to detection in which only a single marker is used. However, careful selection must be made on a particular tumor marker that is uniquely expressed in breast cancer, but not in normal epithelial cells or normal blood cells.

Survivin is a novel member of the inhibitor of apoptosis protein (IAP) family having 1.5 kb located at chromosome 17q25 with four exons and three introns. This form was originally described by Ambrosini *et al.* (1997) and was named survivin wild-type (survivin-wt). Transcription of the survivin gene produces a 489 bp mRNA that translates into a cytoplasmic protein having 140 amino acids with a molecular weight of 16.5 Kd. Four additional splice variants named survivin-ΔEx3, survivin-2α, survivin-2B and survivin-3B are also known to exist. However, as Span *et al.* (2006) have pointed out, survivin-wt appears to be the dominant form in breast cancer tissues.

The study by Johnson and Howerth (2004) has indicated that survivin is a bifunctional protein that suppresses apoptosis and regulates cell division. Survivin regulates the G2/M phase of the cell cycle by associating with mitotic spindle microtubules and directly inhibiting caspases analogous to other IAPs. Studies by Adida *et al.* (1998) and Konno *et al.* (2000) mention that survivin is present and functional during fetal development, but it does not seem to be involved in the physiological regulation of apoptosis after birth. However, a recent review by Li and Brattain (2006) has shown that survivin might also play a role in certain physiological processes in many human organs/cells although through somewhat different mechanisms.

Most importantly, survivin is prominently expressed in all of the most common types of human carcinoma, but is undetectable in most normal adult tissues. This trait makes it the most desirable tumor-specific marker up to date. By using various methods of molecular biology and immunology such as RT-PCR, Northern blot, immunocytochemistry, Western blot, and flow cytometry, survivin has been found in nearly all of the common types of cancer including breast cancer (Zaffaroni *et al.*, 2005). Moreover, Chiou *et al.* (2003) have stated that accumulated data have shown survivin overexpression in the primary tumor is almost invariably associated with a poor prognosis for patients with various cancer types.

Studies by Nasu *et al.* (2002), Span *et al.* (2004) and Ryan *et al.* (2005) have reported that the expression of survivin mRNA was detected in the majority of cancer tissues found in patients with breast carcinoma. Additionally, survivin is one of the most uniformly upregulated genes in tumor tissues when compared to healthy ones. Uncontrolled growth of cancerous cells requires anti-apoptotic strategies to extend an otherwise limited lifespan and to counter the customary apoptotic triggers. Thus, overexpression of survivin as noted by Pizzoferrato *et al.* (2004) may provide breast cancer cells with a distinct *in situ* survival advantage and/or allow them more able to spread. Furthermore, survivin-expressing circulating breast cancer cells might be more capable of seeding metastatic sites, and tend to be more successful in establishing metastastic tumors. Zaffaroni and Daidone (2002) have remarked that cells that are unresponsive to apoptotic triggers will also be more resistant to radiation and

chemotherapy. Therefore, survivin mRNA in the peripheral blood might be an ideal tumor marker to identify circulating breast cancer cells which can then be used in predicting the development of metastases and recurrences, estimating prognosis, deciding treatment stratagem, and monitoring the efficacy of adjuvant treatment.

Potential Clinical Applications

Experiments conducted by Yie *et al*. (2006) showed that in 130 healthy controls, all samples analyzed using RT-PCR were negative when evaluated with a 1.2% agarose gel stained with ethidium bromide. However, a single 439 bp survivin-wt fragment and other splice variants were detected in 28.3% of the 69 breast cancer patients. By using the RT-PCR-ELISA, it was found that there was a high statistical difference ($P < 0.001$) in survivin mRNA concentrations between breast cancer patients and healthy controls (Figure 13.1A). A normal range and a cutoff point for the positivity of survivin mRNA in peripheral blood specimens were also established after performing a receiver operating characteristic

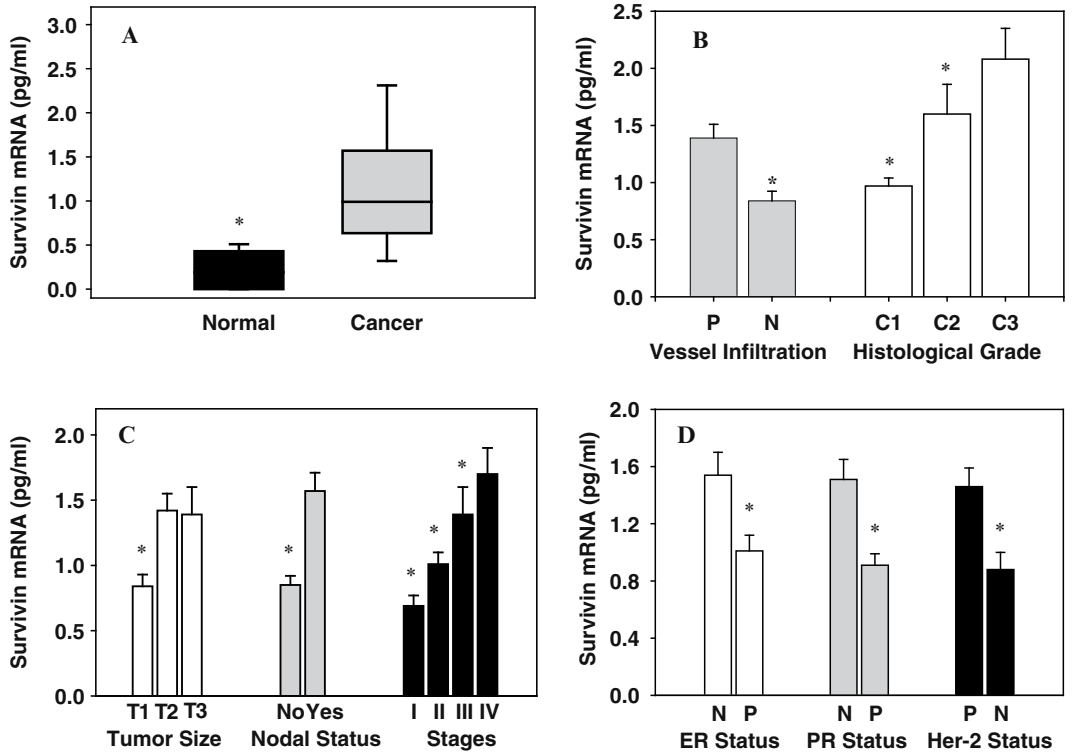

FIGURE 13.1. **Association of survivin-expressing circulating breast cancer cells with clinicopathological parameters.** (A) Comparison of survivin mRNA concentrations in peripheral blood samples between 67 breast cancer patients and 135 healthy controls in which a significant statistical difference was observed (Mann-Whitney rank sum test: $T = 10{,}822$, $P < 0.001$). A cutoff value was established by ROC analysis. Associations of survivin with (B) histological grade and vessel infiltration; (C) tumor size, nodal status and clinical stages; (D) ER, PgR, and HER2 statuses were analyzed by either the Kruskal-Wallis one-way analysis of variance on ranks test or the Mann-Whitney rank sum test: $P < 0.002$, 0.0001, 0.001, 0.0001, 0.03, 0.009, 0.002, and 0.006 respectively for the RT-PCR-ELISA. (Yie *et al*. 2006.)

(ROC) curve analysis. In general, ~ 50.7% of the patients were determined to be positive in having survivin-expressing circulating breast cancer cells.

Clinicopathological parameters including tumor histological grade, vessel infiltration, tumor size, nodal status, ER/PgR status, HER2 status, and clinical stages have shown to be significantly associated with survivin mRNA concentrations (Figure 13.1B–D). In addition, these well-established prognostic/predictive factors were shown to be significantly associated with positive detection rates in relation to the cutoff value. Thus, the quantitative assessment of survivin mRNA with the RT-PCR-ELISA not only accurately detects the presence of circulating breast cancer cells, but also significantly increases the rate of positive detection over conventional RT-PCR in clinical applications.

Furthermore, after the initial assay test, ~ 17 available patients who were treated with similar adjuvant chemotherapy regimens were followed up for a period of 2–36 months. Survivin-expressing circulating cancer cells were detected in the peripheral blood samples of 11 patients (64.7%), 6 of whom had metastasized and 5 were in the early stages of the disease at the time of the assay. Nine out of the 11 patients eventually suffered a relapse within the 36-months follow-up period. In comparison, for the six patients who did not show any presence of survivin-expressing circulating cancer cells in their peripheral blood samples, only two out of the six (33.3%) suffered a relapse. These two patients were diagnosed as in stages II and IV, respectively, at the time of the initial assay.

Interestingly, survivin mRNA was detected in the peripheral blood samples obtained from 11 out of 37 patients (29.7%) with early stages of breast cancer. Five of these survivin-positive patients were available for a follow-up in which three out of the five (60%) patients developed a relapse of the disease. This outcome could indicate that some breast carcinomas may have a higher potential to cause hematogenous metastasis even in the early stage of the disease. To further confirm the predictive value of detecting survivin-expressing circulating breast cancer cells, a ROC curve analysis was performed (Figure 13.2). Results showed that the area under the curve (AUC) was 0.852 and $P < 0.0001$. When survivin mRNA concentration was at the cutoff point of 1.01 pg/ml, the specificity of predicting

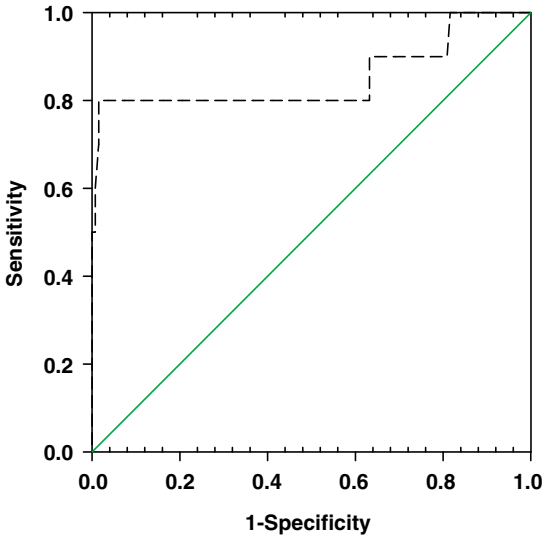

FIGURE 13.2. **Predictive value of the detection of survivin-expressing breast cancer cells using RT-PCR-ELISA evaluated by ROC curve analysis.** Use of ROC curve analysis in the prediction of recurrence by detecting survivin-expressing circulating cancer cells in patients with breast cancer. The value of AUC was 0.85 (95% confidence interval; 0.65–1.03, $P < 0.0001$). At the survivin mRNA concentration cutoff value of 1.01 pg/ml, specificity was 100% and sensitivity was 80%. (Yie *et al*. 2006.)

recurrence was 100% and the sensitivity was 80%. From these findings, it can be concluded that the detection of survivin-expressing circulating cancer cells is a viable method for predicting cancer recurrence in patients with breast cancer. It may also suggest that the presence of survivin mRNA in the peripheral blood carries the risk of hematogenous spread regardless of the clinical stages of the disease. Also, a significant correlation between survivin expression in the blood and the prognostic/predictive factors of nodal status and vessel infiltration was established. The consequence of this discovery indicates that the testing of blood for the presence of circulating cancer cells may provide a complementary technique in enhancing current methods of cancer prognosis.

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION-ELISA METHODOLOGY

Preparation of RT-PCR-ELISA System

Cell Culture

Breast cancer cell line MCF-7 (American Type Culture Collection, Rockville, MD, USA) was purchased and cultured in a RPMI1640 medium supplemented with 100 U/ml of penicillin, 50 µg/ml of streptomycin and 10% of heat-inactivated fetal bovine serum (Sigma, St. Louis, MO, USA). The culture was maintained in 10 × 20 cm tissue culture plates (BD Biosciences, Bedford, MA, USA) at 37°C in a 5% CO_2 environment. Log phase cells were collected at 90% confluence by 0.25% of trypsin digestion and centrifuged for 5 min at 1,000 rpm. They were resuspended in a phosphate buffered saline (PBS) solution and counted using a hemocytometer. The cells were cultured in preparation for the RT-PCR-ELISA system as well as in its validation.

Extraction of Total RNA from MCF-7 Cell Line

Total RNA of the cultured MCF-7 breast cancer cells was extracted using a Trizol Kit (Gibco, Burlington, ON, Canada) in accordance with the manufacturer's instructions. The quality of the extracted total RNA was estimated by an agarose formaldehyde gel electrophoresis and its concentration was determined by a spectrophotometer reading at 260/280 nm.

Synthesis of cDNA

An amount of 5 µg of the total RNA was added into test tubes containing a pre-prepared reverse transcription (RT) mixture that included 500 ng of Oligo $(dT)_{12–18}$ primer, 0.5 U of RNase inhibitor, 0.5 mM dNTPs, 50 mM DTT, 5x RT buffer, and 5 U of MMLV-reverse transcriptase (Epicentre Technologies, Madison, WI, USA) for a total reaction volume of 20 µl. The contents of the tubes were mixed thoroughly and placed in a thermocycler (Eppendorf, Hamburg, Germany) which was programmed for one cycle at 37°C for 60 min followed by one cycle at 90°C for 5 min to inactivate the reverse transcriptase. The synthesized cDNA was either immediately used for the PCR or stored at −20°C until needed.

First-Round PCR

To prepare for the first-round PCR, an aliquot of cDNA (2 µl) was mixed with 12.5 pmol of primers and a *Taq* DNA

polymerase kit containing 2.5 µl of 10x buffer, 5 µl of 5x Q-solution, 200 µM dNTPs, 1.5 mM $MgCl_2$ and 0.625 U of *Taq* DNA polymerase (Qiagen, Hilden, Germany). Diethyl pyrocarbonate (DEPC)-treated water was added to comprise a final volume of 25 µl. The mixture was incubated for the indicated cycles: denaturing at 94°C for 1 min, annealing at 62°C for 1 min and extension at 72°C for 2 min for 30 cycles, followed by incubation at 72°C for 10 min in the thermocycler. In the first-round PCR, a 439 bp survivin fragment was amplified with a forward primer (SUF) corresponding to survivin mRNA nucleotides 47–66 (5'-GGC ATG GGT GCC CCG ACG TT-3'), and a backward primer (SUB) complementary to survivin mRNA nucleotides 466–485 (5'-AGA GGC CTC AAT CCA TGG CA-3'). *See Note 1 on page 169.*

Second-Round PCR (Nested PCR)

To prepare for the second-round PCR, an aliquot (2 µl) of 1:100 of the first-round PCR product was mixed with 12.5 pmol of primers and a *Taq* DNA polymerase kit containing 10 µl of 10x buffer, 20 µl of 5x Q-solution, 200 µM dNTPs, 1.5 mM $MgCl_2$ and 2.5 U of *Taq* DNA polymerase (Qiagen). A final volume of 100 µl was created after adding DEPC-treated water. The mixture was incubated for the indicated cycles: denaturing at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min for 30 cycles, followed by incubation at 72°C for 10 min in the thermocycler. In the nested PCR process, a 339 bp survivin fragment within the 439 bp survivin fragment was amplified by using primer sets SUF1 corresponding to survivin mRNA nucleotides 99–118 (5'-GGA CCA CCG CAT CTC TAC AT-3'), and SUB1 complementary to survivin nucleotides 419–438 (5'-GCA CTT TCT TCG CAG TTT CC-3'). *See Note 1 on page 169.*

Construction of Survivin-Plasmid

The 439 bp survivin fragment amplified from the first-round PCR was ligated into a pQE-30UA vector (Qiagen) at a ratio of 5:1 by using 5 U of T4 DNA ligase (Invitrogen, Carlsbad, CA, USA) held at 16°C for 2 h. This recombinant plasmid was named pQE-30UA/survivin. The bacteria *E. coli M15* transformed with pQE-30UA/survivin were grown in a Luria-Bertain (LB) medium with 100 µg/ml of ampicillin and 50 µg/ml of karamycin overnight at 37°C. The recombinant plasmid was subsequently extracted with an extraction kit (Qiagen) by following the manufacturer's instructions. Thereafter, it was subjected to a DNA sequencing confirmation and its concentration was determined by a spectrophotometer reading at 260/280 nm.

Preparation of Nuclear Hybridization Platform

The 339 bp survivin fragment that was prepared earlier in the second-round PCR was utilized as a nucleic acid hybridization probe by means of purification with a QIAquick PCR Purification Kit (Qiagen) in accordance with the manufacturer's instructions. The quality of the purified probe was estimated by a 1.2% agarose gel electrophoresis and its concentration was determined by a spectrophotometer reading at 260/280 nm.

The probe was then denatured at 95°C for 5 min and cooled on ice for 2 min. Afterwards, 1 µg/ml of the probe was

directly coated on microtiter plates/strips (Thermo Electron Corporation, Milford, MA, USA) with 50 µl per well in a PBS coating buffer (pH = 7.2) and incubated overnight at 4°C. Following the incubation, the plates/strips were washed twice with 0.01 M of PBS washing solution containing 0.05% tween-20. Finally, the prepared plates/strips were either immediately used or stored at −20°C until needed. *See Note 2 on page 170.*

Detection of Circulating Cancer Cells by RT-PCR-ELISA System

Sample Preparation, Total RNA Extraction, and cDNA Synthesis

A 2 ml sample of peripheral blood was collected into test tubes containing sodium citrate. The tubes were immediately sent to the laboratory for processing. The samples were centrifuged at 3,000 rpm in room temperature to obtain all the blood cells. Total RNA of whole blood cells were extracted using the Trizol Kit (Gibco) as described previously for the MCF-7 breast cancer cell line. The total RNA concentration was determined by a spectrophotometer reading at 260/280 nm. *See Note 3 on page170.*

An amount of 5 µg of the total RNA from each of the blood cell samples was added into test tubes containing the pre-prepared RT mixture as discussed before in the synthesis of cDNA. The tubes were placed in the thermocycler which was programmed for one cycle at 37°C for 60 min followed by one cycle at 90°C for 5 min to inactivate the reverse transcriptase. *See Note 4 on page 170.*

PCR Amplification

The survivin mRNA fragment in the blood cells was amplified with 12.5 pM of fluorescein-labeled SUF and SUB primers to generate flourescein-labeled PCR products. First, an aliquot of cDNA (2 µl) from each of the blood cell samples was mixed with the pre-prepared PCR mixture containing the same contents as seen previously in the first-round PCR. The total reaction volume was 12.5 µl which was then incubated for the indicated cycles: denaturing at 94°C for 1 min, annealing at 62°C for 1 min and extension at 72°C for 2 min for 26 cycles, followed by incubation at 72°C for 10 min in the thermocycler. The pQE-30UA/survivin plasmid diluted in serial concentrations ranging from 0 to 102.4 fg per tube was used as standards. Finally, the standards were amplified together with the samples in the same batch of assay.

Quantitative Determination of PCR Products by Nuclear Hybridization-based ELISA

An amount of 2.5 µl of each fluorescein-labeled PCR product in duplicate was added into the aforementioned probe-coated microtiter plates/strips to make up a total volume of 10 µl with 1x PCR buffer. The fluorescein-labeled PCR product was first denatured by using 20 µl of 1 N NaOH/0.05% thymol blue solution at room temperature for 10 min, and then hybridized to the coated probe in a hybridization buffer (0.95 M NaCl, 0.5 M NaH_2PO_3, 0.1 M sodium citrate, 1% block solution (Roche, Basel, Switzerland), pH = 4.8) for 2 h at 60°C. *See Note 5 on page 170.*

After hybridization, the plates/strips were washed four times with PBS washing solution (*See Note 6 on page 170*). Subsequently, the plates/strips were added with 50 µl per well of the 1:1,000 anti-fluorescein antibody-HRP conjugate (Roche) diluted

in a sample buffer (0.01 M PBS/150 mM NaCl, 0.5% block solution, 5 mM EDTA and 1% tween-20) and incubated at room temperature for 1 h. Afterwards, they were washed another four times with the washing solution and 100 µl of 3, 3', 5, 5'-Tetramethylbenzidine (TMB) (Sigma) were added. Following a 15 min incubation at room temperature, color reactions were stopped by adding 50 µl per well of 1 M HCl. The plates/strips were read at 450/630 nm using a microplate reader (Bio-TEK, Winooski, VT, USA).

In addition, the standards were hybridized at the same time as the samples in one batch of assay. Survivin mRNA levels in each of the samples were determined by comparing to the standards with the results being expressed in pg/ml (*See Note 7 on page 170*). Positive control was obtained from the MCF-7 breast cancer cell line cDNA while negative control was realized without the cDNA. Both of these were simultaneously amplified by PCR and added to the microtiter plates/strips at each assay.

Validation of RT-PCR-ELISA System

Validation of RT-PCR Process

Specificity of the RT-PCR process was assessed by: (1) observing the 439 bp survivin fragment generated with SUF and SUB primer sets from cultured MDA231, MDA465 and MCF-7 breast cancer cell lines, all 15 samples of breast cancer tissue, and some peripheral blood specimens (*See Note 8 on page 170*); (2) noting that no transcripts were identified in the water control or the RT-negative control samples; (3) confirming the PCR amplification for survivin through a direct sequence analysis. To determine the sensitivity of the RT-PCR process, serial dilutions of RNA extracted from the MCF-7 breast cancer cells were performed. The band of 439 bp survivin fragment was detectable starting from 100 ng of the total RNA isolated from the breast cancer cell lines, breast cancer tissues or positive blood specimens.

To determine the reproducibility of the entire RT-PCR process, separate RT-PCRs were performed on 10 aliquots, 5 µg each, of the total RNA extracted from the MCF-7 breast cancer cell line. The RT-PCR products were subsequently analyzed using ELISA. The coefficient of variation between the cDNA samples was 5%. To further assess the reproducibility of PCR and the measurement steps, excluding the RT stage, 8 aliquots of a single cDNA sample diluted in a ratio of 1:10 were amplified separately and measured using ELISA. Ensuing statistical analysis showed that there were no significant differences amongst the aliquots.

Validation of ELISA Process

The specificity of the ELISA process was assessed by using fluorescein-labeled PCR products of other tumor markers including CK19, β-hCG, EGFR, CEA, HER2 and prostate specific antigen (PSA). No signals were obtained from using these non-survivin fragments in the hybridization step as shown by Yie *et al*. (2006). The linearity and sensitivity of the ELISA system was determined by using serial dilutions of known amounts of pQE-30UA/survivin plasmid as standards and amplifying along with the sample cDNAs in the same batch. The illustration in Figure 13.3 shows the standard curve of optical density (OD) values against the survivin fragment in

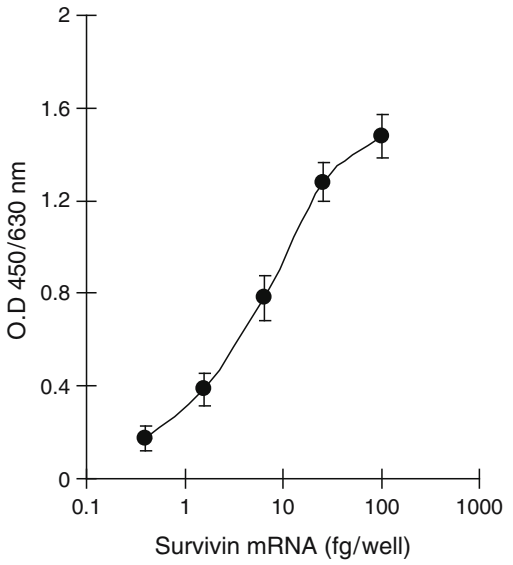

FIGURE 13.3. **RT-PCR-ELISA standard curves.** Linearity and sensitivity of the RT-PCR-ELISA detection system: serial dilutions of known amounts of pQE-30UA/survivin plasmid were amplified using PCR and measured using ELISA. Results were expressed as OD (450/690 nm) values against the survivin fragment in concentrations between 0.4 and 102.4 fg per well in triplicate from 17 assays (mean ± standard error). (Yie *et al*. 2006.)

concentrations between 0.4 and 102.4 fg per well in triplicate from 17 assays. The lowest detection limit of the assay was 0.4 fg per well (mean OD value + 2 standard deviations at zero concentration of the standard template). The reproducibility of the entire RT-PCR-ELISA process demonstrated that the variation coefficients (CV %) within and between batches were 4.5% and 9.2%, respectively.

Additional assessment regarding the sensitivity and specificity of the RT-PCR-ELISA technique for tumor cell detection was carried out in which various number of cultured MCF-7 breast cancer cells were seeded into different test tubes containing 2 ml of whole blood cells from healthy donors. A total of one million cells were present in the tubes with ratios of tumor cells to hematopoietic cells ranging from 1:100 to 1:1,000,000. This mimicked a clinical setting for the detection of breast cancer cells in the peripheral blood. Another 20 non-seeded blood samples obtained from different healthy controls were also processed. Several experiments were conducted to evaluate the detection of MCF-7 breast cancer cells by using the assay with results establishing that the observed sensitivity corresponded to the identification of one cancer cell amongst 1×10^6 hematopoietic cells. With regards to the evaluation of specificity, none of the healthy control samples showed up positive for survivin mRNA.

NOTES

1. The correct size of PCR products (439 and 339 survivin fragments) from the MCF-7 breast cancer cell line, samples of breast cancer tissue, and blood samples with high concentrations of circulating cancer cells was determined by comparison with a DNA standard on a 1.2% agarose gel and the identity was confirmed by a sequence analysis.
2. The coated plates/strips are valid for up to 1 year when stored under −20°C.
3. In order to validate the procedure, total RNA extracted from whole blood cells were compared with those from isolated blood mononuclear cells by using a gradient Percoll centrifugation (Sigma). Results showed that there were no significant statistical differences in either the quality or the concentration between the two extraction procedures.
4. To optimize the reproducibility of cDNA synthesis, an aliquot of a master mix solution containing 500 ng of Oligo $(dT)_{12–18}$ primer, 0.5 U of RNase inhibitor, 0.5 mM dNTPs in 5x reaction buffer and 50 mM DTT was used to reduce any variation between RT processes.
5. Nucleic acid hybridization is sequence-dependent and varies under different environmental parameters. Generally speaking, hybridization temperature is experimentally chosen or selected to be 2°C lower than the thermal melting point (Tm) for some predefined ionic strength and pH level.
6. Low stringency washing conditions (e.g., using more salt and lower temperature) can increase sensitivity, but can also produce nonspecific hybridization and high background signals. High stringency washing

conditions (e.g., using less salt and higher temperature which is closer to the hybridization temperature) tend to lower the background signal with typically only the specific signal remaining.

7. To ensure that survivin expression in samples can be directly calculated from the standard curve, 34 blood samples were collected from breast cancer patients. The total RNA extraction and cDNA synthesis were performed using the already discussed methods. For internal control, β-actin cDNA was introduced and coamplified in the same reaction sample using β-actin control amplimer sets (Clontech, Palo Alto, CA, USA). Survivin expression in each of the samples was represented as a ratio of survivin to β-actin, determined by a 1.2% agarose gel electrophoresis stained with ethidium bromide and analyzed using a laser densitometer (Molecular Dynamics, Sunnydale, CA, USA). Results were then compared with those obtained from the RT-PCR-ELISA process from which a significant correlation ($r = 0.9378$, $P < 0.0001$) between the two methods was evident. Data from Yie *et al*. (2006).
8. In addition, survivin splicing variants (survivin-ΔEx3 and survivin-2B) were also observed in some specimens by using the SUF and SUB primer pairs.

CONCLUSIONS AND PERSPECTIVES

It is now clear that breast cancer cells are occasionally able to shed from the primary lesion very early during the natural history of tumors, and that hematogenous spreading of tumor cells from a primary tumor can be considered as a crucial step in the metastasis cascade leading eventually to the formation of clinically manifested metastases. Current prognostic/predictive factors and detection methods have been shown to be not as sensitive or specific enough for the prediction of metastases and recurrences. Consequently, as outlined by a number of recent studies, detection of disseminated tumor cells in the peripheral blood by using either cytometric or molecular approaches might be of clinical use, especially with respect to predicting clinical outcomes, making therapeutic decisions, and monitoring therapies. Nevertheless, published reports have also revealed certain problems which can inevitably lead to false-positive results that greatly diminish the correlation between tumor markers and some of the well-known clinical and pathological prognostic/predictive factors.

To improve upon the existing methodologies, a novel quantitative technique based on an ELISA detection system for RT-PCR products called RT-PCR-ELISA has been developed and used to detect survivin-expressing circulating breast cancer cells in peripheral blood specimens. Survivin is a member of the IAP family whose mRNA expression is detectable in the majority of primary breast carcinomas, but is rarely expressed in normal breast tissues or hematopoietic cells. This feature makes survivin one of the most desirable tumor-specific markers up to date.

The RT-PCR-ELISA method represents a reliable and reproducible assay to be used in studies geared towards the quantitative analysis of relative changes in survivin gene expression. The findings obtained from the detection of survivin mRNA in the peripheral blood of breast cancer patients through the use of the assay, strongly suggest that the presence of circulating tumor cells is highly associated with clinicopathological parameters and could have a clinical application value in predicting metastasis and recurrence in cancer patients. The results also suggest that circulating breast cancer cells expressing survivin mRNA may denote a characteristic feature of disseminated cancer cells which have the ability to establish metastasis and recurrence.

Even so, the mechanisms by which a malignant tumor cell establishes a metastatic foci

is a complex process wherein to achieve successful macrometastases, a tumor cell must first separate from the primary tumor, gain access to an efferent channel (whether lymphatic or vascular), then survive the transport to a distant capillary bed, exit the vessel at the distant site, and finally obtain a new blood supply within the distant organ. As Lacroix (2006) further elaborates, at each stage, the cancer cell must evade immunological responses and potentially adverse metabolic conditions. Furthermore, Mehes *et al*. (2001) reported that most circulating tumor cells are apoptotic and very few of them that are shed into the blood stream succeed in establishing secondary tumors. Thus, the presence of circulating tumor cells *per se* should not be regarded as indicative of metastatic disease. Nonetheless, of all the metastastic mechanisms, the characteristic/phenotype of spread cancer cells provide the most contribution to the success of macrometastases as demonstrated by Al-Hajj *et al*. (2003), where the breast cancer cells that were capable of establishing tumors were identified as those that expressed CD44, an adhesion molecule and cell surface marker.

In future studies, extensive investigations to assess recurrences and survival rates on a larger number of cohort patients will be needed. Likewise, multiple center studies on the early prediction of disease metastasis and recurrence by way of detecting circulating cancer cells using the RT-PCR-ELISA for survivin should be of great interest. Overall, additional *in vitro* experiments and studies on animal models are also necessary to fully verify the hypothesis that the detection of circulating breast cancer cells expressing survivin mRNA is more successful in establishing metastastic tumors.

REFERENCES

Adida, C., Crotty, P.L., McGrath, J., Berrebi, D., Diebold, J., and Altieri, D.C. 1998. Developmentally regulated expression of the novel cancer anti-apoptosis gene survivin in human and mouse differentiation. *Am. J. Pathol. 152*: 43–49.

Aerts, J., Wynendaele, W., Paridaens, R., Christiaens M.R., van den Bogaert, W., van Oosterom, A.T., and Vandekerckhove, F. 2001. A real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR) to detect breast carcinoma cells in peripheral blood. *Ann. Oncol. 12*: 39–46.

Al-Hajj, M., Wicha, M.S., Benito-Hernandez, A., Morrison S.J., and Clarke, M.F. 2003. Prospective identification of tumorigenic breast cancer cells. *Proc. Natl. Acad. Sci. USA. 100*: 3983–3988.

Ambrosini, G., Adida, C., and Altieri, D.C. 1997. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat. Med. 3*: 917–921.

Bosma, A.J., Weigelt, B., Lambrechts, A.C., Verhagen, O.J., Pruntel, R., Hart, A.A., Rodenhuis, S., and van't Veer, L.J. 2002. Detection of circulating breast tumor cells by differential expression of marker genes. *Clin. Cancer Res. 8*: 1871–1877.

Bray, F., McCarron, P., and Parkin, M.D. 2004. The changing global patterns of female breast cancer incidence and mortality. *Breast Cancer Res. 6*: 229–239.

Chiou, S.K., Jones, M.K., and Tarnawski, A.S. 2003. Survivin–an anti-apoptosis protein: its biological roles and implications for cancer and beyond. *Med. Sci. Monit. 9*: 125–129.

Cianfrocca, M., and Goldstein, L.J. 2004. Prognostic and predictive factors in early stage breast cancer. *Oncologist 9*: 606–616.

Denley, H., Pinder, S.E., Elston, C.W., Lee, A.H.S., and Ellis, I.O. 2002. Preoperative assessment of prognostic factors in breast cancer. *J. Clin. Pathol. 54*: 20–24.

Ferlay, J., Bray, F., Pisani, P., and Parkin, D.M. 2001. GLOBOCAN 2000: Cancer Incidence, Mortality and Prevalence Worldwide, IARC Cancer Base No. 5. Lyon: IARC Press.

Fisher, B., Jeong, J.H., Anderson, S., Bryant, J., Fisher, E.R., and Wolmark, N. 2002. Twenty-five year follow-up of a randomized trial comparing radical mastectomy, total mastectomy, and total mastectomy followed by irradiation. *New Engl. J. Med. 347*: 567–575.

Gilbey, A.M., Burnett, D., Coleman, R.E., and Holen, I. 2004. The detection of circulating breast cancer cells. *J. Clin. Pathol. 57*: 903–911.

Goeminne, J.C., Guillaume, T., Salmon, M., Machiels, J.P., D'Hondt, V., and Symann, M. 1999. Unreliability of carcinoembryonic antigen (CEA) reverse transcriptase-polymerase chain reaction (RT-PCR) in detecting contaminating breast cancer cells in peripheral blood stem cells due to induction of CEA by growth factors. *Bone Marrow Transplant 24*: 769–775.

Gross, H.J., Verwer, B., Houck, D., Hoffman, R.A., and Recktenwald, D. 1995. Model study detecting breast cancer cells in peripheral blood mononuclear cells at frequencies as low as 10^{-7}. *Proc. Natl. Acad. Sci. USA. 92*: 537–541.

Konno, R., Yamakawa, H., Utsunomiya, H., Ito, K., Sato, S., and Yajima, A. 2000. Expression of survivin and Bcl-2 in the normal human endometrium. *Mol. Hum. Reprod. 6*: 529–534.

Johnson, M.E., and Howerth, E.W. 2004. Survivin: a bifunctional inhibitor of apoptosis protein. *Vet. Pathol. 41*: 599–607.

Jung, R., Kruger, W., Hosch, S., Holweg, M., Kroger, N., Gutensohn, K., Wagener, C., Neumaier, M., and Zander, A.R. 1998. Specificity of reverse transcriptase polymerase chain reaction assays designed for the detection of circulating cancer cells is influenced by cytokines in vivo and in vitro. *Br. J. Cancer 78*: 1194–1198.

Lacroix, M. 2006. Significance, detection and markers of disseminated breast cancer cells. *Endoc.-Relat. Cancer 13*: 1033–1067.

Lambrechts, A.C., Bosma, A.J., Klaver, S.G., Top, B., Perebolte, L., van't Veer, L.J., and Rodenhuis, S. 1999. Comparison of immunocytochemistry, reverse transcriptase polymerase chain reaction, and nucleic acid sequence-based amplification for the detection of circulating breast cancer cells. *Breast Cancer Res. Treat. 56*: 219–231.

Li, F., and Brattain, M.G. 2006. Role of the survivin gene in pathophysiology. *Am. J. Pathol. 169*: 1–11.

Manhani, A.R., Manhani, R., Soares, H.P., Bendit, I., Lopes, F., Nicoletti, A.G. Fonseca, F.L., Novaes, M., Zatta, S.M., Arias, V., Giralt, S., and del Giglio, A. 2001. CK-19 expression by RT-PCR in the peripheral blood of breast cancer patients correlates with response to chemotherapy. *Breast Cancer Res. Treat. 66*: 249–254.

Mehes, G., Witt, A., Kubista, E., and Ambros, P.F. 2001. Circulating breast cancer cells are frequently apoptotic. *Am. J. Pathol. 159*: 17–20.

Mirza, A.N., Mirza, N.O., Vlastos, G., and Singletary, S.E. 2002. Prognostic factors in node-negative breast cancer: a review of studies with sample size more than 200 and follow-up more than 5 years. *Ann. Surg. 235*: 10–26.

Muller, V., and Pantel, K. 2004. Bone marrow micrometastases and circulating tumor cells: current aspects and future perspectives. *Breast Cancer Res. 6*: 258–261.

Nasu, S., Yagihashi, A., Izawa, A., Saito, K., Asanuma, K., Nakamura, M., Kobayashi, D., Okazaki, M., and Watanabe, N. 2002. Survivin mRNA expression in patients with breast cancer. *Anticancer Res. 22*: 1839–1843.

Pachmann, K., Heiss, P., Demel, U., and Tilz, G. 2001. Detection and quantification of small numbers of circulating tumor cells in peripheral blood using laser scanning cytometer (LSC). *Clin. Chem. Lab. Med. 39*: 811–817.

Pantel, K., and Brakenhoff, R.H. 2004. Dissecting the metastatic cascade. *Nat. Rev. Cancer 4*: 448–456.

Pizzoferrato, E., Liu, Y., Gambotto, A., Armstrong, M.J., Stang, M.T., Gooding, W.E., Alber, S.M., Shand, S.H., Watkins, S.C., Storkus, W.J., and Yim, J.H. 2004. Ectopic expression of interferon regulatory factor-1 promotes human breast cancer cell death and results in reduced expression of survivin. *Cancer Res. 64*: 8381–8388.

Ring, A., Smith, I.E., and Dowsett, M. 2004. Circulating tumor cells in breast cancer. *Lancet Oncol. 5*: 79–88.

Ryan, B., O'Donovan, N., Browne, B., O'Shea, C., Crown, J., Hill, A.D., McDermott, E., O'Higgins, N., and Duffy, M.J. 2005. Expression of survivin and its splice variants survivin-2B and survivin-DeltaEx3 in breast cancer. *Br. J. Cancer 92*: 120–124.

Smith, B.M., Slade, M.J., English, J., Graham, H., Luchtenborg, M., Sinnett, H.D., Cross, N.C., and Coombes, R.C. 2000. Response of circulating tumor cells to systemic therapy in patients with metastatic breast cancer: comparison of quantitative polymerase chain reaction and immunocytochemical techniques. *J. Clin. Oncol. 18*: 1432–1439.

Span, P.N., Sweep, F.C., Wiegerinck, E.T., Tjan-Heijnen, V.C., Manders, P., Beex, L.V., and de Kok, J.B. 2004. Survivin is an independent

prognostic marker for risk stratification of breast cancer patients. *Clin. Chem. 50*: 1986–1993.

Span, P.N., Tjan-Heijnen, V.C., Heuvel, J.J., de Kok, J.B., Foekens, J.A., and Sweep, F.C. 2006. Do the survivin (BIRC5) splice variants modulate or add to the prognostic value of total survivin in breast cancer? *Clin. Chem. 52*: 1693–1700.

Stathopoulou, A., Vlachonikolis, I., Mavroudis, D., Perraki, M., Kouroussis, C., Apostolaki, S., Malamos, N., Kakolyris, S., Kotsakis, A., Xenidis, N., Reppa, D., and Georgoulias, V. 2002. Molecular detection of cytokeratin-19-positive cells in the peripheral blood of patients with operable breast cancer: valuation of their prognostic significance. *J. Clin. Oncol. 20*: 3404–3412.

Taback, B., Chan, A.D., Kuo, C.T., Bostick, P.J., Wang, H.J., Giuliano, A.E., and Hoon, D.S. 2001. Detection of occult metastatic breast cancer cells in blood by a multimolecular marker assay: correlation with clinical stage of disease. *Cancer Res. 61*: 8845–8850.

Valasek, M.A., and Repa, J.J. 2005. The power of real-time PCR. *Advan. Physiol. Educ. 29*: 151–159.

Yie, S.M., Luo, B., Ye, N.Y., Xie, K., and Ye, S.R. 2006. Detection of Survivin expressing circulating cancer cells in the peripheral blood of breast cancer patients by a RT-PCR ELISA. *Clin. Exp. Metastasis 23*: 279–289.

Zach, O., Kasparu, H., Wagner, H., Krieger, O., and Lutz, D. 2002. Prognostic value of tumour cell detection in peripheral blood of breast cancer patients. *Acta. Med. Austriaca 29 [Suppl 59]*: 32–34.

Zaffaroni, N. and Daidone, M.G. 2002. Survivin expression and resistance to anticancer treatments: perspectives for new therapeutic interventions. *Drug Resist. Updates 5*: 65–72.

Zaffaroni, N., Pennati, M., and Daidone, M.G. 2005. Survivin as a target for new anticancer interventions. *J. Cell. Mol. Med. 9*: 360–372.

Zieglschmid, V., Hollmann, C., and Bocher, O. 2005. Detection of disseminated tumor cells in peripheral blood. *Crit. Rev. Clin. Lab. Sci. 42*: 155–196.

14
Node-Negative Breast Cancer: Predictive and Prognostic Value of Peripheral Blood Cytokeratin-19 mRNA-Positive Cells

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INTRODUCTION

As a result of increased mammography screening programs, the majority of patients with breast cancer in recent years present with early-stage disease because the screening-detected tumors are significantly smaller than symptomatic or palpable tumors and frequently without axillary lymph node involvement. Metastatic involvement in the axillary lymph nodes is a powerful prognostic factor. Although the Early Breast Cancer Trialists' Collaborative Group (2005) has shown a direct relationship between the number of involved nodes and the clinical outcome; nearly 30% of patients with node-negative breast cancer will present distant recurrence and will die as a result of their disseminated disease. This observation suggests that despite the general model of tumor cell dissemination, through the lymphatogenous route in the regional lymph nodes, there is also a direct haematogenous tumor cell dissemination that bypasses the lymphogenous. Thus, breast cancer detection at early stages does not ensure the definitive cure due to unpredictable invasiveness and metastatic potential of tumor cells.

There are several dilemmas concerning the adjuvant treatment of patients with node-negative breast cancer. Expansion of therapeutic opportunities further complicates these decisions. Determination of the likelihood of recurrence for each patient in combination with the estimation of the efficacy of the available therapeutic modalities and the evaluation of potential side effects would assist physicians to determine the probable benefit of adjuvant systemic therapy for individual patient.

PROGNOSTIC AND PREDICTIVE FACTORS

Adjuvant systemic therapy is currently recommended for all node-positive patients with breast cancer as the disease

is not localized anymore. For node-negative patients guidance is not as equivocal and virtually all patients receive systemic chemotherapy. The purpose of adjuvant therapy is to eliminate probable micrometastatic deposits and disseminated tumor cells that have disseminated before or during the surgical excision of the tumor. However, adjuvant systemic therapy is associated with significant side effects; therefore, it would be very important to optimally determine patients who are at high risk for relapse for adjuvant treatment by detecting clinically silent micrometastatic disease. The identification of prognostic factors associated with either the growth or metastatic potential of the primary tumor would also assist in selecting the most favorable adjuvant therapy for an individual patient.

A prognostic factor is defined as any parameter available at the time of diagnosis or surgery that is associated with disease-free or overall survival in the absence of systemic adjuvant therapy and, as a result, is able to correlate with the natural history of the disease. In contrast, a predictive factor is any parameter associated with response or lack of response to a particular therapy. The ideal prognostic marker which could be expressed by tumor cells is that which is strongly associated with early metastases and short survival. Conversely, tumors which do not express the prognostic marker would be associated with an indolent course, the absence of metastases, and prolonged survival in nearly all patients. According to the evaluation guidelines that were defined by McGuire (1991) the minimal criteria that must be considered when one is attempting to evaluate a new prognostic factor are the following:

- The factor to be studied should possess clear biological significance.
- The number of patients in the study must be appropriate according to the kind of study (pilot, definitive or confirmatory).
- There should be an adequate sample size studied for meaningful calculations.
- The patients' population must be defined and not biased.
- There must be methodological validation.
- Clinical cut-off must be defined.
- Assays must be reproducible.

Ideally, a study of prognostic factors should involve only patients who have received no systemic therapy. However, such studies have become nearly impossible to carry out because systemic therapy is now recommended for the vast majority of patients with breast cancer.

There are three categories of prognostic and predictive factors: (1) patient's characteristics that are independent of the disease, such as age; (2) disease's characteristics, such as tumor size and histologic type; and (3) biomarkers (measurable parameters in tissues, cells, or fluids), such as estrogen receptor status, progesterone receptor status, and tumor cell proliferation parameters. Although a large number of various factors have been investigated in early and node-negative breast cancers, presently only tumor size, histological grade, lymphatic and vascular invasion and proliferation rate are widely accepted as clinically relevant prognostic factors. Estrogen and progesterone receptor status as well as HER2/neu expression are both prognostic and predictive factors. On the contrary, ethnicity and patient age at diagnosis, genetic profiling, cell adhesion molecules and proteases, apoptosis-related proteins, cell cycle molecules, angiogenesis-related

proteins, growth factor receptors and presence of minimal residual disease in bone marrow and peripheral blood, have also been proposed as prognostic or predictive factors but their clinical value still remains investigational.

Tumor Size

For node-negative patients, tumor size is the most powerful prognostic factor and is routinely used to make adjuvant treatment decisions. Carter *et al*. (1989) reported, in a population of 13,464 women with node-negative breast cancer, a 5-year overall survival close to 99% in patients with tumors < 1 cm compared with 89% for tumors between 1 and 3 cm and 86% for tumors between 3 and 5 cm. Although the incidence of disease recurrence increases as the tumor size increases, Adair *et al*. (1974) have shown that patients with extremely large tumors tend to have better outcomes than those with tumors of an intermediate size. An explanation could be that tumors that have grown to a large size without resulting in nodal involvement might have a lower potential for metastatic spread.

Histological Grade

The most widely used grading systems for breast cancers are the Scarff-Bloom-Richardson classification and Fisher's nuclear grading system. A correlation between histologic grade, as determined by the Scarff-Bloom-Richardson classification and 5-year disease-free survival has been demonstrated by Le Doussal *et al*. (1989), in a study of 1,262 women. Patients with an Scarff-Bloom-Richardson score of 3 had a 4.4 relative risk of recurrence compared with those with an Scarff-Bloom-Richardson of 1. Although tumor grade has a prognostic significance, a criticism of its prognostic value has been the poor reproducibility and lack of agreement among different observers.

Lymphatic and Vascular Invasion

Vascular invasion refers to the invasion of lymphatic spaces, blood vessels, or both in the peritumoral area by tumor emboli. Lymphatic and vascular invasion does have prognostic significance for the risk of local and distant recurrence and is primarily used to make decisions for lymph node-negative patients with borderline tumor sizes. Lee *et al*. (2006) studied 2,760 women with node-negative invasive breast carcinoma with median follow-up of 13 year and showed that lymphovascular invasion was an independent factor associated with a decreased overall survival.

Proliferation Rate

Various markers associated with the proliferation capacity of tumor cells have been studied as prognostic factors in node-negative breast cancer patients. Among the most intensively investigated are Ki-67, S-phase fraction, and mitotic index. The nuclear antigen Ki-67 is expressed only in proliferating cells (late G1, S, M, and G2 phases of the cell cycle). Brown *et al*. (1996) demonstrated that Ki-67 provides significant independent prognostic information and that high Ki-67 was associated with a 1.8-fold increased risk of recurrence. Recently Baak *et al*. (2007) found that the prognostic significance of Ki-67 staining in node-negative invasive breast cancer is age dependent and that its overexpression is more important in younger patients.

S-phase fraction refers to the proportion of tumor cells that are proliferating and is measured by DNA flow cytometry. A strong association between S-phase fraction and disease-free interval, as well as overall survival, was found by Bryant *et al*. (1998) in a well-defined cohort of over 4,000 breast cancer patients from National Surgical Adjuvant Breast and Bowel Project protocol B-14.

Mitotic index is determined by using light microscopy on paraffin-embedded tumor specimens stained with hematoxylin and eosin to count mitotic figures and is usually expressed as the number of mitoses per high-power field. Only a few studies have assessed the prognostic value of mitotic index for decreased survival in node-negative breast cancer patients. Aaltomaa *et al*. (1992) found that volume-corrected mitotic index is a more powerful predictor of clinical outcome than uncorrected mitotic index; in multivariate analysis mitotic index was emerged as an independent factor associated with a decreased overall survival.

Estrogen and Progesterone Receptors

Estrogen and progesterone receptors are intracellular polypeptides that bind to estrogen, translocate into the nucleus and act as hormone-dependent transcriptional regulators. Overexpression of the progesterone receptor serves as a functional assay because it indicates that the estrogen receptors pathway is intact, even if the tumor is reported as estrogen receptors-negative. The presence of estrogen and progesterone receptors in an invasive breast carcinoma is both prognostic and predictive. Its prognostic effect is difficult to evaluate in that it must be assessed in the absence of adjuvant tamoxifen. Although conflicting results have appeared in the literature, the larger studies with longer follow-up have consistently demonstrated that patients with estrogen receptor-positive tumors have longer disease-free interval than patients with estrogen receptor-negative tumors. Among node-negative patients, small but statistically significant differences in disease-free interval and overall survival times have been found between estrogen receptor-positive cases and estrogen receptor-negative cases. McGuire *et al*. (1990), in a multivariate analysis of prognostic factors including the estrogen receptor status for more than 3,000 patients, showed that in node-negative patients the estrogen receptor status was more important for prognosis than the tumor size. Nevertheless, Donegan (1997) demonstrated that the value of estrogen receptor status as an independent prognostic variable is diminished by its association with other established indicators of favorable prognosis, such as older age, low-grade histology, favorable nuclear grade, low S-phase fraction, normal complement of DNA, and low proliferation index.

The additional knowledge gained by measuring progesterone receptors in patients with node-negative disease is not clear. The presence of progesterone receptors is an indicator of an intact estrogen-response pathway, because it is produced by estrogen stimulation. Although, estrogen receptor status is a stronger predictor of disease-free interval, progesterone receptor status is more closely associated with overall survival, probably because it is a better indicator of response to endocrine therapy after disease recurrence.

The presence of estrogen or progesterone receptors is, however, a powerful predictive factor for the likelihood of benefit from adjuvant endocrine therapy. The most recent Early Breast Cancer Trialists' Collaborative Group (1998) update of randomized trials using adjuvant tamoxifen, included 37,000 women; it was shown that 5 years of adjuvant tamoxifen led to a 47% proportional reductions in the risk of recurrence and a 26% decrease of the mortality risk in patients with estrogen receptor-positive tumors.

Human Epidermal Growth Factor Receptor-2 (HER2/*neu*)

HER2/*neu* (also known as c-erbB-2) is a proto-oncogene that encodes a 185-kDa tyrosine kinase glycoprotein. HER2/*neu* is overexpressed in 20–30% of breast cancers and almost always results from gene amplification. Overexpression is inversely correlated with ER expression and is associated with poorly differentiated tumors with a high proliferation rate. Overexpression of this gene is associated with a more aggressive clinical course, a higher incidence of clinical relapse, and a decrease of overall survival in node-positive cases. However, Yamauchi *et al*. (2001) have reported that, in node-negative patients, HER2/*neu* expression is not a strong prognostic factor. Prognostic value of HER2/*neu* expression in node-negative breast cancers seems to become more reliable by measuring its amplification with fluorescence *in situ* hybridization (FISH), as mentioned by Press *et al*. (1997). Recent data by Seidman *et al*. (2001) indicate that HER2/*neu* gene overexpression, evaluated with FISH, is the major predictive factor for the efficacy of trastuzumab, monoclonal antibody against extracellular domain of the HER2/*neu* receptor. HER2/*neu* expression may also predict benefit from adjuvant anthracyclines as have been reported by Ravdin (2001). There are also data suggesting that HER2/*neu* overexpression may confer "resistance" to adjuvant endocrine therapy (tamoxifen) (De Placido *et al*., 2003).

MINIMAL RESIDUAL DISEASE

The investigation and evaluation of the above mentioned factors as well as other not fully validated prognostic factors, may improve our ability to accurately determine high risk for relapse patients with early breast cancer. The development of metastases is the final step in the natural history of the disease and the definitive confirmation of tumor aggressiveness. Between these two steps lies the detection of minimal residual disease. Presence of disseminated or circulating tumor cells in bone marrow aspirates and the peripheral blood, respectively, is the result of poor prognostic parameters of the primary tumor, and indicates, quite early, its metastatic potential.

The detection of occult tumor cells in the bone marrow (disseminated tumor cells) of patients with early stage breast cancer has been shown by several investigators to be an independent predictive and prognostic factor for early disease recurrence and decreased overall survival. Braun *et al*. (2005) reported that the immunohistochemical detection of bone marrow disseminated tumor cells in 4,703 patients with operable breast cancer was an independent prognostic parameter for early relapse and death during the first 5 years

of follow-up. However, bone marrow is not an easy source for monitoring disseminated tumor cells. Peripheral blood could be an ideal source for the detection of circulating tumor cells due to the simple sampling procedure.

CIRCULATING TUMOR CELLS

In many patients with breast cancer circulating cells with the characteristics of tumor cells can be identified in the peripheral blood. These cells are present not only in patients with advanced disease, but also in those whose tumors are apparently localized.

The development of distant metastasis is a cascade of linked sequential steps involving multiple host-tumor interactions. Cancer cells, acquiring certain genetic abnormalities, grow unregulated and eventually lose the ability to adhere to one another. Thus, tumor cells detach from the primary tumor, migrate through the basement membrane and the extracellular matrix, penetrate the surrounding vessels and travel in the lymphatic and/or blood systems to a new site. These particular cells possess many differences from the main cell population of the primary tumor.

Indeed, there are several references in literature about the phenotype dissimilarity between circulating or disseminated tumor cells and the primary tumor cells. Thiery (2002) described changes in cytoskeleton which lead to loss of epithelial cell polarity, disassembly of tight junctions, adherent junctions, and desmosomes, as well as increase of cell motility. Alterations occur in a variety of genes that encode molecules such as plakoglobin, as mentioned by Braun and Pantel (1999), and Ep-CAM, as described by Thurm *et al*. (2003), molecules which participate in adhesion between cells and between cells and extracellular matrix. Roetger *et al*. (1998) found that the expression of MMP-2, CD44 and integrins $\alpha_v\beta_3$ or $\alpha_6\beta_1$ also are altered in circulating tumor cells, facilitating cell movement through the extracellular matrix. In addition, Braun and Pantel (1999), described modifications in ICAM-1 and HLA-1 expression which help cells to escape from immune system as well as in proteins like α_5 and β_1 integrins or STAT-1 and (2′–5′) oligoadenylate synthetase-1, which prevent apoptosis. Although the fraction of apoptotic circulating tumor cells rises after chemotherapy, as demonstrated by Fehm *et al*. (2006), data by Balic *et al*. (2006) indicate that a high percentage of circulating cells demonstrate a CD44+CD24−/low phenotype, representative of putative stem cells with self-renewal and tumorigenic potential.

DETECTION OF CIRCULATING TUMOR CELLS

According to the literature, the detection rate of circulating tumor cells in early-stage breast cancer varies between 0% and 88%. This variability (0–88%) of results seems to be the consequence of variations in methodology. Factors that may influence the results include: (i) heterogeneity of the studied populations according to the stage since positivity increases as clinical stage rises, (ii) the interval of time separating surgery from the obtaining of blood samples, (iii) Hu *et al*. (2003) demonstrated that surgery may increase the number of

circulating tumor cells in the peripheral blood. Other causes of conflicted results include delay between collection and analysis, incorrect conditions of sample storage, contamination with normal epithelial cells during venipuncture, different criteria or threshold of positivity and variability of sensitivity and specificity of various markers for detecting circulating tumor cells.

Techniques for Detecting Circulating Tumor Cells

Methods which can be used for the identification of circulating tumor cells must mainly distinguish between epithelial and other cells, and secondarily, cancer and normal epithelial cells. Techniques to detect circulating tumor cells can be roughly divided, into cytometric and nucleic acid-based approaches. Cytometric approaches use immunocytochemical methods to detect circulating cells based upon the expression of specific cell surface markers. Nucleic-acid-based approaches detect DNA or RNA sequences that are differentially expressed in tumor cells and normal blood components. Even in metastatic patients, the number of circulating tumor cells in peripheral blood is very low when compared with the surrounding blood cells. In addition to the implementation of more sensitive molecular techniques, enrichment for the tumor cell population from the blood may increase the sensitivity for the detection of circulating tumor cells.

Enrichment Techniques for Preanalytical Circulating Tumor Cells

The enrichment circulating tumor cells is usually performed by using density gradients (Ficoll/Hypaque, OncoQuick), porous membranes, or immunomagnetic techniques (using magnetic affinity cell sorting, magnetic beads or ferrofluid-based systems). Density gradients allow the isolation of mononuclear cells, which are believed to contain the circulating tumor cells fraction; however, tumor cell losses may occur in the granulocyte fraction. Porous membranes, with pore sizes chosen such that smaller leukocytes pass through, are also available for circulating tumor cells enrichment. Magnetic affinity cell sorting and magnetic beads use antibodies, linked to small paramagnetic beads, with an affinity for specific cells. The cells can then be selected with a powerful magnet. Commercially available beads are linked to antiepithelial antibodies for positive selection (HEA125, cytokeratins, BerEP4) or linked to a monoclonal antibody directed against CD45 for negative selection of leukocytes. The ferrofluid-based system uses EP-CAM (epithelial-cell adhesion molecule) coupled to colloids of 1 nm (ferrofluids) followed by magnetic separation.

Cytometric Techniques

Immunocytochemistry, immunofluorescence and flow cytometry analysis can be used for the detection and isolation of individual tumor cells. Immunocytochemical methods are based on monoclonal antibodies against various epithelial specific antigens. For breast tumors, the most used targets for antibody-based techniques are cytokeratins. Malignant cells derived from cells of epithelial origin tend to retain the intermediate filaments of their progenitor cell type. Therefore, the detection of cytokeratins in an environment where no cytokeratin expression is expected (such as in the mesenclymal-derived peripheral blood cells) has been proposed as a

surrogate marker for epithelial tumor cells. Nevertheless, as reported by Zieglschmid *et al*. (2005), because of the use of illegitimate antibodies, a false-positive detection rate of 1–3% can be expected, since an expression of cytokeratin has been observed in hematopoietic cells.

Immunocytochemistry and immunofluorescence are time consuming, relatively expensive and difficult to use for routine implementation. In contrast, flow cytometry offers the advantage of a fully automated technique allowing quantitative measurements with high sensitivity, good resolution, speed, reproducibility and statistical reliability. However, an advantage of immunocytochemistry is that it may allow further characterization of the cells at a molecular level, in terms of expression of key biological markers.

Nucleic Acid-Based Techniques

Polymerase chain reaction (PCR) has been used to detect free DNA and also to identify and characterize circulating cancer cells through the detection of genetic (allele-specific expression, micro-satellite instability, loss of heterozygosity) and epigenetic alterations (methylation status), that are specifically associated with cancer cells, as have been reviewed by Sidransky (1997). Although the routes by which tumor DNA in such patients enters the circulation currently remain unknown, cell lysis, tumor necrosis, apoptosis, and active cell shedding have been hypothesized by Anker *et al*. (1999) to be involved in this process. However, this use of PCR is limited by poor specificity. This is due in part to the high stability of DNA in plasma and therefore, the detection and measurement of circulating tumor DNA may not be a reliable marker indicative of viable tumor cells actively shedding nucleic acids and, thus, the potential of a particular patient to develop metastatic disease.

Reverse transcription (RT)-PCR has been used to identify circulating tumor cells through their expression of breast cancer-associated mRNA transcripts. The advantage of RNA-based approaches is that the viability of RNA in clinical samples once released from cells is poor because it is highly susceptible to degradation by blood RNAses. Thus, detection of an RNA transcript in a blood sample suggests that it is present in the context of a viable tumor cell.

The first step in RT-PCR is isolation of peripheral-blood mononuclear cells from the blood sample. The usual approach is density-gradient centrifugation, although some investigators have used immunomagnetic separation to enrich samples for epithelial cells. After RNA extraction from the cells, reverse transcriptase is used to transcribe target transcripts into cDNA. The cDNA is then subjected to PCR amplification with primers specific to the transcript of interest. To increase sensitivity, a second PCR reaction with different primers can be done on the amplification product of the first reaction (nested PCR). Reactions are run with both positive controls (RNA from a breast-cancer cell line in most cases) and negative controls. The quality of the RNA and efficiency of the reaction are controlled by co-amplification of transcripts of house-keeping genes such as those for glyceraldehyde-3-phosphate dehydrogenase or β-actin. Products of the RT-PCR reaction are then separated by agarose-gel electrophoresis and stained with ethidium bromide for detection. The sensitivity of the assay can be assessed

by analysis of serial dilutions of a breast-cancer cell line in blood from a healthy volunteer.

To improve the reliability, especially the specificity of RT-PCR assays, quantitative real-time RT-PCR may be used. An additional advantage of real-time PCR is that a normal range of values can be established if the assay is done for some controls; a cut-off point for positivity can then be identified, improving the specificity of the test. Moreover, when compared with 'conventional' RT-PCR, real-time RT-PCR relies not only on primers, but also on internal probes that specifically hybridize to the amplified sequences. In addition, as described by Zieglschmid *et al*. (2005), due to the continuous measurement of the amplified signal, false-positive results which could produce an abnormally shaped and non-linear amplification curve that could be easily identified and removed. Although inhibitors of the PCR reaction present in blood could limit the *in vivo* sensitivity of nucleic-acid-based techniques, in comparative studies RT-PCR has higher rates of positivity than cytometric assays as have been shown by Lambrechts *et al*. (1999).

Molecular Markers for Detecting Circulating Tumor Cells

Both cytometric and nucleic-acid-based techniques use molecular markers for the identification of circulating tumor cells. An ideal marker should be universally expressed on all breast cancer cells (avoidance of false-negative results), but exclusively on them (avoidance of false-positive results); moreover, it should be easily detectable, with little variance and bear clinical relevance.

Markers with Low Breast Cancer Specificity

Cytokeratins are the most widely used markers for circulating breast cancer cells. Various types of cytokeratins are expressed in all epithelial tumors cells, but they have also been detected in normal and neoplastic non-epithelial cell types. Cytokeratin-19 (CK-19), a cytoplasmic intermediate filament expressed primarily on normal and tumor cells of epithelial origin has been studied extensively as a potential marker for minimal residual disease in blood. It is a very sensitive but not a breast cancer-specific marker. False-positive results may be obtained as a consequence of its expression in normal tissues of small amounts (illegitimate expression) as reported by Zieglschmid *et al*. (2005) in hematological disorders; this is due to the induction of its expression by cytokines and growth factors, which circulate at higher concentrations in inflammatory conditions and neutropenia. Another reason for false-positive results is the presence of pseudogenes which have significant sequence homology to CK-19 mRNA. Two CK-19 pseudogenes, CK-19a and CK-19b have been described by Ruud *et al*. (1999). Other markers that have been tested are cytokeratins 8, 18 and 20, cell adhesion molecules (EpCAM), CEA, growth factor receptors EGFR and ERBB2 and the glycosylated protein mucin-1.

Markers with High Breast Cancer Specificity

In order to increase the specificity of detection methods, many investigators evaluated several markers with almost exclusive expression on breast tissues. Molecules such as mammaglobins A and B,

maspin, BS106 protein, transcription factor ESR1 and trefoil peptides TFF1 and TFF3, although have high diagnostic accuracy for the detection of circulating breast cancer cells, they are not universally overexpressed in breast cancer cells. Moreover, evaluation of their expression in primary tumor is required before blood testing for circulating tumor cells.

CLINICAL RELEVANCE OF CIRCULATING TUMOR CELLS

Although the importance of disseminated tumor cells in bone marrow is well established as has been demonstrated by many authors, the significance of circulating tumor cells is less investigated and still uncertain. Stathopoulou *et al.* (2002) evaluated the presence of circulating tumor cells expressing CK-19 mRNA by nested RT-PCR in the peripheral blood of patients with stage I and II breast cancer before the initiation of any adjuvant cytotoxic or hormone therapy. Circulating CK-19 mRNA-positive cells could be detected in almost 30% of patients, and multivariate analyses demonstrated that the presence of these cells was an independent prognostic factor for decreased disease-free interval and overall survival.

Our group has recently found that persistence of CK-19 mRNA-positive cells after the completion of adjuvant chemotherapy had a negative effect on disease-free interval, which was dependent upon the number of involved lymph nodes (Xenidis *et al.*, 2003). More specifically, the hazard of relapse for patients with ≤ three involved axillary lymph nodes and detectable CK-19 mRNA-positive cells after adjuvant chemotherapy was 3.81 times higher than that of patients with CK-19 mRNA-negative cells. In contrast, Wiedswang *et al.* (2006) reported that in lymph-negative patients, disseminated tumor cell status in bone marrow, but not in peripheral blood, predicted differences in disease-free interval.

Predictive and Prognostic Value of Circulating Tumor Cells in Node-Negative Breast Cancer Patients

In an effort to elucidate the importance of circulating tumor cells in the peripheral blood of node-negative breast cancer patients before the initiation of any adjuvant treatment, a prospective study has been conducted in the Department of Medical Oncology of University General Hospital of Crete (Xenidis *et al.*, 2006). Peripheral blood was obtained from 167 patients with N0 breast cancer before the initiation of adjuvant treatment (usually 3–4 weeks after primary surgery). There was no clinical or radiological evidence of distant metastasis in all enrolled patients. Immunostaining with an anti-CK-19 mouse antihuman monoclonal antibody was performed in all paraffin-embedded axillary lymph nodes in order to ensure that all patients had node-negative disease.

Most of the patients received adjuvant chemotherapy in the context of research protocols especially designed for patients with high-or low-risk node-negative breast cancer. Adjuvant chemotherapy consisted of FEC (fluorouracil, epirubicin and cyclophosphamide), T/EC (epirubicin, cyclophosphamide followed by docetaxel) as well as classical CMF (cyclophosphamide, methotrexate and fluorouracil). Tamoxifen was administered to all patients with estrogen-or progesterone-positive tumors.

All blood samples tested for the presence of CK-19 mRNA-positive cells, using a real time PCR assay. Detection of CK-19 mRNA-positive circulating tumor cells was significantly associated with HER2/neu-positivity ($p = 0.033$) but not with other patients' or tumors' clinicopathologic characteristics. The median follow-up period for the whole group of patients was 32 months. During this period, 20 patients (12%) presented a distant or/and locoregional recurrence. Clinical recurrence was significantly more frequent in patients with (44.4%) than without (3%) detectable CK-19 mRNA-positive circulating cells ($p < 0.000001$). The median number of CK-19 mRNA-positive cells was 0.8 MCF-7 cell equivalents/5 µg RNA and 0.0 MCF-7 cell equivalents/5 µg RNA in relapsed and non-relapsed patients, respectively (Mann-Whitney test, $p < 0.00001$). The median disease-free interval for patients with detectable CK-19 mRNA-positive circulating tumor cells was 55 months, whereas a disease-free interval has not yet been reached for patients without CK-19 mRNA-positive cells (log-rank $p < 0.00005$). During the follow-up period, eight patients (4.8%) died as a result of disease progression. Seven (87.5%) of these patients had detectable CK-19 mRNA-positive circulating cells compared with only one death observed in the group of patients without detectable cells ($p < 0.00005$). Although, the median overall survival has not yet been reached, patients with detectable circulating cells had a significantly shorter overall survival compared with patients with undetectable cells (log-rank $p < 0.00005$). Cox regression analysis revealed that detection of circulating cells remained the only independent prognostic factor for overall survival. Multivariate analysis revealed that detection of circulating cells, as well as estrogen receptor–negative tumors histopathologic grade 3, premenopausal status and the administration of CMF were all found to be independent predictive factors for early relapse.

CONCLUSION

The TNM system is incapable of identifying a subgroup of women who, although they have an early-stage breast cancer, may be at high risk of relapse and death. This is especially true for patients with small and node-negative tumors; therefore, it is important to develop new predictive and prognostic markers. Detection of minimal residual disease, either as disseminated tumor cells in bone marrow or as circulating tumor cells in peripheral blood, comprises an independent predictive and prognostic factor for early relapse and disease-specific death, respectively. However, there are discrepancies in the literature concerning the prognostic and predictive value of disseminated tumor cells in node-negative breast cancer patients. We cannot exclude methodologic reasons that may influence the detection limits of the used assays that may account for this phenomenon. Some of these problems may be eliminated by the development of standardized assays that are sensitive enough to detect a low load of occult tumor cells, facilitating thus the evaluation of the clinical significance of occult tumor cells in node-negative breast cancer patients.

Peripheral blood could be a valuable source for monitoring circulating tumor cells due to the simple sampling procedure. This opens the way to further investigate

important questions such as whether the detection of circulating tumor cells should be performed in all patients at the time of primary diagnosis to identify high-risk patients or circulating tumor cells detection at diagnosis should modify the adjuvant therapeutic strategy. Finally, it is very important to understand the clinical importance of the detection of circulating tumor cells during the administration of adjuvant chemotherapy or hormone-therapy which would allow the development of secondary adjuvant therapeutic strategies.

REFERENCES

Aaltomaa, S., Lipponen, P., Eskelinen, M., Kosma, V.M., Marin, S., Alhava, E., and Syrjanen, K. 1992. Mitotic indexes as prognostic predictors in female breast cancer. *J. Cancer Res. Clin. Oncol. 118*: 75–81.

Adair, F., Berg, J., Joubert, L., and Robbins, G.F. 1974. Long-term followup of breast cancer patients: the 30-year report. *Cancer 33*: 1145–1150.

Anker, P., Mulcahy, H., Chen, X.Q., and Stroun, M. 1999. Detection of circulating tumour DNA in the blood (plasma/serum) of cancer patients. *Cancer Metastasis Rev. 18*: 65–73.

Baak, J.P., van Diest, P.J., Voorhorst, F.J., der Wall, E., Beex, L.V., Vermorken, J.B., Janssen, E.A., and Gudlaugsson, E. 2007. The prognostic value of proliferation in lymph node-negative breast cancer patients is age dependent. *Eur. J. Cancer 43*: 527–535.

Balic, M., Lin, H., Young, L., Hawes, D., Giuliano, A., McNamara, G., Datar, R.H., and Cote, R.J. 2006. Most early disseminated cancer cells detected in bone marrow of breast cancer patients have a putative breast cancer stem cell phenotype. *Clin. Cancer Res. 12*: 5615–5621.

Braun, S. and Pantel, K. 1999. Biological characteristics of micrometastatic cancer cells in bone marrow. *Cancer Metastasis Rev. 18*: 75–90.

Braun, S., Vogl, F.D., Naume, B., Janni, W., Osborne, M.P., Coombes, R.C., Schlimok, G., Diel, I.J., Gerber, B., Gebauer, G., Pierga, J.Y., Marth, C., Oruzio, D., Wiedswang, G., Solomayer, E.F., Kundt, G., Strobl, B., Fehm, T., Wong, G.Y., Bliss, J., Vincent-Salomon, A., and Pantel, K. 2005. A pooled analysis of bone marrow micrometastasis in breast cancer. *N. Engl. J. Med. 353*: 793–802.

Brown, R.W., Allred, C.D., Clark, G.M., Osborne, C.K., and Hilsenbeck, S.G. 1996. Prognostic value of Ki-67 compared to S-phase fraction in axillary node-negative breast cancer. *Clin. Cancer Res. 2*: 585–592.

Bryant, J., Fisher, B., Gunduz, N., Costantino, J.P., and Emir, B. 1998. S-phase fraction combined with other patient and tumor characteristics for the prognosis of node-negative, estrogen-receptor-positive breast cancer. *Breast Cancer Res. Treat. 51*: 239–253.

Carter, C.L., Allen, C., and Henson, D.E. 1989. Relation of tumor size, lymph node status and survival in 24,740 breast cancer cases. *Cancer 63*: 181–187.

De Placido, S., De Laurentiis, M., Carlomagno, C., Gallo, C., Perrone, F., Pepe, S., Ruggiero, A., Marinelli, A., Pagliarulo, C., Panico, L., Pettinato, G., Petrella, G., and Bianco, A.R. 2003. Twenty-year results of the Naples GUN randomized trial: predictive factors of adjuvant tamoxifen efficacy in early breast cancer. *Clin. Cancer Res. 9*: 1039–1046.

Donegan, W.L. (1997) Tumor-related prognostic factors for breast cancer. *C.A. Cancer J. Clin. 47*: 28–51.

Early Breast Cancer Trialists' Collaborative Group. 1998. Tamoxifen for early breast cancer: an overview of the randomized trials. *Lancet 351*: 1451–1467.

Early Breast Cancer Trialists' Collaborative Group. 2005. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomized trials. *Lancet 365*: 1687–1717.

Fehm, T., Becker, S., Becker-Pergola, G., Sotlar, K., Gebauer, G., Durr-Storzer, S., Neubauer, H., Wallwiener, D., and Solomayer, E.F. 2006. Presence of apoptotic and non apoptotic disseminated tumor cells reflect response to neoadjuvant systemic therapy (NST) in breast cancer. *Breast Cancer. Res. 8*: R60.

Hu, X.C., Loo, W.T., and Chow, L.W. 2003. Surgery related shedding of breast cancer cells

as determined by RT-PCR assay. *J. Surg. Oncol. 82*: 228–232.

Lambrechts, A.C., Bosma, A.J., Klaver, S.G., Top, B., Perebolte, L., van' t Veer, L.J., and Rodenhuis, S. 1999. Comparison of immunocytochemistry, reverse transcriptase polymerase chain reaction, and nucleic acid sequence-based amplification for the detection of circulating breast cancer cells. *Breast Cancer Res. Treat. 56*: 219–231.

Le Doussal, V., Tubiana-Hulin, M., Friedman, S., Hacene, K., Spyratos, F., and Brunet, M. 1989. Prognostic value of histologic grade nuclear components of Scarff-Bloom-Richardson (SBR). An improved score modification based on a multivariate analysis of 1262 invasive ductal breast carcinomas. *Cancer 64*: 1914–1921.

Lee, A.H., Pinder, S.E., Macmillan, R.D., Mitchell, M., Ellis, I.O., Elston, C.W., and Blamey, R.W. 2006. Prognostic value of lymphovascular invasion in women with lymph node negative invasive breast carcinoma. *Eur. J. Cancer 42*: 357–362.

McGuire, W.L. 1991. Breast cancer prognostic factors: evaluation guidelines. *J. Natl. Cancer Inst. 83*: 154–155.

McGuire, W.L., Tandon, A.K., Allred, D.C., Chamness, G.C., and Clark, G.M. 1990. How to use prognostic factors in axillary node-negative breast cancer patients. *J. Natl. Cancer Inst. 82*: 1006–1015.

Press, M.F., Bernstein, L., Thomas, P.A., Meisner, L.F., Zhou, J.Y., Ma, Y., Hung, G., Robinson, R.A., Harris, C., El-Naggar, A., Slamon, D.J., Phillips, R.N., Ross, J.S., Wolman, S.R., and Flom, K.J. 1997. HER-2/neu gene amplification characterized by fluorescence in situ hybridization: poor prognosis in node-negative breast carcinomas. *J. Clin. Oncol. 15*: 2894–2904.

Ravdin, P.M. 2001. Is Her2 of value in identifying patients who particularly benefit from anthracyclines during adjuvant therapy? A qualified yes. *J. Natl. Cancer Inst. Monogr. 30*: 80–84.

Roetger, A., Merschjann, A., Dittmar, T., Jackisch, C., Barnekow, A., and Brandt, B. 1998. Selection of potentially metastatic subpopulations expressing c-erbB-2 from breast cancer tissue by use of an extravasation model. *Am. J. Pathol. 153*: 1797–1806.

Ruud, P., Fodstad, O., and Hovig, E. 1999. Identification of a novel cytokeratin 19 pseudogene that may interfere with reverse transcriptase-polymerase chain reaction assays used to detect micrometastatic tumor cells. *Int. J. Cancer 80*: 119–125.

Seidman, A.D., Fornier, M., Esteva, F.J., Tan, L., Kaptain, S., Bach, A., Panageas, K.S., Arroyo, C., Valero, V., Currie, V., Gilewski, T., Theodoulou, M., Moynahan, M.E., Moasser, M., Sklarin, N., Dickler, M., D'Andrea, G., Cristofanilli, M., Rivera, E., Hortobagyi, G.N., Norton, L., and Hudis, C. 2001. Weekly trastuzumab and paclitaxel therapy for metastatic breast cancer with analysis of efficacy by HER2 immunophenotype and gene amplification. *J. Clin. Oncol. 19*: 2587–2595.

Sidransky, D. 1997. Nucleic acid-based methods for the detection of cancer. *Science 278*: 1054–1059.

Stathopoulou, A., Vlachonikolis, I., Mavroudis, D., Perraki, M., Kouroussis, C., Apostolaki, S., Malamos, N., Kakolyris, S., Kotsakis, A., Xenidis, N., Reppa, D., and Georgoulias, V. 2002. Molecular detection of cytokeratin-19-positive cells in the peripheral blood of patients with operable breast cancer: evaluation of their prognostic significance. *J. Clin. Oncol. 20*: 3404–3412.

Thiery, J.P. 2002. Epithelial-mesenchymal transitions in tumour progression. *Nat. Rev. Cancer 2*: 442–454.

Thurm, H., Ebel, S., Kentenich, C., Hemsen, A., Riethdorf, S., Coith, C., Wallwiener, D., Braun, S., Oberhoff, C., Janicke, F., and Pantel, K. 2003. Rare expression of epithelial cell adhesion molecule on residual micrometastatic breast cancer cells after adjuvant chemotherapy. *Clin. Cancer Res. 9*: 2598–2604.

Wiedswang, G., Borgen, E., Schirmer, C., Karesen, R., Kvalheim, G., Nesland, J.M., and Naume, B. 2006. Comparison of the clinical significance of occult tumor cells in blood and bone marrow in breast cancer. *Int. J. Cancer. 118*: 2013–2019.

Xenidis, N., Vlachonikolis, I., Mavroudis, D., Perraki, M., Stathopoulou, A., Malamos, N., Kouroussis, C., Kakolyris, S., Apostolaki, S., Vardakis, N., Lianidou, E., and Georgoulias, V. 2003. Peripheral blood circulating cytokeratin-19 mRNA-positive cells after the completion of adjuvant chemotherapy in patients

with operable breast cancer. *Ann. Oncol. 14*: 849–55.

Xenidis, N., Perraki, M., Kafousi, M., Apostolaki, S., Bolonaki, I., Stathopoulou, A., Kalbakis, K., Androulakis, N., Kouroussis, C., Pallis, T., Christophylakis, C., Argyraki, K., Lianidou, E.S., Stathopoulos, S., Georgoulias, V., and Mavroudis, D. 2006. Predictive and prognostic value of peripheral blood cytokeratin-19 mRNA-positive cells detected by real-time polymerase chain reaction in node-negative breast cancer patients. *J. Clin. Oncol. 24*: 3756–3762.

Yamauchi, H., Stearns, V., and Hayes, D.F. 2001. When is a tumor marker ready for prime time? A case study of c-erbB-2 as a predictive factor in breast cancer. *J. Clin. Oncol. 19*: 2334–2356.

Zieglschmid, V., Hollmann, C., and Bocher, O. 2005. Detection of disseminated tumor cells in peripheral blood. *Crit. Rev. Clin. Lab. Sci. 42*: 155–196.

15
Breast and Colon Carcinomas: Detection with Plasma CRIPTO-1

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INTRODUCTION

Human Cripto-1 (CR-1), also known as teratocarcinoma-derived growth factor-1 (TDGF-1) is a member of the Epidermal Growth Factor (EGF)-Cripto-1/FRL-1/ Cryptic (CFC) protein family (Bianco *et al*., 2005a; Strizzi *et al*., 2005). Structurally the EGF-CFC family consists of extracellular soluble or cell membrane-associated proteins that contain an NH_2-terminal signal peptide, a modified EGF-like region, a conserved cysteine-rich domain (the CFC motif), and a short hydrophobic COOH-terminus which, with the exception of FRL-1, contains additional sequences for glycosylphosphatidylinositol (GPI) cleavage and attachment (Bianco *et al*., 2005a). Unlike the canonical EGF motif that contains three disulfide loops (A, B and C), the variant EGF-like motif in the EGF-CFC proteins lacks the A loop, possesses a truncated B loop and has a complete C loop. Because the EGF-CFC peptides lack the A loop, these proteins do not directly bind to any of the known *HER*-related tyrosine kinase receptors either as homodimers or heterodimers (Bianco *et al*., 1999, 2005a). The CFC domain of human CR-1 contains three disulfide bonds in a pattern which structurally resembles the von Willebrand factor C (VWFC)-like domains found within the COOH-terminal extracellular portions of the Notch ligands, Jagged1, and Jagged2 (Foley *et al*., 2003). Like several of the Notch receptor proteins, all of the EGF-CFC proteins contain a consensus O-linked fucosylation site within the EGF-like motif initially thought to be necessary for their ability to function as a coreceptor for the transforming growth factor β (TGFβ)-related protein, Nodal (Schiffer *et al*., 2001). However, a recent study demonstrates that it is the amino acid threonine to which fucose is bound and not fucose *per se* that is required for CR-1 coreceptor activity with Nodal (Shi *et al*., 2007). Biochemical characterization of human CR-1 identified Asn-79 as being an N-linked glycosylation site with > 90% occupancy, and Ser-40 and Ser-161 as being *O*-linked glycosylation sites with 80% and 40% occupancy, respectively

(Bianco *et al*., 2005a). Whether mutation of these other glycosylation sites can affect the biological activity of CR-1 is presently unknown.

FUNCTION OF CRIPTO-1 DURING EMBRYONIC DEVELOPMENT

Mouse Cripto-1 (Cr-1) mRNA expression is found in the mouse embryonic ectoderm following implantation of the blastocyst (Bianco *et al*., 2005a; Strizzi *et al*., 2005). Expression increases on day 6.5 of gestation and is found in the ingressing epiblast cells in the nascent primitive streak and increases dramatically in the developing mesoderm cells as the epiblast cells undergo epithelial mesenchymal transition (EMT) (Bianco *et al*., 2005a; Strizzi *et al*., 2005). During EMT, intercellular contacts between epithelial cells are lost due to decreased E-cadherin expression and disruption of the adherens junction complex. As a result, cells become less adhesive and potentially more motile. Also, during EMT, alteration in architecture and molecular composition of the cytoskeleton causes epithelial cells to acquire a more spindle-shaped, mesenchyme-like phenotype (Boyer *et al*., 2000). Expression of Cr-1 then decreases in the embryo by day 7. Subsequent fetal stages show expression of Cr-1 in myocardial tissue of the developing heart. Cr-1, therefore, plays a vital role during embryogenesis and fetal development because Cr-1 null mice ($Cr\text{-}1^{-/-}$) die at day 7.5 due to their inability to gastrulate and form appropriate germ layers (Bianco *et al*., 2005a).

CRIPTO-1 IN HUMAN MAMMARY GLAND DEVELOPMENT AND TUMORIGENESIS

In the postnatal mouse, Cr-1 has been detected at variable levels during different stages of mammary gland development with the highest levels reported during pregnancy and lactation (Bianco *et al*., 2005a). Elevated levels of Cr-1 mRNA and protein were detected in mammary gland epithelium of older breeder Balb/c mice that have a relatively higher incidence of developing mammary tumors (Herrington *et al*., 1997). Our group has also detected biologically active CR-1 in human milk demonstrating that CR-1 is a secretory component of the mammary gland and that a soluble form of CR-1 may play a role in the regulation of proliferation and differentiation of milk producing cells (Bianco *et al*., 2001).

Native mouse and human Cripto-1 proteins are 24, 28, and 36 kDa in size, and additional proteins ranging from 14 to 60 kDa have also been identified in mouse and human normal tissues and carcinomas (Bianco *et al*., 2005a). The variation in size of these protein species may be due to the removal of the hydrophobic signal peptide and/or to additional post-translational modifications, such as glycosylation, myristylation, or phosphorylation of the core protein (Bianco *et al*., 2005a). Mouse Cr-1 protein can be released by treatment of cells with phosphatidylinositol-specific phospholipase C (PI-PLC) (Minchiotti *et al*., 2000). Removal of the COOH-terminal portion of mouse and human Cripto-1 generates soluble forms of this protein. These soluble forms of Cripto-1 are biologically active and can function as

coligands for Nodal in a number of different assays *in vivo* in zebrafish, *Xenopus*, and mice, and *in vitro* in reporter assays (Minchiotti *et al*., 2001; Bianco *et al*., 2005a). Full activity requires the presence of a peptide containing only an intact EGF domain and CFC domain (Minchiotti *et al*., 2001; Bianco *et al*., 2005a).

To investigate the potential role of Cr-1 during mouse mammary gland tumorigenesis, Cr-1 expression has been investigated in different mammary gland tumors from transgenic mice overexpressing either *HER-2/neu*, transforming growth factor α (TGFα), int-3, polyoma middle T (PyMT), or simian virus 40 large T antigen, as the expression of these transgenes has been shown to lead to the spontaneous development of mammary gland tumors (Bianco *et al*., 2005a; Strizzi *et al*., 2005). In fact, all of these tumors expressed significant levels of Cr-1 as determined by reverse transcription-polymerase chain reaction, Western blot analysis, and immunohistochemistry.

Expression of Cr-1 was also consistently expressed in hyperplastic mammary glands of PyMT, *HER-2/neu*, and TGFα mouse transgenic models, suggesting a role for Cr-1 in progression and deregulation of mammary epithelial proliferation (Niemeyer *et al*., 1999; Bianco *et al*., 2005a; Strizzi *et al*., 2005). Furthermore, overexpression of a human CR-1 transgene in the mouse mammary gland under the control of the mouse mammary tumor virus (MMTV) or the whey acidic protein (WAP) promoter results in mammary multifocal hyperplasias and adenocarcinomas in ~30–50% of multiparous mice (Sun *et al*., 2005; Wechselberger *et al*., 2005).

Although estrogen receptor-positive MCF-7 human breast cancer cells overexpressing CR-1 failed to grow in the absence of estrogens, they showed higher proliferation rates in serum-free medium, increased colony formation in soft agar, increased resistance to apoptosis induced by disruptions in cell-matrix interactions (known as anoikis), and increased invasiveness as compared to control MCF-7 cells (Normanno *et al.*, 2004a). Overexpression of Cr-1 in the mouse mammary epithelial cell line EpH4 also caused increased cell proliferation and anchorage-independent growth in soft agar. EpH4 overexpressing Cr-1 also formed duct-like structures when grown in collagen type I matrix and exhibited enhanced chemotaxsis and migration when cultured on plastic or on porous filters coated with Matrigel (Wechselberger *et al.*, 2001). The effects of Cr-1 in EpH4 cells also suggest that Cr-1 might play a role in inducing EMT of mammary epithelial cells. This has been further validated in human cervical carcinoma cells overexpressing Cr-1 which showed increased expression of vimentin and increased migration and invasion through matrix-coated membranes, suggesting that CR-1-induced vimentin expression may contribute to a more aggressive phenotype of cervical carcinoma (Ebert *et al.*, 2000). In fact, vimentin is an important cytoskeletal component of the mesenchymal cell cytoskeleton, and is characteristically increased in epithelial cells and tumors during EMT (Steinert and Roop, 1988).

Further evidence of the role that Cr-1 might play in the induction of EMT is based on a study by Strizzi *et al*. (2004). In this regard, decreased expression of E-cadherin and increased expression of N-cadherin, vimentin and several different types of integrins was observed in mammary gland hyperplasias and tumors from

MMTV-CR-1 transgenic mice and in the mouse mammary epithelial cell line, HC-11, overexpressing CR-1 (HC-11/CR-1). Also, in the CR-1 transgenic mammary gland tumors and HC-11/CR-1 cells, the zinc-finger repressor transcription factor, snail, which is known to down-regulate E-cadherin expression, was found to be expressed at significantly higher levels as compared to control nontumorigenic mammary tissue (Strizzi *et al*., 2004).

Cripto-1 has been shown to be a target gene during canonical *wnt*/β-catenin signaling (Morkel *et al*., 2003). Both the CR-1 transgenic mammary tumors and the HC-11/CR-1 cells were found to express the phosphorylated inactive form of glycogen synthease kinase 3β (GSK-3β) as well as the non-phosphorylated active form of β-catenin, suggesting a possible link between CR-1 and a canonical *wnt* signaling pathway. In fact, during *wnt* signaling, non-phosphorylated β-catenin is translocated to the nucleus in a complex with Tcf/Lef-1, and functions as a transcription factor activating gene such as *c-myc*, *cyclin-D1* and *slug* (that is related to snail) that has been shown to be involved in increased cell survival, proliferation, and migration (Polakis, 2000). Several of these *wnt* signaling target genes have been shown to be upregulated in mouse mammary epithelial cells overexpressiong CR-1 and in mammary tumor tissues of MMTV-CR-1 transgenic mice (Strizzi *et al*., 2004).

INTRACELLULAR SIGNALING PATHWAYS ACTIVATED BY CRIPTO-1

Until recently, Cripto-1 had been considered as an orphan ligand. Several studies have implicated Cripto-1 in the activation of multiple intracellular signaling pathways revealing a complex interplay of signaling molecules activated by Cripto-1 during embryonic development and cellular transformation. These pathways are extensively reviewed by Bianco *et al*. (2005a). It suffices to highlight that the major signaling pathways activated by Cripto-1 are a Nodal/ALK4/ALK7/Smad-2 signaling pathway and a Nodal-independent Glypican-1/c-src/mitogen activated protein kinase (MAPK)/AKT signaling pathway, which are known to induce certain cellular activities such as proliferation, survival, and migration. Activation of these signaling pathways can be achieved by a soluble GPI-truncated human CR-1 recombinant protein or by a refolded peptide containing only the EGF-like domain, suggesting that CR-1 can function as a soluble growth factor in mammalian cells (Bianco *et al*., 2002, 2005a). In this respect, it has been reported that cleavage of the GPI-linkage from the mouse Cr-1 protein with PI-PLC generates a functional soluble protein (Minchiotti *et al*., 2000). Furthermore, CR-1 can also be detected in the conditioned medium of several human carcinoma cell lines overexpressing CR-1, suggesting that a soluble isoform of CR-1 is naturally cleaved from the cell membrane (Normanno *et al*., 2004a).

We have previously shown that a soluble CR-1 recombinant protein can enhance the tyrosine phosphorylation of the SH2-adapter protein Shc, triggering the downstream activation of the *ras*/*raf*/MAPK and phosphatidylinositol 3′ kinase (PI3K)/AKT/GSK-3β signaling pathways in several different mouse and human cell lines (Bianco *et al*., 2005a). Surprisingly, activation of these two intracellular signaling

pathways is independent of Nodal and ALK4. In fact, CR-1 can enhance the phosphorylation of MAPK and AKT in EpH4 mouse mammary epithelial cells and MC3T3-E1 osteoblast cells that lack Nodal and ALK4 expression, respectively (Bianco *et al*., 2002). Activation of these two signaling pathways is mediated by direct binding of CR-1 to the GPI-linked heparan sulphate proteoglycan (HSPG) Glypican-1, which can then activate the cytoplasmic tyrosine kinase c-src triggering activation of MAPK and AKT (Bianco *et al*., 2003). Moreover, Glypican-1 and c-src are required by CR-1 to stimulate MAPK and AKT phosphorylation in mammary epithelial cells. Reciprocally, CR-1 can enhance Smad-2 phosphorylation in mammary epithelial cells independently of Glypican-1 and c-src, suggesting that these are two distinct pathways, which are activated by CR-1 in mammalian cells. Finally, an intact c-src kinase is required by CR-1 to induce *in vitro* transformation and to enhance migration in mammary epithelial cells, suggesting that inappropriate activation of c-src by CR-1 in a Nodal and ALK4 independent manner may play a key role in promoting cellular transformation (Bianco *et al*., 2003).

EXPRESSION OF CRIPTO-1 IN HUMAN COLON AND BREAST CARCINOMAS

Several studies have demonstrated that CR-1 is overexpressed in a number of human breast, colon, gastric, and pancreatic cancer cell lines, suggesting that this protein may function as an autocrine growth factor in these tumor cells (Bianco *et al*., 2005a). More recently, a wide, comparative screening for CR-1 expression in cell lines from different carcinoma types has been completed (Normanno *et al*., 2004b). CR-1 mRNA and/or immunoreactive protein was detected in cells derived from non-small cell lung cancer (NSCLC) and from ovarian, testicular, and renal carcinomas.

Expression of CR-1 mRNA and/or immunoreactive protein in primary human colon and breast carcinomas has been confirmed by several studies. High levels of CR-1 mRNA and protein have been found in 73% of primary and metastatic colorectal cancers. In particular, CR-1 mRNA expression by Northern blot analysis was detected in 68% of primary or metastatic human colorectal cancers, but only in 3% of noninvolved adjacent colon mucosa (Ciardiello *et al*., 1991). Expression of the CR-1 protein was demonstrated by using immunohistochemistry in 79% of colon tumors, in 57% of tubulovillous or tubular adenomas and in 12% of the noninvolved normal colonic mucosa adjacent to tumors or adenomas, but not in normal colon mucosa specimens (Saeki *et al*., 1992). These data have been confirmed in additional studies in which immunoreactive CR-1 was detected in 67–84% of colorectal carcinomas and in 42–55% of colon adenomas (De Angelis *et al*., 1999; Saeki *et al*., 1995). In the study by Saeki *et al*. (1995), expression of CR-1 in normal colonic mucosa was not observed, whereas 22% of hyperplastic polyps were found to express significant levels of CR-1 protein. Furthermore, the frequency of CR-1 expression in the adenomas correlated with the degree of dysplasia, and with the size and the histological subtype of colon adenomas (Saeki *et al*., 1994).

Non-polypoid flat adenomas had a higher frequency of CR-1 expression than polypoid lesions. Therefore, all these studies suggest that CR-1 may be a tumor marker for human colorectal cancer and may be involved in the early events of colon carcinogenesis (Saeki *et al*., 1995). In agreement with this hypothesis, CR-1 expression was detected in 62% of normal colon mucosa specimens from individuals that belong to families with a high incidence of colorectal carcinomas, whereas only 20% of colon mucosa from low risk patients specimens were positive for CR-1 (De Angelis *et al*., 1999). In an additional study, CR-1 immunoreactivity was found in 71% of primary rectal carcinomas and in 37% of normal rectal mucosa adjacent to carcinoma (Gagliardi *et al*., 1994). Tumors with CR-1 immunoreactivity extending to the adjacent normal mucosa showed an increase in the incidence of bowel wall penetration, lymph node involvement and a recurrence rate of 100%, suggesting a correlation between CR-1 immunoreactivity in the non-involved adjacent mucosa and prognosis and survival of patients with rectal tumors.

CR-1 mRNA and/or protein can be detected in ~ 80% of primary human infiltrating breast carcinomas, in 47% of ductal carcinoma *in situ* (DCIS), in 13% of non-involved adjacent breast tissue samples, and in ~ 6% of normal breast specimens (Normanno *et al*., 1995; Panico *et al*., 1996). In the normal mucosa specimens, a limited number of epithelial cells stain for CR-1 (< 25%) (Panico *et al*., 1996). In these studies, no significant correlations were observed between CR-1 mRNA expression or immunoreactivity and various clinicopathological parameters such as tumor stage, estrogen receptor status, lymph node involvement, histologic grade, proliferative index as assessed by Ki-67 staining, flow cytometry or loss of heterozygosity (LOH) on chromosome 17p (Normanno *et al*., 1995; Panico *et al*., 1996). More recently, a cohort of 120 patients with operable breast cancer were analysed for expression of CR-1 by using tissue micorarrays (Gong *et al*., 2007). Expression of CR-1 was detected in 47.5% of patients.

A significant association was found between CR-1 immunostaining and several clinicopathological features of the tumors. In particular, expression of CR-1 was more frequent in patients with poor prognosis according to the Nottingham Prognostic Index, histological grade 3 tumors as compared with grade 1 lesions, and high cell proliferation Ki-67 index as compared with low index. A long follow up was available for these patients (median 125 months). Univariate analysis revealed a significant correlation between overexpression of CR-1 and poor prognosis. Multivariate analysis confirmed that CR-1 expression is an independent prognostic factor in breast cancer patients. Finally, in human primary breast carcinomas, CR-1 is frequently coexpressed with other EGF-related peptides, such as TGFα, amphiregulin (AR), and heregulin, implying that different growth factors might cooperate in supporting the autonomous proliferation of breast cancer cells. A positive correlation between nuclear *HER-4* expression and CR-1 expression in primary human breast carcinomas has also been described (Srinivasan *et al*., 2000). This finding is intriguing because it has been shown that CR-1 can indirectly enhance the tyrosine phosphorylation of *HER-4* (Bianco *et al*., 1999).

CRIPTO-1 AS TARGET FOR THERAPEUTIC INTERVENTION IN HUMAN CARCINOMAS

The high frequency of CR-1 expression in human carcinomas and lack or low levels of expression in normal tissues make it a potential target for therapeutic intervention. Different approaches can be used to inhibit the activity of a growth factor: block its synthesis, neutralize its activity, or block the activation of its cognate receptor. CR-1 can activate different receptor signaling pathways through binding with different putative receptor molecules or by interacting with other growth factors such as activin A and B, TGF-β1, and Tomoregulin-1 (Bianco *et al*., 2005a; Gray *et al*., 2006). Therefore, therapeutic approaches aimed to block CR-1 activity have mainly been based upon the use of antisense oligonucleotides to reduce its expression or neutralize antibodies to block the activity of the CR-1 protein.

Sequence specific antisense (AS) oligonucleotides or AS expression vectors can block the expression of specific proteins by binding to the corresponding mRNA and preventing translation. This approach has been successfully utilized to impair CR-1 expression in several different types of human carcinoma cells. In particular, inhibition of CR-1 expression in human GEO and CBS colon cancer cells by using either an amphotropic recombinant CR-1 AS mRNA retroviral expression vector or CR-1 AS phosphorothioate oligonucleotides resulted in a significant growth inhibition *in vitro* (Ciardiello *et al*., 1994). GEO cells that were infected with the CR-1 AS retroviral vector exhibited a reduced tumorigenicity in nude mice. CR-1 infected GEO cells formed tumors that were smaller and appeared after a longer latency period as compared with non-infected colon cancer cells. Similar results were obtained in NTERA2 human embryonal carcinoma cells (Baldassarre *et al*., 1996). A superadditive effect was observed in reducing the growth of GEO cells *in vitro* when a CR-1 AS oligonucleotide was combined with TGFα AS and AR AS oligonucleotides, suggesting that different growth factors contribute to regulate the proliferation of colon cancer cells (Normanno *et al*., 1996). Similarly, an additive growth inhibitory effect was observed in MDA-MB-468 human breast cancer cells that had been treated with a combination of a CR-1 AS oligonucleotide and both TGFα AS and AR AS oligonucleotides (De Luca *et al*., 1999). Provocative data have also been obtained using combinations of CR-1 AS oligonucleotides with conventional chemotherapeutic drugs in colon cancer cells (De Luca *et al*., 1997). In a clonogenic assay, pretreatment of GEO cells with different concentrations of 5-fluorouracil, adriamycin, mitomycin C, or cis-platinum induced an additive growth inhibitory effect when the cells were subsequently treated with a CR-1 AS oligonucleotide.

Anti-CR-1 second generation antisense oligonucleotides have also been developed. These molecules contain phosphorothioate backbone, and segment of 2′-O-methylribonucleosides modified at both the 5′ and 3′ ends of the oligonucleotide (MBOs). MBOs show increased affinity to target mRNA, increased biological activity, reduced polyanionic-related side effects, and increased *in vivo* stability as compared

with phosphorothioate oligonucleotides (Agrawal and Zhao, 1998). Furthermore, it has been shown that these compounds are active following oral administration (Agrawal and Zhao, 1998). CR-1 AS MBOs were able to block the *in vitro* growth of carcinoma cell lines derived from different carcinoma types, including colon and breast cancer (De Luca *et al*., 2000; Normanno *et al*., 2004b). Treatment of carcinoma cells with CR-1 AS oligonucleotides resulted in a significant reduction in the levels of expression of CR-1 mRNA and protein. Furthermore, treatment with CR-1 AS MBOs produced in colon carcinoma cells a significant reduction in the levels of activation of AKT but not of p42/p44 MAPK. Administration of these agents by either the intra-peritoneal or the oral route resulted in a significant reduction in the growth of colon cancer xenografts in immunocompromised mice (De Luca *et al*., 2000; Normanno *et al*., 2004b). Interestingly, treatment with the combination of CR-1, AR, and TGFα MBOs produced a more significant growth inhibition of colon cancer xenografts as compared with treatment with a single oligonucleotide. The combination also produced a significant reduction in the levels of microvessels in the treated tumors as compared with untreated tumors or tumors from animal treated with a single AS oligonucleotide. This observation suggests that the anti-tumor effect of CR-1-targeting agents might be at least in part related to the pro-angiogenetic properties of CR-1 (Bianco *et al*., 2005b).

Finally, CR-1 AS oligonucleotides have also been used in combination with agents that block different intracellular signal transduction pathways (Normanno *et al*., 1999). In particular, a CR-1 AS MBO has been used in combination with a humanized anti-human epidermal growth factor receptor monoclonal antibody (mAb) C225 and with 8-Cl-cAMP, a specific analog that inhibits type I protein kinase A. Low doses of each agent produced only a 15–35% growth inhibition in GEO cells *in vitro*, but when a CR-1 AS oligonucleotide was combined with either the MAb C225 or with 8-Cl-cAMP, a synergistic antiproliferative effect occurred. Moreover, when the three agents were added together, a nearly complete suppression in the ability of GEO cells to grow in soft agar occurred. Interestingly, treatment with all three compounds induced apoptosis in GEO cells whereas single treatment or a combination of only two agents failed to induce any apoptosis.

More recently, monoclonal blocking antibodies directed against CR-1 have been developed. In particular, Adkins *et al*. (2003) have generated mouse mAbs that were able to prevent the binding of CR-1 to Activin B and, therefore, to reverse the CR-1 blockade of Activin B-induced growth suppression in human breast carcinoma cells. The anti-CR-1 antibodies were able to inhibit tumor cell growth up to 70% in two xenograft models of testicular and colon cancers. Both Nodal and Activin B were found to be expressed in the tumor xenografts, suggesting that the anti-tumor activity of the anti-CR-1 mAb might be related to its ability to block the interactions of CR-1 with both signal transduction pathways. Finally, rat monoclonal antibodies directed against the EGF-like domain of the CR-1 peptide produced a significant inhibition of the *in vitro* growth of different carcinoma cell lines (Xing *et al*., 2004). The anti-CR-1 mAbs also prevented tumor development *in vivo* and inhibited the growth of

established tumors of LS174T colon xenografts in immunocompromised mice. Treatment with the anti-EGF-like domain antibodies produced a significant reduction in the levels of activation of AKT, activation of c-Jun-NH2-terminal kinase and p38 kinase signaling pathways, and ultimately apoptosis in cancer cells. In agreement with previous findings by De Luca *et al.* (1997), treatment of colon cancer cells with a combination of the rat anti-CR-1 antibodies and conventional cytotoxic drugs, such as 5-fluorouracil, epirubicin or cis-platinum, resulted in a more significant inhibition of tumor cell growth as compared with treatment with a single agent.

DETECTION OF CRIPTO-1 IN THE PLASMA OF BREAST AND COLON CANCER PATIENTS WITH ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Because CR-1 is expressed at high levels in different types of human malignancies, detection and measurement of CR-1 levels in human plasma/and or serum may have clinical significance. We, therefore, developed a sandwich-type enzyme-linked immunosorbent assay (ELISA) to analyze CR-1 levels in plasma samples from patients with colon and breast cancers although this methodology can be applied to a wide variety of human biological fluids containing CR-1 protein, as we have previously shown for human milk (Bianco *et al.*, 2001, 2006). Sandwich ELISA has been shown to be more sensitive than ELISA, in which the antigen is directly bound to the plate (Borgono *et al.*, 2003). In a sandwich ELISA, a monoclonal antibody directed against a target antigen (capture antibody) is usually captured on a 96-well microtiter plates and another antibody of different species is used as the detection antibody. In our sandwich ELISA for CR-1, an anti-CR-1 mouse monoclonal antibody (A10. B2.18, Biogen-Idec, Cambridge, MA) was used as the capture antibody and an anti-CR-1 rabbit polyclonal antibody (Biocon, Frederick, MD) was used as the detection antibody.

Sandwich ELISA method for detecting CR-1 in biological fluids

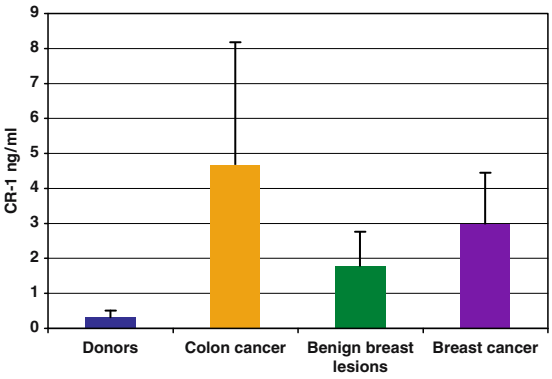
1. Adsorb anti-CR-1 mouse monoclonal antibody A10.B2.18 (1 µg/well) to 96-wellmicrotiter plates and incubate for 1 h at room temperature.
2. Block the plates with 2% milk (200 µl/well) (Kirkergaard & Perry Laboratories, Gaithersburg, MD) for 1 h at room temperature.
3. Wash the plates three times with washing buffer (200 µl/well) containing 0.002 M imidazole buffered saline with 0.02% Tween-20 (Kirkergaard & Perry Laboratories).
4. Add 100–200 µl of undiluted samples (i.e., plasma, milk, etc.) per well and incubate overnight at 4°C.
5. For the standard curve use CR-1 recombinant protein (R&D Biosystems, Minneapolis, MN) adsorbed at concentrations ranging from 30 pg to 1 µg/well and for negative controls use 2% milk blocking buffer (100–200 µl according to the volume of the samples).
6. Remove unbound CR-1 by washing the plates five times with washing buffer and add anti-CR-1 rabbit polyclonal antibody (Biocon) diluted 1:3,000 in 1% milk diluent buffer containing 1%

non-fat dry milk in a borate buffer for 1 h at room temperature (Kirkergaard & Perry Laboratories).
7. Wash plates five times with washing buffer and add goat anti-rabbit IgG conjugated to horseradish peroxidase (1:3000; Amersham Pharmacia Biotech, Piscataway, NJ) for 1 h at room temperature.
8. Wash plates five times with washing buffer and add 100 µl/well of TMB peroxidase substrate containing 3, 3', 5, 5'-tetramethylbenzidine in an acidic buffer (Kirkergaard & Perry Laboratories). Incubate plates in the dark for 10 min to allow color to develop.
9. Add TMB stop solution (1% HCl) (Kirkergaard & Perry Laboratories) in the same volume of the TMB peroxidase substrate buffer and after 5 min read the absorbance at 450 nm.

This sandwich ELISA for CR-1 is highly specific, because no cross-reactivity of the mouse monoclonal and rabbit polyclonal anti-CR-1 antibodies with members of the EGF-family of peptides was detected. In fact, EGF, AR, heparin-binding EGF, and heregulin-β1, which share amino acid sequence similarities within the EGF-like domain with CR-1 protein, were not detected in this sandwich ELISA for CR-1, with readings comparable with the background signal. Furthermore, using this sandwich ELISA for CR-1 we were able to detect human CR-1 in the plasma of MMTV-CR-1 transgenic mice. No CR-1 protein was detected in the plasma of FVB/N control mice because the anti-CR-1 mouse monoclonal antibody (A10. B2.18) used in the sandwich ELISA could only react with the human CR-1 transgene and not with the endogenous mouse Cr-1 protein (Adkins *et al.*, 2003). CR-1 was detected in the plasma of MMTV-CR-1 mice during pregnancy (0.047 ± 0.0035 ng/ml), and in mice with hyperplastic lesions of the mammary gland (0.041 ± 0.0056 ng/ml) or with mammary adenocarcinomas (0.052 ± 0.0021 ng/ml). No statistically significant differences were observed among these three groups of animals, probably due to the constitutive expression of high levels of CR-1 in the mammary gland driven by the MMTV promoter.

CR-1 has also been detected by Western blot analysis and ELISA in 24 human milk samples with concentrations between 62 and 118 ng/ml (Bianco *et al.*, 2001). CR-1 purified from human milk by immunoaffinity chromatography was biologically active and its presence in human milk might be physiologically important for mammary epithelial cell growth and differentiation. The presence of CR-1 in human milk prompted us to investigate the possibility that CR-1, expressed at high levels by different types of tumors, might be released by cancer cells and reach the blood circulation. We, therefore, analyzed CR-1 levels in plasma samples derived from 33 colon cancer or 54 breast carcinoma patients at different clinical stages compared to 21 healthy volunteers. CR-1 levels in the plasma of patients with colon (4.68 ng/ml) or breast cancer (2.97 ng/ml) were significantly higher when compared with the control group (0.32 ng/ml) (Figure 14.1). At a cut-off level of 0.7 ng.ml the ELISA test had 100% sensitivity (all cancer patients were positive to the test) and 95% specificity (only 1 out of 21 controls was positive in the ELISA test). High CR-1 plasma levels were detected also in breast cancer patients at an early stage, suggesting that CR-1 might be useful in the early diagnosis

of this disease. No significant correlation between CR-1 levels in the plasma of colon and breast cancer patients and various clinicopathologic parameters, such as tumor size, lymph node involvement, proliferative index, estrogen and progesterone receptor status or *HER-2* status was found. Furthermore, the levels of CR-1 in the plasma of patients with colon and breast cancer were not found to be associated with the degree of CR-1 positivity in tumor sections, as assessed by immunohistochemistry analysis. Moderate levels of CR-1 were also found in the plasma of 21 women with benign breast lesions, including hyperplasia and atypical hyperplasia (Figure 15.1). However, the mean CR-1 levels in the plasma of patients with benign breast lesions (1.7 ng/ml) were significantly lower that the mean CR-1 plasma levels detected in patients with breast carcinomas (2.97 ng/ml). Within the benign breast lesions, CR-1 plasma levels were higher in lesions characterized by the presence of sclerosis and in fibroadenomas, a lesion characterized by the presence of epithelial hyperplasia and fibrosis. Because pathological fibrosis, including renal, lung, and liver fibrosis, have been associated with morphological and functional modification of epithelial cells that acquire a fibroblastic-mesenchymal phenotype through a phenomenon known as EMT, it is possible that CR-1 might have a role in inducing EMT in mammary epithelial cells of non-malignant breast lesions (Zeisberg and Kalluri, 2004). In agreement with the results from the plasma analysis for CR-1 in patients with breast cancer, real-time PCR showed a large increase in the levels of CR-1 mRNA expression in six human breast carcinomas as compared with normal breast tissue.



CR-1 concentration in plasma (ng/ml ± SD)

Donors (n=21)	0.32 ± 0.19
Colon cancer (n=33)	4.68 ± 3.5
Benign breast lesions (n=21)	1.77 ± 0.99
Breast cancer (n=54)	2.97 ± 1.48

FIGURE 15.1. Plasma CR-1 levels, detected by sandwich ELISA for CR-1, in patients with colon cancer, benign breast lesions and breast cancer compared with normal donors. n, number of patients; SD, standard deviation

In conclusion, recent findings demonstrating that CR-1 expression (as detected by immunostaining of sections from biopsy specimens) showed positive correlation with increased tumor aggressiveness and poor survival in breast cancer patients, suggest the potential application of CR-1 as a prognostic marker (Gong *et al*., 2007). However, this method for detecting CR-1 is dependent on the relatively invasive procedure of biopsy sampling, the availability of a histopathological service for sample processing, quality of primary antibodies used for immunostaining, and potential interobserver variation of interpretation of the degree of immunostaining results. We have demonstrated the capability to detect CR-1 in human plasma by employing an ELISA-based assay platform (Bianco *et al*., 2006). More importantly, we have shown that CR-1 levels are significantly higher in cancer patients as compared to

healthy control subjects. Therefore, this simple and practical method for detecting plasma CR-1 levels described in detail in this chapter could potentially serve as a useful tool for the diagnosis and prognosis of certain types of human cancers.

REFERENCES

Adkins, H.B., Bianco, C., Schiffer, S.G., Rayhorn, P., Zafari, M., Cheung, A.E., Orozco, O., Olson, D., De Luca, A., Chen, L.L., Miatkowski, K., Benjamin, C., Normanno, N., Williams, K.P., Jarpe, M., LePage, D., Salomon, D., and Sanicola, M. 2003. Antibody blockade of the Cripto CFC domain suppresses tumor cell growth in vivo. *J. Clin. Invest. 112*: 575–587.

Agrawal, S., and Zhao, Q. 1998. Mixed backbone oligonucleotides: improvement in oligonucleotide-induced toxicity in vivo. *Antisense Nucleic Acid Drug Dev. 8*: 135–139.

Baldassarre, G., Bianco, C., Tortora, G., Ruggiero, A., Moasser, M., Dmitrovsky, E., Bianco, A.R., and Ciardiello, F. 1996. Transfection with a CRIPTO anti-sense plasmid suppresses endogenous CRIPTO expression and inhibits transformation in a human embryonal carcinoma cell line. *Int. J. Cancer 66*: 538–543.

Bianco, C., Kannan, S., De Santis, M., Seno, M., Tang, C.K., Martinez-Lacaci, I., Kim, N., Wallace-Jones, B., Lippman, M.E., Ebert, A.D., Wechselberger, C., and Salomon, D.S. 1999. Cripto-1 indirectly stimulates the tyrosine phosphorylation of erb B-4 through a novel receptor. *J. Biol. Chem. 274*: 8624–8629.

Bianco, C., Wechselberger, C., Ebert, A., Khan, N., Sun, Y., and Salomon D.S. 2001. Identification of Cripto-1 in human milk. *Breast Cancer Res. Treat. 66*: 1–7.

Bianco, C., Adkins, H.B., Wechselberger, C., Seno, M., Normanno, N., De Luca, A., Sun, Y., Khan, N., Kenney, N., Ebert, A., Williams, K.P., Sanicola, M., and Salomon, D.S. 2002. Cripto-1 activates nodal- and ALK4-dependent and -independent signaling pathways in mammary epithelial cells. *Mol. Cell. Biol. 22*: 2586–2597.

Bianco, C., Strizzi, L., Rehman, A., Normanno, N., Wechselberger, C., Sun, Y., Khan, N., Hirota, M., Adkins, H., Williams, K., Margolis, R.U., Sanicola, M., and Salomon D.S. 2003. A Nodal- and ALK4-independent signaling pathway activated by Cripto-1 through Glypican-1 and c-Src. *Cancer Res. 63*: 1192–1197.

Bianco, C., Strizzi, L., Normanno, N., Khan, N., and Salomon D.S. 2005a. Cripto-1: an oncofetal gene with many faces. *Curr. Top. Dev. Biol. 67*: 85–133.

Bianco, C., Strizzi, L., Ebert, A., Chang, C., Rehman, A., Normanno, N., Guedez, L., Salloum, R., Ginsburg, E., Sun, Y., Khan, N., Hirota, M., Wallace-Jones, B., Wechselberger, C., Vonderhaar, B.K., Tosato, G., Stetler-Stevenson, W.G., Sanicola, M., and Salomon D.S. 2005b. Role of human cripto-1 in tumor angiogenesis. *J. Natl. Cancer Inst. 97*: 132–141.

Bianco, C., Strizzi, L., Mancino, M., Rehman, A., Hamada, S., Watanabe, K., De Luca, A., Jones, B., Balogh, G., Russo, J., Mailo, D., Palaia, R., D'Aiuto, G., Botti, G., Perrone, F., Salomon, D.S., and Normanno, N. 2006. Identification of cripto-1 as a novel serologic marker for breast and colon cancer. *Clin. Cancer Res. 12*: 5158–5164.

Borgono, C.A., Grass, L., Soosaipillai, A., Yousef, G.M., Petraki, C.D., Howarth, D.H., Fracchioli, S., Katsaros, D., and Diamandis, E.P. 2003. Human kallikrein 14: a new potential biomarker for ovarian and breast cancer. *Cancer Res. 63*: 9032–9041.

Boyer, B., Valles, A.M., and Edme, N. 2000. Induction and regulation of epithelial-mesenchymal transitions. *Biochem. Pharmacol. 60*: 1091–1099.

Ciardiello, F., Kim, N., Saeki, T., Dono, R., Persico, M.G., Plowman, G.D., Garrigues, J., Radke, S., Todaro, G.J., and Salomon, D.S. 1991. Differential expression of epidermal growth factor-related proteins in human colorectal tumors. *Proc. Natl. Acad. Sci. U S A. 88*: 7792–7796.

Ciardiello, F., Tortora, G., Bianco, C., Selvam, M.P., Basolo, F., Fontanini, G., Pacifico, F., Normanno, N., Brandt, R., Persico, M.G., Salomon, D.S., and Bianco, A.R. 1994. Inhibition of CRIPTO expression and tumorigenicity in human colon cancer cells by antisense RNA and oligodeoxynucleotides. *Oncogene 9*: 291–298.

De Angelis, E., Grassi, M., Gullick, W.J., Johnson, G.R., Rossi, G.B., Tempesta, A., De Angelis, F., De Luca, A., Salomon, D.S., and Normanno, N. 1999. Expression of cripto and amphiregulin in

colon mucosa from high risk colon cancer families. *Int. J. Oncol. 14*: 437–440.

De Luca, A., Selvam, M.P., Sandomenico, C., Pepe, S., Bianco, A.R., Ciardiello, F., Salomon, D.S., and Normanno, N. 1997. Anti-sense oligonucleotides directed against EGF-related growth factors enhance anti-proliferative effect of conventional anti-tumor drugs in human colon-cancer cells. *Int. J. Cancer 73*: 277–282.

De Luca, A., Casamassimi, A., Selvam, M.P., Losito, S., Ciardiello, F., Agrawal, S., Salomon, D.S., and Normanno, N. 1999. EGF-related peptides are involved in the proliferation and survival of MDA-MB-468 human breast carcinoma cells. *Int. J. Cancer 80*: 589–594.

De Luca, A., Arra, C., D'Antonio, A., Casamassimi, A., Losito, S., Ferraro, P., Ciardiello, F., Salomon, D.S., and Normanno, N. 2000. Simultaneous blockage of different EGF-like growth factors results in efficient growth inhibition of human colon carcinoma xenografts. *Oncogene 19*: 5863–5871.

Ebert, A.D., Wechselberger, C., Nees, M., Clair, T., Schaller, G., Martinez-Lacaci, I., Wallace-Jones, B., Bianco, C., Weitzel, H.K., and Salomon, D.S. 2000. Cripto-1-induced increase in vimentin expression is associated with enhanced migration of human Caski cervical carcinoma cells. *Exp. Cell Res. 257*: 223–229.

Foley, S.F., van Vlijmen, H.W., Boynton, R.E., Adkins, H.B., Cheung, A.E., Singh, J., Sanicola, M., Young, C.N., and Wen, D. 2003. The CRIPTO/FRL-1/CRYPTIC (CFC) domain of human Cripto. Functional and structural insights through disulfide structure analysis. *Eur. J. Biochem. 270*: 3610–3618.

Gagliardi, G., Talbot, I. C.,Northover, J.M.A., Warre, A., Stamp, G.W.H., Lalani, E.-N., Gullick, W.J., and Pignatelli, M. 1994. Cripto, a member of the epidermal growth factor family, is overexpressed in human pancreatic cancer and chronic pancreatitis. *Int. J. Cancer 56*: 668–674.

Gong, Y.P., Yarrow, P.M., Carmalt, H.L., Kwun, S.Y., Kennedy, C.W., Lin, B.P., Xing, P.X., and Gillett, D.J. 2007. Overexpression of Cripto and its prognostic significance in breast cancer: a study with long-term survival. *Eur. J. Surg. Oncol. 33*: 438–443.

Gray, P.C., Shani, G., Aung, K., Kelber, J., and Vale, W. 2006. Cripto binds transforming growth factor beta (TGF-beta) and inhibits TGF-beta signaling. *Mol. Cell. Biol. 26*: 9268–9278.

Herrington, E.E., Ram, T.G., Salomon, D.S., Johnson, G.R., Gullick, W.J., Kenney, N., and Hosick, H.L. 1997. Expression of epidermal growth factor-related proteins in the aged adult mouse mammary gland and their relationship to tumorigenesis. *J. Cell. Physiol. 170*: 47–56.

Minchiotti, G., Parisi, S., Liguori, G., Signore, M., Lania, G., Adamson, E.D., Lago, C.T., and Persico, M.G. 2000. Membrane-anchorage of Cripto protein by glycosylphosphatidylinositol and its distribution during early mouse development. *Mech. Dev. 90*: 133–142.

Minchiotti, G., Manco, G., Parisi, S., Lago, C.T., Rosa, F., and Persico, M.G. 2001. Structure-function analysis of the EGF-CFC family member Cripto identifies residues essential for nodal signalling. *Development 128*: 4501–4510.

Morkel, M., Huelsken, J., Wakamiya, M., Ding, J., van de Wetering, M., Clevers, H., Taketo, M.M., Behringer, R.R., Shen, M.M., and Birchmeier, W. 2003. Beta-catenin regulates Cripto- and Wnt3-dependent gene expression programs in mouse axis and mesoderm formation. *Development 130*: 6283–6294.

Niemeyer, C.C., Spencer-Dene, B., Wu, J.X., and Adamson, E.D. 1999. Preneoplastic mammary tumor markers: Cripto and Amphiregulin are overexpressed in hyperplastic stages of tumor progression in transgenic mice. *Int. J. Cancer 81*: 588–591.

Normanno, N., Kim, N., Wen, D., Smith, K., Harris, A.L., Plowman, G., Colletta, G., Ciardiello, F., and Salomon, D.S. 1995. Expression of messenger RNA for amphiregulin, heregulin, and cripto-1, three new members of the epidermal growth factor family, in human breast carcinomas. *Breast Cancer Res. Treat. 35*: 293–297.

Normanno, N., Bianco, C., Damiano, V., de Angelis, E., Selvam, M.P., Grassi, M., Magliulo, G., Tortora, G., Bianco, A.R., Mendelsohn, J., Salomon, D.S., and Ciardiello, F. 1996. Growth inhibition of human colon carcinoma cells by combinations of anti-epidermal growth factor-related growth factor antisense oligonucleotides. *Clin. Cancer Res. 2*: 601–609.

Normanno, N., Tortora, G., De Luca, A., Pomatico, G., Casamassimi, A., Agrawal, S., Mendelsohn, J., Bianco, A.R., and Ciardiello, F. 1999.

Synergistic growth inhibition and induction of apoptosis by a novel mixed backbone antisense oligonucleotide targeting CRIPTO in combination with C225 anti-EGFR monoclonal antibody and 8-Cl-cAMP in human GEO colon cancer cells. *Oncol. Rep. 6*: 1105–1109.

Normanno, N., De Luca, A., Bianco, C., Maiello, M.R., Carriero, M.V., Rehman, A., Wechselberger, C., Arra, C., Strizzi, L., Sanicola, M., and Salomon, D.S. 2004a. Cripto-1 overexpression leads to enhanced invasiveness and resistance to anoikis in human MCF-7 breast cancer cells. *J. Cell. Physiol. 198*: 31–39.

Normanno, N., De Luca, A., Maiello, M. R., Bianco, C., Mancino, M., Strizzi, L., Arra, C., Ciardiello, F., Agrawal, S., and Salomon D.S. 2004b. Cripto-1: a novel target for therapeutic intervention in human carcinoma. *Int. J. Oncol. 25*: 1013–1020.

Panico, L., D'Antonio, A., Salvatore, G., Mezza, E., Tortora, G., De Laurentiis, M., De Placido, S., Giordano, T., Merino, M., Salomon, D.S., Mullick, W.J., Pettinato, G., Schnitt, S.J., Bianco, A.R., and Ciardiello, F. 1996. Differential immunohistochemical detection of transforming growth factor alpha, amphiregulin and CRIPTO in human normal and malignant breast tissues. *Int. J. Cancer 65*: 51–56.

Polakis, P. 2000. Wnt signaling and cancer. *Genes Dev. 14*: 1837–1851.

Saeki, T., Stromberg, K., Qi, C.F., Gullick, W.J., Tahara, E., Normanno, N., Ciardiello, F., Kenney, N., Johnson, G.R., and Salomon, D.S. 1992. Differential immunohistochemical detection of amphiregulin and cripto in human normal colon and colorectal tumors. *Cancer Res. 52*: 3467–3473.

Saeki, T., Salomon, D.S., Gullick, W.J., Mandai, K., Yamagami, K., Moriwaki, S., Takashima, S., Nishikawa, Y., and Tahara, E. 1994. Expression of cripto-1 in human colorectal adenomas and carcinomas is related to degree of dysplasia. *Int. J. Oncol. 5*: 445–452.

Saeki, T., Salomon, D.S., Johnson, G.R., Gullick, W.J., Mandai, K., Yamagami, K., Moriwaki, S., Tanada, M., Takashima, S., and Tahara, E. 1995. Association of epidermal growth factor-related peptides and type I receptor tyrosine kinase receptors with prognosis of human colorectal carcinomas. *Jpn. J. Clin. Oncol. 25*: 240–249.

Schiffer, S.G., Foley, S., Kaffashan, A., Hronowski, X., Zichittella, A.E., Yeo, C.Y., Miatkowski, K., Adkins, H.B., Damon, B., Whitman, M., Salomon, D.S., Sanicola, M., and Williams, K.P. 2001. Fucosylation of Cripto is required for its ability to facilitate nodal signaling. *J. Biol. Chem. 276*: 37769–3778.

Shi, S., Ge, C., Luo, Y., Hou, X., Haltiwanger, R.S., and Stanley, P. 2007. The threonine that carries fucose, but not fucose, is required for cripto to facilitate nodal signaling. *J. Biol. Chem*. (in press).

Srinivasan, R., Gillett, C.E., Barnes, D.M., and Gullick, W.J. 2000. Nuclear expression of the c-erbB-4/HER-4 growth factor receptor in invasive breast cancers. *Cancer Res. 60*: 1483–1487.

Steinert, P.M., and Roop, D.R. 1988. Molecular and cellular biology of intermediate filaments. *Annu. Rev. Biochem. 57*: 593–625.

Strizzi, L., Bianco, C., Normanno, N., Seno, M., Wechselberger, C., Wallace-Jones, B., Khan, N.I., Hirota, M., Sun, Y., Sanicola, M., and Salomon, D.S. 2004. Epithelial mesenchymal transition is a characteristic of hyperplasias and tumors in mammary gland from MMTV-Cripto-1 transgenic mice. *J. Cell. Physiol. 201*: 266–276.

Strizzi, L., Bianco, C., Normanno, N., and Salomon, D. 2005. Cripto-1: a multifunctional modulator during embryogenesis and oncogenesis. *Oncogene 24*: 5731–5741.

Sun, Y., Strizzi, L., Raafat, A., Hirota, M., Bianco, C., Feigenbaum, L., Kenney, N., Wechselberger, C., Callahan, R., and Salomon, D.S. 2005. Overexpression of human Cripto-1 in transgenic mice delays mammary gland development and differentiation and induces mammary tumorigenesis. *Am. J. Pathol. 167*: 585–597.

Wechselberger, C., Ebert, A.D., Bianco, C., Khan, N.I., Sun, Y., Wallace-Jones, B., Montesano, R., and Salomon, D.S. 2001. Cripto-1 enhances migration and branching morphogenesis of mouse mammary epithelial cells. *Exp. Cell Res. 266*: 95–105.

Wechselberger, C., Strizzi, L., Kenney, N., Hirota, M., Sun, Y., Ebert, A., Orozco, O., Bianco, C., Khan, N.I., Wallace-Jones, B., Normanno, N., Adkins, H., Sanicola, M., and Salomon, D.S. 2005. Human Cripto-1 overexpression in the mouse mammary gland results in the development of hyperplasia and adenocarcinoma. *Oncogene 24*: 4094–4105.

Xing, P.X., Hu, X.F., Pietersz, G.A., Hosick, H.L., and McKenzie, I.F. 2004. Cripto: a novel target for antibody-based cancer immunotherapy. *Cancer Res. 64*: 4018–4023.

Zeisberg, M., and Kalluri, R. 2004. The role of epithelial-to-mesenchymal transition in renal fibrosis. *J. Mol. Med. 82*: 175–181.

16
Breast Cancer Risk in Women with Abnormal Cytology in Nipple Aspirate Fluid

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INTRODUCTION

Fear of developing breast cancer is well-founded among women. Breast cancer is the leading cause of death among women ages 20–59 years of age, and the second-leading cause of death in women over 50 years of age (Jemal *et al*., 2005). Approximately 40,000 women will die of this disease in the United States this year. Refining the science of breast cancer risk assessment has become more important with the development of medications to reduce breast cancer risk, the addition of expensive imaging (magnetic resonance imaging (MRI), and prophylactic surgery (Hollingsworth *et al*., 2002; Saslow *et al*., 2007). A standardized algorithm for breast cancer risk assessment is not available at this time in the clinical setting. Women are categorized as either having possible genetic or hereditary risk, or as having risk factors unrelated to a family history of breast cancer. Genetic testing is limited as a risk assessment tool, as only a small percentage of women carry known genetic mutations which result in an increased risk of breast cancer development. Mathematical models (i.e., Gail index) calculate probabilities of developing breast cancer during specified periods of time; however, the factors included in the models contribute a relatively small degree of risk for the eventual development of breast cancer. Hollingsworth *et al*. (2002) suggested that tissue or serum-based strategies should be the next step in refining risk assessment, considering that 70% of women who develop breast cancer have no identifiable risk factors.

Because approximately 95% of breast tumors begin in the lining of the milk ducts of the breast, it is logical to explore these ducts as a means of identifying abnormal cells which may eventually progress to cancer. Nipple aspiration is a minimally invasive technique to obtain fluid from mammary ducts. This chapter will explore current methods of assessing breast cancer risk and the role of nipple aspiration in

enhancing risk assessment. A review of ductal anatomy will be followed by the definition of nipple aspirate fluid (NAF), including information on the cytologic evaluation of this fluid. Research findings demonstrating a relationship between abnormal cytologic results and subsequent breast cancer development will be discussed.

CURRENT METHODS OF ASSESSING BREAST CANCER RISK

DEFINING HIGH RISK

In order to understand the value of tools such as NAF, it is important to understand how breast cancer risk is currently evaluated. How is "high risk" defined when evaluating women for possible breast cancer development? The well-established risk factors include: current age of > 65 years, early menarche (< 12 years of age), nulliparity or first child after age 30, a history of breast biopsy, and family history of breast cancer (Singletary, 2003). Radiation exposure, specifically related to Hodgkin's disease treatment, fluoroscopy for tuberculosis and treatment of chest acne, have also been determined to increase breast cancer risk. Recent research has identified mammographic breast density as a powerful tool for breast cancer risk assessment (Kerlikowske *et al*., 2007). Risk factors contributing smaller degrees of risk include: alcohol intake of > 2 drinks per day, high body mass index (BMI) in women over 55 years of age, use of hormone replacement therapy, and menopause at > 55 years of age (Table 16.1). It is believed that the greater the number of the above-mentioned factors present, the greater the risk of eventual breast cancer development. Despite years of research dedicated to articulating the risk factors leading to breast cancer development, no risk assessment model or test completely calculates a woman's risk with great accuracy. The relatively recent identification of two mutations in women at risk for breast and ovarian cancer has honed in on women with exceptional risk, a 50–85% lifetime risk of developing breast cancer (Winer *et al*., 2001). However, these mutations are believed to be responsible for only 5–10% of breast cancer cases diagnosed.

The tools developed to manage established risk include chemoprevention, MRI and prophylactic surgery (Saslow *et al*., 2007; Singletary, 2003). Chemoprevention refers to the use of selective estrogen receptor modulators (SERMs) such as tamoxifen and raloxifen. Recent studies demonstrating the effectiveness of using MRI for high risk women resulted in the inclusion of this imaging technique in the American Cancer Society (ACS) guidelines (Saslow *et al*., 2007). Prophylactic mastectomy is associated with a greater than 90% reduction in risk in women with extensive family histories of breast cancer (Hartmann *et al*., 1999). Given the potential psychological consequences of this risk reduction strategy, studies suggest that the evidence for this recommendation should be as concrete as possible (Love *et al*., 2002).

RISK ASSESSMENT MODELS

This section will briefly review current mathematical models used to calculate actual risk of breast cancer, as well as genetic tests which evaluate the possibility of having

TABLE 16.1. Risk factors for breast cancer. (Singletary, 2003.)

Risk factor	Category at risk	Comparison category	Relative risk
Alcohol intake	Two drinks per day	Nondrinker	1.2
Body Mass Index	80th percentile, age 55 or greater	20th percentile	1.2
Hormone replacement therapy, with estrogen and progesterone	Current user for at least 5 years	Never used	1.3
Radiation exposure	Repeated fluoroscopy/chest radiation for acne/Radiation therapy for Hodgkin's disease	No exposure	1.6–5.2
Early menarche	Younger than 12 years	Older than 15 years	1.3
Late menopause	Older than 55 years	Younger than 45 years	1.2–1.5
Age at first childbirth	Nulliparous or first child after 30	First child before 20	1.7–1.9
Breast density	BI-RADS density = 4	BI-RADS density = 1	9.1–13.4
Current age	65 or older	Less than 65	5.8
Past history of breast cancer	Invasive breast carcinoma	No history of invasive breast carcinoma	6.8
Other histologic findings	Lobular carcinoma in situ	No abnormality detected	16.4
	Ductal carcinoma in situ	No abnormality detected	17.3
Breast biopsy	Hyperplasia w/out atypia[a]	No hyperplasia	1.9
	Hyperplasia with atypia	No hyperplasia	5.3
	Hyperplasia with atypia and positive family history	No hyperplasia, negative family history	11
Cytology (fine-needle aspiration or FNA, nipple aspiration fluid)	Proliferation w/out atypia[a]	No abnormality detected	2.5
	Proliferation with atypia	No abnormality detected	4.9–5.0
	Proliferation with atypia and positive family history	No abnormality detected	18.1
Family history	1st-degree relative 50 years or older with postmenopausal breast cancer	No 1st- or 2nd-degree relative with breast cancer	1.8
	1st-degree relative with premenopausal breast cancer	No 1st- or 2nd-degree relative with breast cancer	3.3
	2nd-degree relative with breast cancer	No 1st- or 2nd-degree relative with breast cancer	1.5
	Two 1st-dgree relatives with breast cancer	No 1st- or 2nd-degree relative with breast cancer	3.6
Germline mutation	Heterozygous for BRCA1, age < 40	Not heterozygous for BRCA1, age < 40	200[b]
	Heterozygous for BRCA1, age 60–69	Not heterozygous for BRCA1, age 60–69	15[b]

[a] There is controversy over whether pathologic hyperplasia detected in breast biopsy samples is directly equivalent to cytologic hyperplasia detected in samples obtained through FNA or nipple aspiration.
[b] Relative risks are subject to ascertainment bias and may overestimate the true risk associated with germline mutations in BRCA genes.

breast cancer gene mutations. For purposes of this chapter, the term models will refer to both mathematical models of risk assessment as well as commonly used genetic tests that predict the probability of having a *BRCA1* or *BRCA2* mutation. These genetic models do not directly test for genetic mutations, but are used to help decide who should be referred for genetic testing. The models discussed in this section include the Gail model, the Claus model, BRCAPRO, and the Tyrer-Cuzick model.

The most commonly employed breast cancer risk assessment model is the Gail

model. This mathematical model was originally designed using information from 284,780 Caucasian women participating in a breast cancer detection project between 1973–1980 (Gail *et al*., 1989). The risk factors chosen for this model include a combination of reproductive risk factors and first-degree family history of breast cancer. These factors are: age, age at menarche, number of prior breast biopsies, age at first live birth, and number of first-degree relatives affected with breast cancer. These factors produce absolute risk figures at both 5 years from the time of assessment and lifetime risk up to the age of 90. More recently, the presence of proliferative cellular conditions such as hyperplasia or atypical ductal hyperplasia, if known from breast biopsy, was added to the model to enhance accuracy (Euhus *et al*., 2002). The Gail model offers both strengths and weaknesses. The model was found to be useful in specialized clinic settings and appears to be most applicable to women without a strong family history of breast cancer, suggesting minimal risk of an inherited genetic mutation (Sakorafas *et al*., 2002). However, it has limited usefulness for women with an extensive history of breast cancer, as all relevant family history is not included in the model.

The Claus model, on the other hand, is designed to evaluate breast cancer risk by incorporating extensive family history of breast cancer development into risk calculations (Claus *et al*., 1993). Both the Gail and the Claus models were developed prior to genetic testing, and the Claus model gave credence to the idea of inherited genetic mutations and future risk of breast cancer development (Euhus, 2001). The Claus model incorporates breast cancer information on mothers, sisters, aunts, and grandmothers as well as the age of any affected family members at diagnosis. Unlike the Gail model, this model does not use reproductive risk factors to determine breast cancer risk and is best suited for women with an extensive family history of breast cancer.

BRCAPRO is considered to be the most comprehensive tool used to determine the probability of having a *BRCA1* or *BRCA2* mutation (Allain *et al*., 2002). One study found it to be highly sensitive for predicting the presence of a *BRCA1* or *BRCA2* mutation, missing only 15% of mutations present (Berry *et al*., 2002). This tool does not evaluate risk factors unrelated to family history, such as reproductive risk factors or the presence of atypical ductal hyperplasia from breast biopsy. It also underestimates risk in women with familial clustering not related to *BRCA1* or *BRCA2* mutations (Euhus, 2001).

A recently developed model, the Tyrer-Cuzick model, attempts to merge the concepts in the models discussed above. This model was developed based on the fact that many risk assessment models look at either possible genetic risk of breast cancer or risk associated with reproductive factors, but no models have incorporated both personal risk factors and genetic analysis (Tyrer *et al*., 2004). The Tyrer-Cuzick model inputs information on *BRCA* genes, personal risk factors and a low penetrance gene into a computer program which offers an individualized risk estimate. In a recent study, this model was found to correlate most strongly with BRCAPRO (Fasching *et al*., 2007). The Tyrer-Cuzick model was conceptualized to provide more accurate feedback for women considering genetic analysis for possible breast cancer gene mutations (Beckmann *et al*., 2007). The use

of these models and tools varies with the provider's knowledge and familiarity. Nipple aspirate fluid may provide easy access to biomarkers found in the breast ductal system, allowing for individual biologic risk rather than the probability of risk.

BREAST DUCT ANATOMY

DUCTAL ANATOMY

Incorporating the use of NAF into breast cancer risk assessment requires a thorough understanding of the breast ductal system. Although structures in the breast have been identified, there remain questions regarding the ductal system. The source of NAF has never been clearly identified. Does the fluid come from a large ductal system or a blind accessory duct or both? Is it collected and stored as a stagnant pool in a lactiferous sinus or reservoir just underneath the nipple? Or is it secreted or diffused from a segmental branch close to the nipple? The contents of NAF have been examined and thought to represent ductal fluid or at least the hormonal milieu of the breast although no ductal fluid to NAF orifice correlation has been done (Khan *et al*., 2005). With advances in surgical therapy (i.e., nipple sparing mastectomy, lumpectomies, quadrantectomies), imaging and ductal lavage as a method to acquire cells from distal areas of the duct, it has become imperative to better understand the normal anatomy of the breast and in particular the structure of a ductal system.

Nomenclature regarding breast anatomy is confusing, especially in relation to the ducts. The breast is considered one organ or gland but each duct is also often referred to as a gland. However, most will agree that a breast ductal system, also referred to as a lobe, is a unit consisting of an opening on the surface of the nipple which leads to a lactiferous duct, which branches into segmental, then subsegmental ducts, then terminal ducts that end as thousands of lobules and alveoli which secrete milk (Harris *et al*., 2004). Most surgical textbooks and atlases depict 15–20 ducts in the breast which radiate out from the nipple in fairly equal sizes like segments of a cut grapefruit. Often the breast is divided into quadrants although it is now known that this has no correlation to the actual anatomy of the ductal systems.

Cooper (1840), a prolific pathologist and master anatomist, believed that a comprehensive understanding of the natural structures of the breast was necessary to explain the pathologic changes that occur. In his original work from 1840, he injected wax into the ducts and found that the most he could inject was 12 ducts. He carefully separated all the ducts; however, again he did not find any communication. His drawings show them to be more separate than they are in reality. Subsequently, Cooper's work was reduced to a few pages in textbooks, particularly absent were any diagrams of the nipple (Anderson, 1978).

Recent advances in software technology, such as ultrasound, 3D MRI, and stereomicroscopy, have revived the field of breast anatomy. Going and Moffat (2004) examined the anatomy of the breast ducts in an autopsy of a 19 year-old woman along with mastectomy specimens and constructed a 3D image (Figure 16.1). They found large variations in size and volume of each ductal system. One duct drained almost a quarter of the total breast volume. The largest six systems comprised 75% of the breast. The ducts clearly intermingled

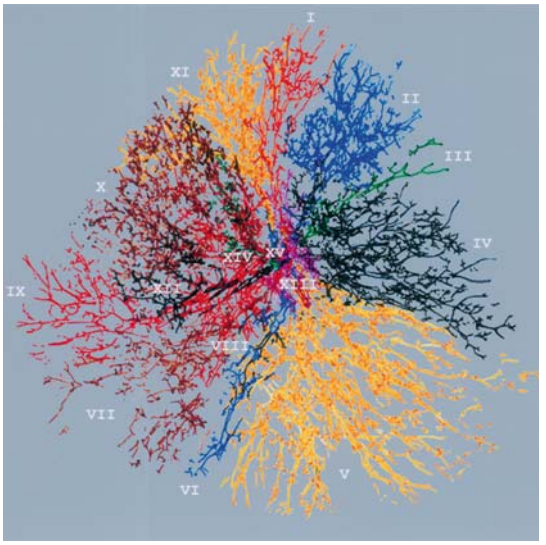

FIGURE 16.1. Three-dimensional image-ductal system (Going & Moffat, 2004)

and interdigitated but did not anastomose. They confirmed Cooper's (1840) finding that two to three ducts head directly backwards to the muscle and several ducts head toward the sternum while the majority are arranged inferiorly and toward the axilla. Tot (2005) describes the segments as pyramidal systems and has coined the phrase "sick lobe" as the area affected by ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS).

The original source for the number of 15–20 ductal systems cannot be ascertained although the figure continues to be quoted while more recent data supports the finding of only 5–12 significant independent, arborizing lactiferous ductal systems, each of which covers a finite portion of the breast and can be assessed from the nipple (King and Love, 2006). It is important to note that studies indicating the presence of 15–20 ducts have used cross sections for analysis, whereas findings of 6–8 ducts were derived from studies using ductal cannulation. While the number of ducts varies from woman to woman, the exact figure remains constant over the life of a woman.

THE NIPPLE

The number of ductal orifices is also controversial. Because they cannot easily be seen with the naked eye or other imaging device on the surface of a living non-lactating woman, they have not been counted. A cross section of a cadaver nipple under its surface shows 15–50 lumina but these have not been traced into the breast nor seen to be part of a ductal system, and thus some are thought to be blind. Love and Barsky (2004) observed > 200 lactating women in order to map milk duct orifices and identified an average of 5–9 openings. Teboul and Halliwell (1995) reported on $> 6{,}000$ ultrasound studies of the breast ducts and described 5–8 milk pores in the nipple. Finally, Ramsay *et al*. (2005) studied 21 lactating women with ultrasound and described 6–12 main ducts.

Pathologists have noted that the lactiferous ducts are conical in shape narrowing to a minute orifice in a cleft of the nipple. Smooth muscle surrounds the ducts and provides continuance of secretions and enables the nipple to become erect. The outer lining of the duct is quite elastic and can dilate with the pressure from milk ejection. These ducts are thought to dilate into ampullas, reservoirs or lactiferous sinus immediately beneath the nipple. This point is being challenged by Ramsay *et al*. (2005) as they did not find any enlargement in lactating women; however, this may be related to real-time imaging versus autopsy specimen and the fact that her work is in breast-feeding women. Nejad *et al*. (2008) have found

evidence of an "udder" on some non-lactating women undergoing ductal lavage. Going and Moffat (2004) identified three types of duct-like structures behind the nipple. The first group was identified as the major group which opened into the nipple, a second group diminished in caliber and terminated beneath the surface. A third group of accessory ducts originated from the skin at the base. This nomenclature has recently been disputed by Rusby *et al*. (2007) who found that many ducts joined and shared a few common openings. Others have described additional non-arborizing structures varying from 1–4 cm in length found in the nipple. Some of these structures, referred to as tubercles, sebaceous glands, rudimentary ducts or atrophic ducts, open into the nipple and some do not. None of them seems to aborize or function due to their lack of lobules. Hopefully, this controversy will be solved in the near future by on-going research. This confusion regarding the internal anatomy of the nipple is obviously relevant to its aspirate fluid.

NIPPLE ASPIRATE FLUID

COLLECTION OF NIPPLE ASPIRATE FLUID

In the study of breast carcinogenesis, the primary assumption made is that cells progress on a continuum (Martin, 1996). The ability to invade the surrounding breast tissue and metastasize is believed to be present in 20–50% of breast precancers (O'Shaughnessy, 2000). Studying breast epithelial cells for precancerous changes is important for evaluating where in the carcinogenic continuum intervention may be most effective. Breast precancers are defined as having varying degrees of cellular atypias, obtained from various cellular retrieval methods including tissue biopsy, ductal lavage, periareolar fine needle aspiration (FNA), and nipple aspiration. Biopsy, ductal lavage, and FNA have been described in detail elsewhere (Baltzell *et al*., 2005). This chapter will focus solely on nipple aspiration and the resulting NAF.

Obtaining breast epithelial cells through a simple suction technique is known as nipple aspiration. This technique was pioneered by Papanicolaou *et al*. (1958), based on cytopathologic evaluation of cervical specimens and their relationship to cervical cancer. The technique involves a number of noninvasive steps, beginning with the scrubbing of the nipple surface with 2% acetic acid. This step is necessary to remove any keratin blocking the ductal openings. After the nipple is dried, an aspiration device is placed over the nipple and negative pressure is applied with a syringe attached to a central chamber. The patient is asked to massage the breast during this step, beginning at the base of the breast and moving toward the nipple area. This helps to mobilize the fluid toward the nipple surface (Figure 16.2). Squeezing the base of the nipple can express the fluid from the lactiferous sinus. Once fluid is observed, the aspiration device is removed and the fluid is collected in capillary tubes for analysis (Sartorius *et al*., 1977).

Studies have shown varying degrees of success in obtaining NAF using aspiration. Sauter *et al*. (1997) concluded that NAF can be obtained in essentially all eligible subjects; other studies have reported nipple aspiration is far inferior to other techniques such as ductal lavage in obtaining an adequate number of cells for evaluation (Dooley *et al*., 2001). Rose *et al*. (1986)

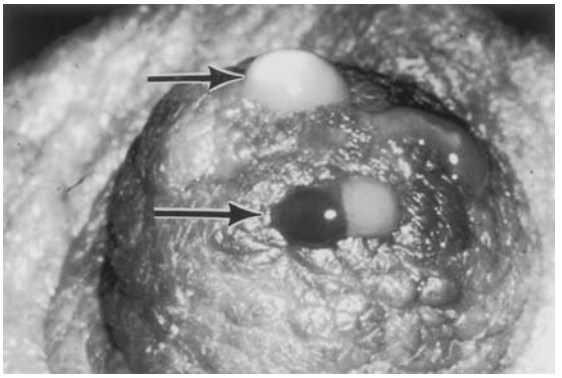

FIGURE 16.2. Nipple aspiration fluid at ductal orifices. (Photo courtesy of Dr. Susan Love Research Foundation.)

reviewed studies which obtained NAF from as few as 25% to as many as 95% of study subjects. Wrensch *et al*. (2001) wrote that obtaining fluid depends on the quantity of underlying fluid present, duct and nipple characteristics, subject age, and the skill of the technician collecting the fluid. A 1990 study found four important factors positively related to the ability to obtain breast fluid (Wrensch *et al*., 2001). These factors are: age between 35 to 50 years, early age at menarche, non-Asian compared to Asian ethnicity, and history of lactation. Of interest is the finding that women who do not yield fluid may be less likely to develop breast cancer than women who do yield fluid (Wrensch *et al*., 1992). A more recent study found younger age (< 50 years), being married, a history of pregnancy, a history of tranquilizer use, and a history of endocrine disorders were all positively associated with the ability to obtain NAF (Baltzell *et al*., 2006).

COMPONENTS OF NIPPLE ASPIRATE FLUID

The fluid obtained by using the basic technique described above is believed to reflect the cellular environment within the breast duct system as well as illuminate changes in the secretory processes of the breast (Klein and Lawrence, 2002). This allows for the investigation of not only the cells, but also the physical environment that surrounds the cells. Cytology is the main technique by which NAF is examined, and at least ten epithelial cells are required for cytological analysis. This minimum has been observed in 53–83% of cases and women under 50 are most likely to yield fluid, as noted above (Dooley *et al*., 2001; Wrensch *et al*., 2001). The cells from the ductolobular units which may be present in NAF include: columnar epithelial cells, macrophages, foam cells, leukocytes, and cellular debris (Dua *et al*., 2006). It is the epithelial cells that are categorized relative to future breast cancer risk. These cytologic categories are generally defined as normal cells and mild as well as marked atypia. On very rare occasions frankly malignant cells may be encountered. In addition to the cellular content of NAF, a wide variety of non-cellular materials of both exogenous and endogenous origin may also be present. Exogenous substances include nicotine, caffeine, pesticides, and oral medications (Petrakis, 1993). Endogenous substances include immunoglobulins, estrogen and progesterone, androgens, prolactin, prostate-specific antigen (PSA), and carcinoembryonic antigen (CEA) (Petrakis, 1986, 1993; Zhao *et al*., 2001).

CYTOLOGIC EVALUATION OF NAF

The diagnostic categories for the evaluation of NAF cytology specimens are similar to those of FNA specimens from breast lesions described in the 1997 consensus criteria for breast fine-needle biopsy samples

published by the National Cancer Institute ("The uniform approach to breast fine-needle aspiration biopsy. NIH Consensus Development Conference," 1997). The five diagnostic categories which were created include: inadequate cellular material for diagnosis (samples with < 10 epithelial cells per sample or unacceptable technical quality), benign, mild atypia, marked atypia, and malignant. The cell characteristics most helpful in identifying abnormalities were related to cell arrangement, cell size, nuclear size, and size variation, nuclear membrane irregularity, chromatin granularity, and the presence of large nucleoli (Ljung *et al*., 2004).

Benign, unremarkable cases generally present primarily with scattered single normal epithelial cells in most cases (Figure 16.3A). The cells are small and have round or oval-shaped nuclei with either finely granular or condensed chromatin. The nuclear membranes are smooth and regular and the cytoplasm is generally moderately abundant. Benign cases may also contain epithelial clusters that are usually small in size and arranged as orderly monolayers. In many cases defined as mildly atypical, ductal cells are moderately enlarged (Figure 16.3B). The majority of mildly atypical ductal cells show a small degree of nuclear enlargement with cells occurring singly in either monolayer arrangements or small clusters. Like benign cases, nuclear membranes are regular and the chromatin is finely granular. Included in this mildly atypical category are cases with features typical of papillomas. These features include mild nuclear enlargement and size variability, and fairly abundant, dense (metaplastic appearing) cytoplasm focally containing large vacuoles. These cells from papillomas are primarily seen in clusters of small to medium size.

Moderate or marked enlargement of nuclei is the hallmark of ductal cells defined as markedly atypical (Figure 16.3C). These markedly atypical cells may be found singly and/or in varying size clusters that typically show crowding of cells rather than orderly monolayer arrangement. Irregular nuclear membranes and coarse rather than finely granular chromatin are characteristic. While nuclear-to-cytoplasmic ratio may also be increased in some cases other markedly atypical cases and even outright cancer cases may show fairly abundant cytoplasm.

A diagnosis of frank malignancy is extremely rare in NAF specimens. If observed, the familiar features of cancer are found in malignant NAF cells (Figure 16.3D). Depending on the nuclear grade of the cancer, the overall enlargement of cells as well as nuclei may be moderate to marked. Typically the cells will appear both singly and in usually small clusters with nuclear crowding and overlap. A hallmark of diagnosis is irregular outline of nuclear membranes and clumped, coarse chromatin pattern. The nuclear-to-cytoplasmic ratios may be markedly increased but a significant subset of even high grade cancers has fairly abundant cytoplasm. Necrosis is a strong indicator of a malignant process and suggests significant presence of DCIS.

An important study in 1983 established the association between atypical cell findings in NAF specimens and atypical ductal hyperplasia found in breast biopsy (King *et al*., 1983). This study was the first of its kind to compare specific morphologic findings in cytologic specimens from nipple fluid with corresponding histologic biopsies. This work was crucial for establishing that the study of breast fluids such as NAF could provide information usable for

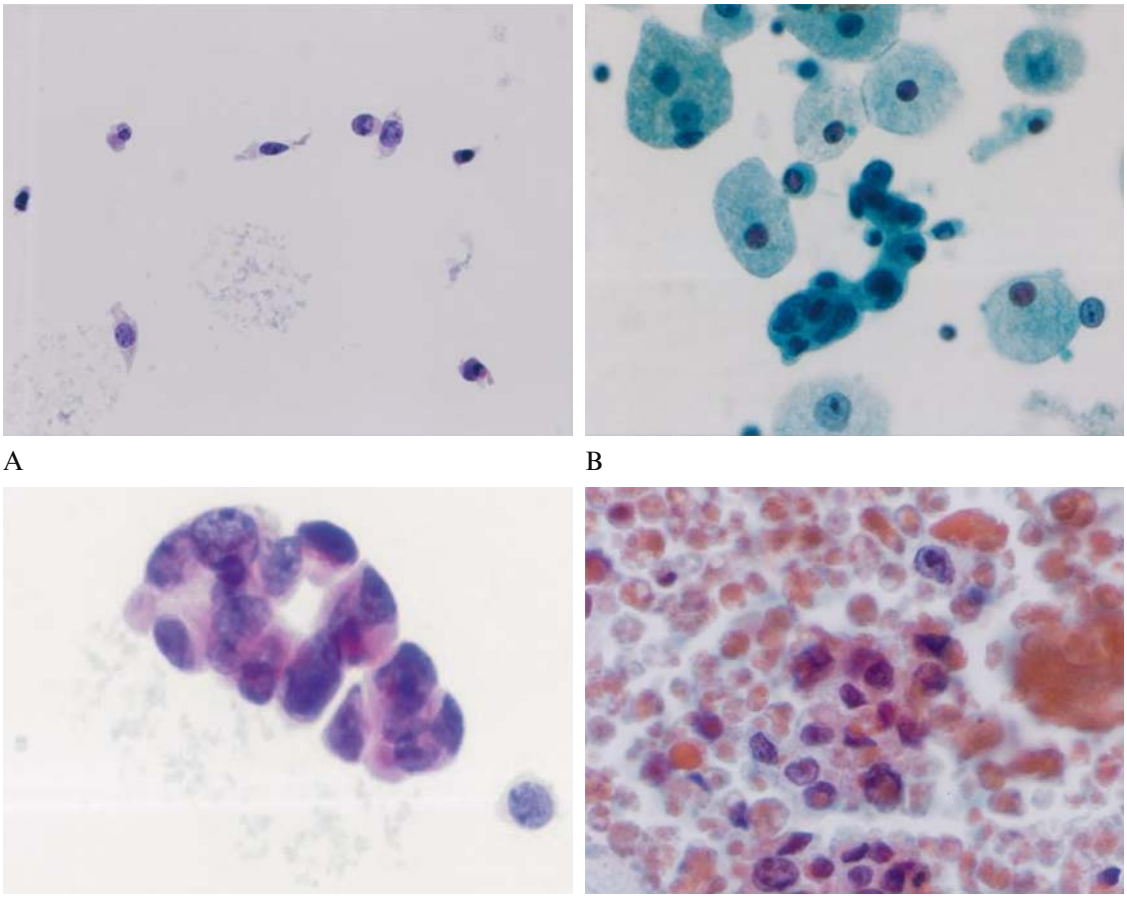

FIGURE 16.3 A. Normal/benign ductal cells. B. Mildly atypical ductal cells. C. Markedly atypical ductal cells. D. Malignant, high grade ductal cancer

breast cancer risk assessment. Furthermore, reviewing repeat NAF samples appears to enhance the accuracy of a cytologic diagnosis of NAF cells (King *et al*., 2004).

THE RELATIONSHIP BETWEEN ABNORMAL CYTOLOGY IN NIPPLE ASPIRATE FLUID AND BREAST CANCER RISK

Earlier in this chapter the current tools for assessing breast cancer risk were outlined. The interest in using NAF as an adjunct to these models is based on the thinking that the components of NAF available for evaluation have been in direct proximity to the lining of the breast ducts, the site of 90% of breast cancer origins (Dua *et al*., 2006). Interestingly, the analysis of NAF may be more informative for finding breast cancer precursors or DCIS, because the duct integrity is no longer intact once an invasive cancer is present. Once the duct is disrupted, the fluids available for analysis via NAF may be absorbed into the surrounding breast tissue.

Several large epidemiologic studies of women without breast cancer found that

the presence of atypical cells in NAF is associated with a four- to fivefold greater risk of subsequent breast cancer development (Wrensch *et al*., 1992, 2001). This risk rises to between 11- to 18-fold when the presence of atypical hyperplasia, found in histologic specimens, is coupled with a family history of breast cancer (Dupont and Page, 1985; Singletary, 2003). A 2006 study found that the mere presence of epithelial cells in NAF was positively associated with subsequent breast cancer risk, regardless of cytologic category (Buehring *et al*., 2006). The addition of NAF cytologic results to the Gail model enhanced the prediction model significantly, particularly for high-risk women (Tice *et al*., 2005).

In addition to NAF cell cytology, other markers of interest found in NAF have been studied recently. The color of NAF has been studied for significance and specimens have been defined as clear, white, yellow, green, and red/brown. It is the red/brown color which has been associated with the presence of an underlying breast tumor (Sauter *et al*., 2006). When NAF color was considered along with age and NAF cytology, the prediction model used in the study was 92% sensitive and 61% specific in predicting if a woman had breast cancer. Prostate-specific antigen (PSA) is also a marker of interest in breast cancer studies. It has been shown that there is an inverse relationship between the presence of PSA found in NAF and the presence of breast cancer (Sauter *et al*., 2002). Furthermore, PSA has been shown to decrease as tumor size, nodal involvement, and disease stage increase in breast cancer patients, providing useful prognostic information for treatment direction (Sauter *et al*., 2004). Further studies have identified basic fibroblast growth factor (bFGF) as a useful enhancement to breast cancer prediction models, with high bFGF levels positively associated with the presence of cancer (Sauter *et al*., 2007). A breast cancer prediction model including cytology, bFGF and age offered an 88% sensitivity and 57% specificity.

Identifying factors which influence NAF production is important if this technique is to be used more frequently in the clinical setting. Several dietary factors have been shown to influence the amount of NAF available for evaluation. These factors include soy and fat intake (Kato *et al*., 2006; Petrakis *et al*., 1996). An increase in soy intake resulted in an increase in NAF production and an increase in the presence of atypical cell findings found in NAF in premenopausal women (Petrakis *et al*., 1996). Also of interest is a study indicating that women who had increased amounts of total fat intake had a higher probability of producing epithelial cell-positive NAF (Kato *et al*., 2006). Another study found that women taking SERMS such as tamoxifen or rafoxifene may have a decrease in NAF production (Higgins *et al*., 2005).

There are other promising NAF markers currently under study in the hopes of ascertaining who is at risk for breast cancer development. The future of breast cancer risk assessment will undoubtedly involve a biomarker to enhance precision and individualized risk assessment. Nipple aspiration is a technique which shows great potential in providing this much needed information.

REFERENCES

Allain, D., Gilligan, M., & Redlich, P. 2002. Genetics and genetic counseling for breast cancer. In W. Donegan & J. Spratt (Eds.), *Cancer of the Breast* (pp. 249–268). Philadelphia: Saunders.

Anderson, J. E. 1978. *Grant's Atlas of Anatomy* (7th ed.). Baltimore: Williams & Wilkins.

Baltzell, K., Eder, S., & Wrensch, M. 2005. Breast carcinogenesis - can the examination of ductal fluid enhance our understanding? *Oncol. Nurs. Forum. 32*: 33–39.

Baltzell, K. A., Wrensch, M., & Sison, J. D. 2006. A descriptive study of variables associated with obtaining nipple aspirate fluid in a cohort of non-lactating women. *BMC Womens Hlth. 6*: 15.

Beckmann, M. W., Bani, M. R., Fasching, P. A., Strick, R., & Lux, M. P. 2007. Risk and risk assessment for breast cancer: molecular and clinical aspects. *Maturitas. 57*: 56–60.

Berry, D., Iversen, E., Gudbjartsson, D., Hiller, E., Garber, J., Peshkin, B., Lerman, C., Watson, P., & Lynch, H. 2002. BRCAPRO validation, sensitivity of genetic testing of BRCA1/BRCA2, and prevalance of other breast cancer susceptibility genes. *J. Clin. Oncol. 20*: 2701–2712.

Buehring, G., Letscher, A., McGirr, K., Khandhar, S., Che, L., Nguyen, C., & Hackett, A. 2006. Presence of epithelial cells in nipple aspirate fluid is associated with subsequent breast cancer: a 25-year prospective study. *Breast Cancer Res. Treat*. *98*: 63–70

Claus, E., Risch, N., & Thompson, W. 1993. The calculation of breast cancer risk for women with a first degree family history of ovarian cancer. *Breast Cancer Res. Treat. 28*: 115–120.

Cooper, A. P. 1840. *On the Anatomy of the Breast*. London: Longmans.

Dooley, W., Ljung, B., Veronesi, U., Cazzaniga, M., Elledge, R., O'Shaughnessy, J., Kuerer, H., & Khan, S. 2001. Ductal lavage for detection of cellular atypia in women at high risk for breast cancer. *J. Natl. Cancer Inst. 93*: 1624–1632.

Dua, R. S., Isacke, C. M., & Gui, G. P. 2006. The intraductal approach to breast cancer biomarker discovery. *J. Clin. Oncol. 24*: 1209–1216.

Dupont, W., & Page, D. 1985. Risk factors for breast cancer in women with proliferative breast disease. *N. Engl. J. Med. 312*: 146–151.

Euhus, D. 2001. Understanding mathematical models for breast cancer risk assessment and counseling. *Breast J. 7*: 224–232.

Euhus, D. M., Leitch, A. M., Huth, J. F., & Peters, G. N. 2002. Limitations of the Gail model in the specialized breast cancer risk assessment clinic. *Breast J. 8*: 23–27.

Fasching, P. A., Bani, M. R., Nestle-Kramling, C., Goecke, T. O., Niederacher, D., Beckmann, M. W., & Lux, M. P. 2007. Evaluation of mathematical models for breast cancer risk assessment in routine clinical use. *Eur. J. Cancer Prev. 16*: 216–224.

Gail, M., Brinton, L., Byar, D., Corle, D., Green, S., Schairer, C., & Mulvihill, J. J. 1989. Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. *J. Natl. Cancer Inst. 81*: 1879–1886.

Going, J. J., & Moffat, D. F. 2004. Escaping from Flatland: clinical and biological aspects of human mammary duct anatomy in three dimensions. *J. Pathol. 203*: 538–544.

Harris, J., Lippman, M., Morrow, M., & Osborne, C. (Eds.). 2004. *Diseases of the Breast* (3rd ed.). Philadelphia: Lippincott Williams & Wilkins.

Hartmann, L., Schaid, D., Woods, J., Crotty, T., Myers, J., Arnold, P., & Petty, P. 1999. Efficacy of bilateral prophylactic mastectomy in women with a family history of breast cancer. *N. Engl. J. Med. 340*: 77–84.

Higgins, S. A., Matloff, E. T., Rimm, D. L., Dziura, J., Haffty, B. G., & King, B. L. 2005. Patterns of reduced nipple aspirate fluid production and ductal lavage cellularity in women at high risk for breast cancer. *Breast Cancer Res. 7*: R1017–R1022.

Hollingsworth, A. B., Nall, S., & Dill, D. 2002. The evolution of breast cancer risk assessment. *J. Okla. State Med. Assoc. 95*: 639–644.

Jemal, A., Murray, T., Ward, E., Samuels, A., Tiwari, R., Ghafoor, A., Feuer, E., & Thun, M. J. 2005. Cancer statistics, 2005. *CA: Cancer J. Clin. 55*: 10–30.

Kato, I., Ren, J., Visscher, D. W., & Djuric, Z. 2006. Nutritional predictors for cellular nipple aspirate fluid: nutrition and breast health study. *Breast Cancer Res. Treat. 97*: 33–39.

Kerlikowske, K., Ichikawa, L., Miglioretti, D. L., Buist, D. S., Vacek, P. M., Smith-Bindman, R., Yankaskas, B., Carney, P. A., & Ballard-Barbash, R. 2007. Longitudinal measurement of clinical mammographic breast density to improve estimation of breast cancer risk. *J. Natl. Cancer Inst. 99*: 386–395.

Khan, S. A., Bhandare, D., & Chatterton, R. T., Jr. 2005. The local hormonal environment and related biomarkers in the normal breast. *Endocr. Relat. Cancer 12*: 497–510.

King, B. L., & Love, S. M. 2006. The intraductal approach to the breast: raison d'etre. *Breast Cancer Res. 8*: 206.

King, E. B., Chew, K. L., Petrakis, N. L., & Ernster, V. L. 1983. Nipple aspirate cytology for the study of breast cancer precursors. *J. Natl. Cancer Inst. 71*: 1115–1121.

King, E. B., Chew, K. L., Hom, J. D., Miike, R., Wrensch, M. R., & Petrakis, N. L. 2004. Multiple sampling for increasing the diagnostic sensitivity of nipple aspirate fluid for atypical cytology. *Acta. Cytol. 48*: 813–817.

Klein, P. M., & Lawrence, J. A. 2002. Lavage and nipple aspiration of breast ductal fluids: a source of biomarkers for environmental mutagenesis. *Environ. Mol. Mutagen. 39*: 127–133.

Ljung, B. M., Chew, K. L., Moore, D. H., II, & King, E. B. 2004. Cytology of ductal lavage fluid of the breast. *Diag. Cytopathol. 30*: 143–150.

Love, R., Newcomb, P., & Trentham-Dietz, A. (Eds.). 2002. *Prevention of Breast Cancer*. Philadelphia: Saunders.

Love, S. M., & Barsky, S. H. 2004. Anatomy of the nipple and breast ducts revisited. *Cancer 101*: 1947–1957.

Martin, G. 1996. Normal cells and cancer cells. In J. W. Bishop, R. (Ed.), *Molecular Oncology*. (pp. 13–40). New York: Scientific-American.

Nejad, M., Tondre, J., Mills, D., & Love, S.M. 2008. Technical enhancements to breast ductal lavage. *Ann. Surg, Onc. 15*(2): 78.

O'Shaughnessy, J. A. 2000. Treating breast precancer. *Clin. Breast Cancer 1 Suppl 1*: S74–S79.

Papanicolaou, G., Holmquist, D., Bader, G., & Falk, E. 1958. Exfoliative cytology of the human mammary gland and its value in the diagnosis of cancer and other diseases of the breast. *Cancer 11*: 377–409.

Petrakis, N. L. 1986. Physiologic, biochemical, and cytologic aspects of nipple aspirate fluid. *Breast Cancer Res. Treat. 8*: 7–19.

Petrakis, N. L. 1993. Nipple aspirate fluid in epidemiologic studies of breast disease. *Epidemiol. Rev. 15*: 188–195.

Petrakis, N. L., Barnes, S., King, E. B., Lowenstein, J., Wiencke, J., Lee, M. M., Miike, R., Kirk, M., & Coward, L. 1996. Stimulatory influence of soy protein isolate on breast secretion in pre- and postmenopausal women. *Cancer Epidemiol. Biomarkers Prev. 5*: 785–794.

Ramsay, D. T., Kent, J. C., Hartmann, R. A., & Hartmann, P. E. 2005. Anatomy of the lactating human breast redefined with ultrasound imaging. *J. Anat. 206*: 525–534.

Rose, D., Lahti, H., Laakso, K., Kettunen, K., & Wynder, E. 1986. Serum and breast duct fluid prolactin and estrogen levels in healthy Finnish and American women and patients with fibrocystic disease. *Cancer 57*: 1550–1554.

Rusby, J. E., Brachtel, E. F., Michaelson, J. S., Koerner, F. C., & Smith, B. L. 2007. Breast duct anatomy in the human nipple: three-dimensional patterns and clinical implications. *Breast Cancer Res. Treat. 106*: 171–179.

Sakorafas, G. H., Krespis, E., & Pavlakis, G. 2002. Risk estimation for breast cancer development; a clinical perspective. *Surg. Oncol. 10*: 183–192.

Sartorius, O., Smith, H., Morris, P., Benedict, D., & Friesen, L. 1977. Cytologic evaluation of breast fluid in the detection of breast disease. *J. Natl. Cancer Inst. 59*: 1073–1078.

Saslow, D., Boetes, C., Burke, W., Harms, S., Leach, M. O., Lehman, C. D., Morris, E., Pisano, E., Schnall, M., Sener, S., Smith, R. A., Warner, E., Yaffe, M., Andrews, K. S., & Russell, C. A. 2007. American cancer society guidelines for breast screening with MRI as an adjunct to mammography. *CA Cancer J. Clin. 57*: 75–89.

Sauter, E. R., Chervoneva, I., Diamandis, A., Khosravi, J. M., Litwin, S., & Diamandis, E. P. 2002. Prostate-specific antigen and insulin-like growth factor binding protein-3 in nipple aspirate fluid are associated with breast cancer. *Cancer Detect. Prev. 26*: 149–157.

Sauter, E. R., Ross, E., Daly, M., Klein-Szanto, A., Engstrom, P. F., Sorling, A., Malick, J., & Ehya, H. 1997. Nipple aspirate fluid: a promising non-invasive method to identify cellular markers of breast cancer risk. *Br. J. Cancer 76:* 494–501.

Sauter, E. R., Klein, G., Wagner-Mann, C., & Diamandis, E. P. 2004. Prostate-specific antigen expression in nipple aspirate fluid is associated with advanced breast cancer. *Cancer Detect. Prev. 28*: 27–31.

Sauter, E. R., Winn, J. N., Dale, P. S., & Wagner-Mann, C. 2006. Nipple aspirate fluid color is associated with breast cancer. *Cancer Detect. Prev. 30*: 322–328.

Sauter, E. R., Wagner-Mann, C., Ehya, H., & Klein-Szanto, A. 2007. Biologic markers of breast cancer in nipple aspirate fluid and nipple discharge are associated with clinical findings. *Cancer Detect. Prev. 31*: 50–58.

Singletary, S. E. 2003. Rating the risk factors for breast cancer. *Ann. Surg. 237*: 474–482.

Teboul, M., & Halliwell, M. 1995. *Atlas of Ultrasound and Ductal Echography of the Breast: the Introduction of Anatomic Intelligence into Breast Imaging*. Oxford: Blackwell Science.

Tice, J. A., Miike, R., Adduci, K., Petrakis, N. L., King, E., & Wrensch, M. R. 2005. Nipple aspirate fluid cytology and the Gail model for breast cancer risk assessment in a screening population. *Cancer Epidemiol. Biomarkers Prev. 14*: 324–328.

Tot, T. 2005. DCIS, cytokeratins, and the theory of the sick lobe. *Virchows Arch. 447*: 1–8.

Tyrer, J., Duffy, S. W., & Cuzick, J. 2004. A breast cancer prediction model incorporating familial and personal risk factors. *Stat. Med. 23*: 1111–1130.

1997. The uniform approach to breast fine-needle aspiration biopsy. NIH Consensus Development Conference.*Am. J. Surg. 174*: 371–385.

Winer, E., Morrow, M., Osborne, C., & Harris, J. 2001. Malignant tumors of the breast. In V. Devita, S. Hellman, & S. Rosenberg (Eds.), *Cancer: Principles & Practice of Oncology* (pp. 1651–1716). Phildelphia: Lippincott.

Wrensch, M. R., Petrakis, N. L., King, E. B., Miike, R., Mason, L., Chew, K. L., Lee, M. M., Ernster, V. L., Hilton, J. F., Schweitzer, R., *et al.* 1992. Breast cancer incidence in women with abnormal cytology in nipple aspirates of breast fluid. *Am. J. Epidemiol. 135*: 130–141.

Wrensch, M. R., Petrakis, N. L., Miike, R., King, E. B., Chew, K., Neuhaus, J., Lee, M. M., & Rhys, M. 2001. Breast cancer risk in women with abnormal cytology in nipple aspirates of breast fluid. *J. Natl. Cancer Inst. 93*: 1791–1798.

Zhao, Y., Verselis, S. J., Klar, N., Sadowsky, N. L., Kaelin, C. M., Smith, B., Foretova, L., & Li, F. P. 2001. Nipple fluid carcinoembryonic antigen and prostate-specific antigen in cancer-bearing and tumor-free breasts. *J. Clin. Oncol. 19*: 1462–1467.

17
Tissue Microarrays: Construction and Utilization for Biomarker Studies

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INTRODUCTION

The capacity of information generated from the Human Genome Project has significantly impacted our understanding of disease pathogenesis (Subramanian *et al*., 2001; Venter *et al*., 2001). These data, in combination with advances in molecular biology techniques, including cDNA microarrays and proteomics, have facilitated the identification of numerous molecular biomarkers that have contributed to the field of cancer biology (DeRisi *et al*., 1996). While cDNA microarrays produce an enormous amount of data from gene expression profiling of normal and disease samples, the technique lacks *in situ* identification and validation of these diagnostic, prognostic, and therapeutic markers in a wide array of human tissue specimens. The need for this type of high-throughput evaluation led to the development of the tissue microarray (TMA).

The first attempt at developing a technique to examine multiple tissue samples under identical conditions was described by Battifora (1986) as the 'sausage' block method. This procedure consisted of wrapping one hundred 1 mm thick "rods' of tissue in a sheet of mammalian small intestine and embedding the resultant 'sausage' of tissues in a paraffin block (Battifora, 1986). This method was useful for testing antibodies because of its identical staining conditions and minimal required reagent; however, the inability to identify individual tissue limited the amount of significant data that could be utilized by the 'sausage' technique. A little over a decade later, the current TMA was developed by Kononen *et al*. (1998) with similar cost-effective principles as the 'sausage' block while also ensuring that individual tissues were easily identifiable. The resulting tissue microarray consists of hundreds of tissue core specimens arranged on an individual slide and assayed simultaneously for immunohistochemistry, *in situ* DNA, or RNA hybridization (Kononen *et al*., 1998). TMAs have proven beneficial by providing clinical validation

*Early Detection Research Network NCI CA-86366, and the Specialized Programs of Research Excellence (SPORE) in Lung Cancer grant P50-CA90388, NCI

of molecular targets identified by cDNA microarrays, which ultimately enhance our understanding of disease prognosis, progression, and therapeutic outcome. This population-based technology has helped translate genomic discoveries into clinical applications. Because of the importance of biomarkers in cancer, this subject has also been discussed by Kelly *et al.*, in this volume.

ADVANTAGES OF TISSUE MICROARRAY TECHNOLOGY

Prior to the development of TMAs, validation of protein expression among clinical specimens was a tedious process that required sectioning and staining of each individual sample. One major advantage of the TMA is the ability to detect the expression of candidate markers in hundreds and even thousands of benign and malignant tissue specimens concurrently. Typically, 200 or more consecutive 4–8 μm thick sections can be cut from one TMA block, which allows for sequential staining of multiple molecular markers generating a large cohort of data. Additionally, each specimen within the TMA receives identical staining conditions because the samples are processed and arrayed together on a single slide. Reagent concentrations, temperatures, incubation times, and wash conditions are the same for each tissue core arrayed in the TMA unlike the slide-to-slide variability that is observed with whole tissue sections. Compared to individual tissue sections, TMAs are much more cost-effective and time efficient without compromising on quality or reliability. TMAs allow for morphological *in situ* assessment of the cellular distribution of molecular markers in a large population of tissue specimens. The amount of tissue used for TMA construction is substantially less than the standard practice of whole tissue sections, which aids in the preservation of the original archived tissue samples for subsequent studies.

CONSTRUCTION AND DEVELOPMENT OF A TISSUE MICROARRAY

The initial step, prior to the actual construction of the TMA, is the collection of the original histopathological tissue blocks that will comprise the array. The decision and identification of which samples will be on the TMA, the retrieval of these archived 'donor' blocks, and the histological determination of the most representative areas are typically among the most time consuming phases of the entire TMA construction process. In general, TMAs are comprised of formalin-fixed, paraffin-embedded tissues; however, several studies also describe the use of frozen tissues embedded in a TMA to improve upon the detection of RNA (Hoos and Cordon-Cardo, 2001; Schoenberg Fejzo and Slamon, 2001). For the most part, the techniques described in this chapter will refer to paraffin-embedded tissues, although information on frozen TMAs will be discussed later in more detail.

Donor Blocks and Core Size and Number. After the collection of the necessary archived paraffin-embedded tissues, a fresh section is cut from each 'donor' block and stained typically with hematoxylin and eosin. The most morphologically representative areas of tissue are marked by a pathologist to assist with the coring

of the 'donor' block. Generally, areas with a high volume of tumor, or of a desired histological category, are used as long as the tissue is well-fixed. Small tissue core biopsies of 0.6 mm diameter and 3–4 mm in height are punched from the 'donor' block, and are precisely arrayed into a recipient paraffin block using a tissue microarrayer (Beecher Instruments, Sun Prairie, WI). While the 0.6 mm diameter core is the most commonly used tissue core size in TMA construction, larger core sizes up to 5 mm in diameter have also been used (Kallioniemi *et al*., 1992; Corzo *et al*., 2006). Each tissue biopsy is usually spaced 0.8 mm apart and the position of every core within the TMA is recorded and linked to a database that contains demographic, clinico-pathological, outcome, and other patient information that can be correlated with the future biomarker data. Asymmetric alignment (e.g., adding 1–2 extra cores at a consistent designated location, Figure 17.1) of tissue cores can help maintain the orientation of the TMA and the inclusion of normal tissue, tissue from other tumor types, or cell lines can serve as internal controls. As many as 1,000 cylindrical tissue cores can be arrayed on a typical 45 × 20 mm recipient block and at least 200 consecutive sections of 4–8 µm thickness can be cut using a standard histopathological microtome. Figure 17.1 outlines the TMA construction process.

There has been some skepticism on whether the small tissue cores sizes within the TMA are representative of the entire tumor, especially when assaying for expression in tumors that have intratumoral heterogeneity. To address this issue, several validation studies have been performed comparing TMAs with individual whole tissue (Gillett *et al*., 2000). The overall consensus among various groups is that there is a strong correlation between the molecular data gathered from staining individual whole tissue sections and TMAs. The inclusion of multiple cores from a single 'donor' block can improve the accuracy of target expression in a TMA in comparison to conventional whole tissue slides. One study examined the expression of estrogen receptor, progesterone receptor, and the *HER-2/neu* oncogene in 38 cases of invasive breast carcinoma by

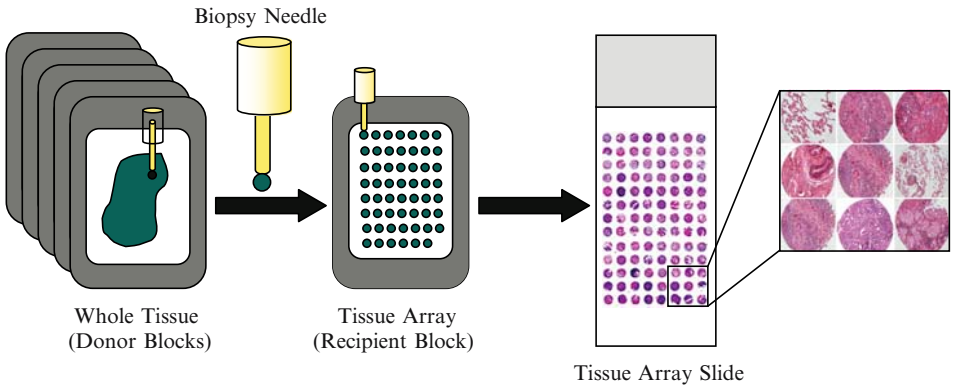

FIGURE 17.1. A schematic of the tissue array construction process. A tissue microarrayer is used to punch core biopsies from 'donor' blocks containing paraffin-embedded tissue. The cores are then transferred from the 'donor' blocks to a 'recipient' tissue array block using a thin biopsy needle and stylet. The tissue array block is cut and the resultant section is transferred to a slide and ready to be assayed. The magnified tissue cores have been stained with hematoxylin and eosin

comparing immunohistochemical staining of 2–10 microarray cores and the original whole tissue sections (Camp *et al*., 2000). In more than 95% of the cases, the analysis of 2 cores was found to be comparable to the expression data observed from the individual whole tissue sections. In cases where TMAs are constructed using particularly heterogeneous tumors, such as prostate cancer, increasing the number of cores per donor sample may minimize the potential variability among expression staining. Rubin *et al*. (2002) showed that 3 cores per donor within a prostate cancer TMA were necessary to reproduce the expression of Ki-67 with respect to the whole tissue sections. Another reason for incorporating multiple tissue cores per sample is the risk of losing tissue during the sectioning and staining process. The use of 3 cores taken from different regions of the donor block can improve the accuracy of array-based data by capturing the heterogeneity of the tumors as well as compensating for tissue loss or unusable cores (Hoos *et al*., 2001).

In addition to increasing the number of cores per sample to counteract concerns of heterogeneity and representativeness, some groups have increased the volume of tissue cores by as large as 5 mm in diameter to preserve histological characteristics (Kallioniemi *et al*., 1992; Corzo *et al*., 2006). For normal or premalignant tissues, the larger core size can be more beneficial because these tissues are harder to sample and generally have other unrelated tissues intermixed, such as the large percentage of adipose tissue within normal breast tissue, that can interfere with adequate representation (Yang *et al*., 2006).

Selecting the Right Tissue from the Correct Patient Population. When considering the assembly of a TMA, there are several different types that can be constructed. Examples include, but are not limited to a multi-tumor, tumor progression, and patient outcome arrays (Nocito *et al*., 2001). A multi-tumor array is comprised of samples from various tumor types and can be used to monitor the expression of a number of molecular markers across different tumor categories. An example of a multi-tumor array was constructed by Schraml *et al*. (1999), whereby gene amplification of several common oncogenes was surveyed using fluorescent *in situ* hybridization (FISH) in 397 tissue samples from 17 different tumor types (Schraml *et al*., 1999). The second category of TMAs is the tumor progression model, which is designed to assess gene expression across various stages of tumor progression (i.e., increasing tumor grades, hormone-sensitive versus hormone-resistant carcinoma) in a single organ. As an example, Callagy *et al*. (2003) constructed a TMA that consisted of various types of breast cancer tumors ranging from grades 1–3, and a collection of 13 biomarkers were analyzed to arrange these breast tumors into biologically and clinically distinct groups (Callagy *et al*., 2003).

The last category of TMAs, and probably the most common type created, is the 'patient outcome' array, which is also termed the prognosis TMA. This array contains samples that are linked to clinical follow-up data that may consist of time to recurrence, therapy response, and/or patient survival. As an example, a breast cancer TMA constructed by Torhorst *et al*. (2001) was intended to determine whether successful associations could be made between molecular alterations and clinical endpoints using the TMA technology (Torhorst *et al*., 2001). In a TMA comprised of 553 breast carcinoma specimens, patient outcome was

found to be clearly linked to the expression analysis of several prognostic markers (estrogen receptor, progesterone receptor, and p53). Therefore, TMAs can provide a straightforward method for connecting expression data on molecular targets with available clinical endpoints.

Available Tissue Microarrays. Constructing an optimally useful TMA is very time consuming and expensive. Therefore, such a process may not be feasible for all laboratories. In this circumstance several companies (i.e., ISU ABXIS AccuMax Array, IMGENEX, NIH/NCI Tissue Array Research Program) have developed commercially available TMAs consisting of various tissue types and core sizes for research use. As a cautionary note, however, very few commercially or institute-available TMAs are linked to a rich data source, thus often limiting their overall utility.

TISSUE MICROARRAY SECTIONING

Sectioning of TMAs can sometimes be more challenging than cutting full tissue blocks. During the cutting process, standard histological sections are floated on a water bath and picked up individually with a slide. However, several groups have found the adhesive-tape based transfer system (Instrumedics Incorporation, Hackensack, NJ) a more reliable method for TMA sections, which minimizes the overall tissue core loss (Rimm *et al*., 2001). The process consists of placing adhesive tape on the face of the tissue section prior to cutting and subsequently the tape and section are removed and positioned on an adhesive-coated slide. The section is then ultraviolet crosslinked to the slide followed by the removal of the tape using a degreasing agent. It is important to ensure the presence and morphology of the tissue cores throughout the sectioning process; therefore, a hematoxylin and eosin staining should be performed after obtaining 50–100 sections. During conventional sectioning, a percentage of the tissue is lost due to "facing" the block each time it is cut. In order to avoid this problem during sectioning of TMAs, multiple sections can be cut at one time and stored for later use. This has generated some concern as to whether the same staining conditions can be achieved after an array has been stored for several weeks or months. A number of groups have reported decreased antigen retrieval capabilities in whole tissue sections stored for less than 1 week prior to staining (Jacobs *et al*., 1996; Bertheau *et al*., 1998). In addition, Fergenbaum *et al*. (2004) showed a reduction in the percentage of immunoreactive cells in a breast cancer TMA stored for 6 months compared to a freshly cut TMA (Fergenbaum *et al*., 2004). In general, TMA sections cut and immunostained on the same day provide the best overall results in terms of degree and intensity of staining. However, Rimm *et al*. (2001) illustrated that recoating array slides with paraffin after sectioning prevents the loss of antigenicity and preserves the slides for up to several months by protecting the tissues from oxidation.

LINKING TISSUE TO A PATIENT AND PATHOLOGY DATABASE

The maximum utility of a TMA is realized when the tissue spots are linked to a rich database of clinical, pathological, and medical history information.

Commercially available software can be used to create such databases. Examples include spreadsheets, such as Microsoft Excel, to more sophisticated relational databases such as Microsoft Access and Filemaker Pro. Other more difficult software such as Oracle can also be used. Microsoft Excel is the most commonly used database system that can link immunohistochemical staining data with patient information to determine the clinical implications of individual biomarkers. During the production of the database, it is critical that the information spreadsheets coincide with the layout of the TMA to ensure proper documentation of the data. That is to say, it must be easy to link tissue to specific database patient information. The information obtained from the staining process is associated with a database that contains relevant patient information corresponding to clinical outcomes. Included in this database is information on the histopathological classifications of the tumor specimens embedded in the TMA. Such classifications include tumor grade, histologic tumor type, tumor size, and lymph node stage (Abd El-Rehim *et al*., 2005). In addition, other clinicopathological variables such as hormone receptor and gene amplification status should be recorded, if available. As an example, it is optimal for a breast cancer TMA to include patient information regarding the status of the estrogen receptor, progesterone receptor, and *HER-2/neu* when available. Another important patient response variable to be incorporated in the database is response to treatment regimens, such as Tamoxifen, Herceptin, and Aromatase inhibitors for breast cancer patients. In order to examine the association between gene expression data and the prognosis of disease, the database needs to contain patient information on tumor recurrence, disease-free survival, and overall survival.

PROTOCOL FOR MARKER ANALYSIS

TMAs are designed to assay DNA, RNA, and protein target expression in a large collection of tissue samples under identical conditions (Figure 17.2). The analysis of DNA and RNA can be performed, for

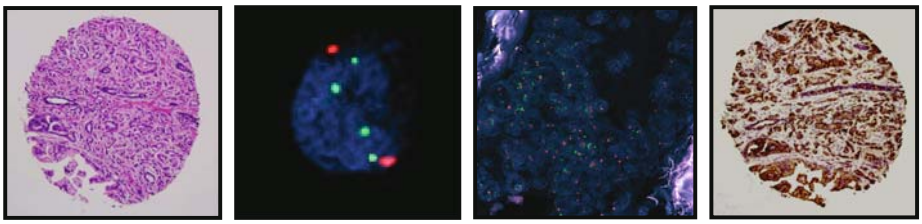

FIGURE 17.2. Various staining methods. A hematoxylin and eosin stain of a TMA will allow one to visualize the morphology of individual tissue cores. TMA's can also be assayed for DNA, RNA, and protein expression. Fluorescent *in situ* hybridization (FISH) is used to identify gene amplifications and DNA rearrangements at the cell level, where mRNA *in situ* hybridization (mRNA ISH) is ideal for detecting RNA transcripts among tissues. Immunohistochemistry (IHC) analysis is the most common and can detect protein expression in various tissue samples

example, by FISH and mRNA *in situ* hybridization (mRNA ISH), respectively. Protein expression is typically determined using immunohistochemistry (IHC), which is generally the most common application for examining expression of molecular markers in a TMA. For detection of protein expression using the IHC application, direct or indirect fluorescence or chemical enzyme-substrate colorimetric detection methods can be applied (Schoenberg Fejzo and Slamon, 2001; Fergenbaum *et al*., 2004). Presented here is a representative method for indirect immunoperoxidase IHC that is often used in our laboratory; however, protocols can vary greatly depending on the specific antibody, tissue types, and/or antigens. The IHC staining protocol for TMAs is similar to the conventional staining method for whole tissue sections and a complete ABC-Elite staining kit (Vector Laboratories, Burlingame, CA) can assist in the staining process.

Sample Immunohistochemistry Protocol

- Heat TMA sections at 56°C for 30 min.
- Incubate sections in xylene overnight and continue with deparaffinization and rehydration steps the next day (xylenes 3×5 min, 100% ethanol 2×5 min, 90% ethanol 1×5 min, 70% ethanol 1×5 min, ddH_2O 1×5 min).
- Rinse in PBS 1× 5 min.
- Submerge the TMA slide in 0.01 M sodium citrate buffer (pH 6.0) for 10 min at 95°C. (Note: The particular antigen of interest will dictate whether this step is required and, if so, which method is most appropriate.)
- Rinse in PBS 1× 5 min, then dH_2O 1× 5 min.
- Quench endogenous peroxidase activity using 3% hydrogen peroxide in methanol for 30 min.
- Rinse in dH_2O 1 × 5 min, then PBS 1× 5 min.
- Block with normal serum at room temperature for 20 min.
- Block with Avidin D solution for 20 min, rinse in PBS 1 × 5 min, then Biotin solution for 20 min, rinse in PBS 1× 5 min.
- Incubate with an unlabeled primary antibody for 60 min at room temperature.
- Rinse in PBS 3 × 5 min.
- Add a corresponding biotin-labeled secondary antibody for 30 min at room temperature.
- Rinse in PBS 3× 5 min.
- Add the avidin:biotinylated enzyme complex (ABC) to the slide and incubate for 30 min at room temperature.
- Rinse in PBS 3× 5 min.
- Develop with an enzyme substrate (i.e., diaminobenzidine, DAB) in order to detect the tissue antigen. Let the reaction continue long enough to get maximal signal to noise ratio. Stop the reaction by submerging slide in dH_2O.
- Counterstain with hematoxylin for ~ 1 min.
- Dehydrate through graded ethanol and xylenes (70% ethanol 1× 5 min, 90% ethanol 1× 5 min, 100% ethanol 1×5 min, xylenes 1× 5 min).
- Coverslip the slide with mounting medium.
- The resultant staining is evaluated by a trained pathologist or an automated program (discussed further in the next section). Issues of background staining versus true staining and edge effects are also reviewed.

FISH analysis is ideally suited for detection of gene amplification and DNA rearrangements at the individual cell level (Kallioniemi *et al*., 1992). In order to achieve the most accurate staining results, uniform fixation of the paraffin-embedded archived tissue blocks is preferable. Studies

performed by Kononen *et al*. (1998) showed that more accurate and reliable results were achieved when using ethanol-fixed tissue for FISH analysis, but formalin-fixed tissue was still able to produce adequate results. When FISH is used in combination with the TMA technology, it is a high-throughput method that can easily determine the amplification frequencies of several genes in a short period of time. This was demonstrated by Kononen *et al*. (1998) using 372 ethanol-fixed primary breast cancer specimens arrayed in a TMA, which were analyzed for the amplification of three main oncogenes involved in breast cancer: *HER-2, MYC*, and *CCND1*.

In order to detect RNA transcripts among tissue samples in a TMA, the mRNA ISH technique is applied (Schoenberg Fejzo and Slamon, 2001; Speel *et al*., 2006); yet, this analysis is used less frequently than FISH and IHC, and the question of whether RNA stays intact and is accessible following formalin fixation and tissue processing, is always an issue. Detailed protocols for IHC, FISH, and mRNA ISH can also be found in the "Theory and Practice of Histological Techniques", 1996 (Bancroft and Stevens, 1996).

TISSUE MICROARRAYS PREPARED FROM FROZEN TISSUE

It has been suggested that the conventional TMAs comprised of paraffin-embedded tissue cores encounter limitations during the detection of RNA and specific proteins (Hoos and Cordon-Cardo, 2001; Schoenberg Fejzo and Slamon, 2001). The fixation and paraffin-embedding processes can lead to mRNA degradation and antigenic alterations in proteins. To overcome this problem, frozen tumor tissue microarrays have been produced by making slight modifications of the standard TMA technology. More specifically, fresh tissue is frozen and embedded in an OCT compound as a 'donor' block and then the individual samples are arrayed into a recipient OCT block. Although frozen tissue microarrays provide a means to overcome the limitations of the paraffin-embedded TMAs, the large number of available archived paraffin-embedded 'donor' blocks will continue to make the paraffin-embedded TMA a more popular technology to employ.

QUANTIFYING BIOMARKER EXPRESSION

Following staining of a TMA, the signal must be quantified. There are a number of ways to accomplish this, but none of which is without flaws. Below we describe common approaches to scoring.

Manual Scoring. To quantify the amount of protein in a given tissue sample, the intensity and frequency of staining are typically scored. Other parameters to consider include whether the staining is cellular or extracellular (i.e., in the stroma or endothelial cells), where the staining is localized with respect to different cellular compartments (e.g., membrane, cytoplasmic, or nuclear), and what types of cells exhibit staining (e.g., epithelial or myoepithelial). For most heterogeneous tissues or tumors, analysis by a pathologist gives the most reliable results and is often required when publishing a study. Scoring is typically done visually by the pathologist who will view each spot on the array under a brightfield microscope with a 20X or 40X objective.

Although there are several different methods and approaches to scoring an array, the following represents one approach. The intensity of expression is evaluated on a 0–3 scale, where 0 is below the level of detection and 3 represents strong expression. The frequency is defined as the percentage of the cells of interest that show a particular intensity of staining. After reviewing each spot, the pathologist will tabulate the results on a spreadsheet (Figure 17.3).

In addition, the pathologist should also note cytological features for each spot such as the histology, tumor architecture, and nuclear grade. Because there may be several histologies present in a given spot, the pathologist can either score each type separately or score only the cells from the histology of interest (for instance, ignoring normal ductal epithelia adjacent to an invasive tumor). The pathologist should also be aware of any staining artifacts such as an overly thick section or 'edge effects' and evaluate accordingly. The ability to intelligently discern these morphologic features and artifacts and score only the regions of interest is a key advantage of scoring manually. The main disadvantage of manual scoring lies in its inherently semi-quantitative nature. Because scoring is done by eye, the visually determined score of each spot may vary from one pathologist to another or even from one session to another. This will at the very least introduce some amount of noise to the data, and at worst lead to biased data and false findings.

Automated Scoring. In response to the inherent disadvantages of manual scoring, several companies currently offer semi-automated image analysis platforms. Examples include platforms offered by Aperio Technologies, Inc. (Vista, CA) and

Case No.	Histology	Grade	% Int 3	% Int 2	% Int 1	% Int 0	Max	Pos	IntMax
C000-001	IDC	2	10	0	0	90	3	10	0.3
C000-001	IDC	3	20	30	50	0	3	100	1.7
C000-001	IDC	3	50	10	0	40	3	60	1.7
C000-001	IDC	2	0	20	40	40	2	60	0.8
C000-002	Normal	0	0	0	0	100	0	0	0
C000-003	LNMET	3	100	0	0	0	3	100	3
C000-003	LNMET	3	100	0	0	0	3	100	3
					C000-001:	*max:*	3	100	1.7
						mean:	2.75	57.5	1.125
						median:	3	60	1.25
						min:	2	10	0.3

FIGURE 17.3. Typical raw scoring sheet and pooling. An example of the raw scoring data is given in the left side of the figure. The spot-level pooled data are generated from the raw scoring data (shaded in light gray) and include Positivity (Pos), Maximum Intensity (Max), and Integrated Intensity (IntMax). All the spots that belong to each case are pooled (dark-gray) to yield case-level pooled data. Abbreviations: IDC, invasive ductal carcinoma; LNMET, lymph node metastasis

HistoRx Inc. (New Haven, CT). Aperio's ScanScope can produce color scans of any slide with a resolution of up to 0.25 µm/pixel (1.57 million pixels per inch) using a 40X objective. The resulting digital slide can be viewed using ImageScope software, or each spot on the TMA slide can be digitally indexed and broken up into discrete spots using TMALab. A mouse or pen tool can be used to manually encircle regions of interest, and various algorithms can be run on these regions. Such algorithms include Positive Pixel Count (an algorithm that measures the area and intensity for a given color, such as brown for DAB) and IHC Nuclear (an algorithm that recognizes nuclei and analyzes only the nuclear staining for each cell). Because a digital scan is taken from each slide, any subsequent analyses on the same scan will be highly consistent with the original (given the same algorithm and parameters) and will be fully quantitative, eliminating any biases. However, in the absence of any intelligent morphometric analysis software that can discern cancerous cells from adjacent normal cells, a pathologist typically still needs to review each spot individually and encircle the regions of interest. There are several studies that have used the Aperio Scanscope System for TMA visualization and/or biomarker expression quantification (Krajewska *et al*., 2007; Stewart *et al*., 2007; Yang *et al*., 2007).

HistoRx's AQUA Analysis platform uses fluorescent tags to define cells or subcellular compartments of interest, for which a protein marker can be quantified (Camp *et al*., 2002). For example, the nuclei can be defined by DAPI staining, epithelial cells defined by pan-cytokeratin, and epithelial membranes outlined by E-cadherin. Measurements of immunoreactivity can be confined only to the cells or subcellular compartments of interest. For example, the nuclear protein expression can be calculated by measuring the fluorescence intensity of a given biomarker only in the areas where DAPI expression is also observed. Fluorescence microscopy using multiple antibodies has the potential for much cleaner, quantitative data than any other technology (Giltnane and Rimm, 2004). However, morophological features often still need to be assessed by a pathologist. Furthermore, the AQUA system currently lacks any method for manually encircling regions of interest, which may introduce noise by including other cells of different, unwanted histopathologies in the analysis. Several studies have been published using the AQUA Analysis platform (Dolled-Filhart *et al*., 2006; Giltnane *et al*., 2006).

DATA ANALYSES: SPOT LEVEL AND POOLED DATA

Spot-Level Analyses. Once each spot on the TMA has been evaluated for its protein expression, several statistical tests can be performed to test its association with any other variable. In 'spot-level' analyses, each spot can be analyzed individually, with respect to its histological type or nuclear grade. In case-level analyses, all the spots belonging to a particular surgical event can be grouped together and tested for association with clinico-pathological variables or clinical outcome of that patient.

Pooled Data. A surgical event in which, for example, breast tissue is removed from an individual patient, is defined as a case. Because a patient may have successive surgeries (e.g., re-excision if the margins were positive for tumor) a patient may conceivably

have more than one case. Moreover, as discussed above, when building the TMA, typically several cores are taken from the 'donor' tissue block to better represent heterogeneity of expression. One patient may have several cases (with one case corresponding to the primary surgical treatment), and each case may have several spots on the TMA. Therefore, in order to relate the many spots that belong to any one given patient, a process called "pooling" is usually employed (Figure 17.3).

For each case, there are three levels of scoring data. At the simplest level are the raw scoring data that the pathologist records, as described above. If scoring is done manually, each spot will have four numbers, each describing the percentage of cells staining at each intensity level (0, 1, 2, and 3). The next level of scoring data are the spot-level pooled data, where the four numbers for each spot are condensed to a single, intuitive number. For example, "Maximum" is the highest intensity present in a given spot, "Positivity" is the percent of cells staining positive at any intensity, and "Integrated Measure" is an integrated measure of expression given by the following formula: [3(%x) + 2(%y) + 1(%z)]/100 where x, y, and z represent the percentage of cells staining at intensity 3, 2 and 1, respectively. The highest, most processed level of scoring data is the case-level pooled data. Because there are several spots for each case, all the spots per given case must be pooled together so that each case has one expression value. This may be done taking the average of all the spot-level values that belong to each case. Ultimately, each case may be represented by the mean Positivity of all the spots that belong to that case. Once a TMA has been scored and pooled for a given biomarker, the case-level pooled data is connected with clinical, pathological and outcome data for statistical analysis as described above.

STATISTICAL TOOLS

There are several statistical tests frequently used for TMA analysis that can handle the challenging characteristics of biomarker data (Table 17.1). Given that the scoring is typically done by a pathologist who qualitatively assigns staining intensity, measurements are done on a non-ordinal scale (i.e., the difference in intensity between 3 and 2 may not be the same as that between 2 and 1 in terms of the actual amount of protein). Furthermore, because certain proteins are over-, under- or sporadically expressed in tumors, data sets tend to be highly skewed (i.e., the histogram of scoring data does not follow a bell-shaped Gaussian curve, but is highly shifted to one side, Figure 17.4). For these reasons, nonparametric statistical tools are used, because they make no assumptions regarding the distribution or nature of the data. The following will briefly cover the basic statistical tools used in TMA biomarker analysis. A summary of statistics used to compare biomarker expression with clinico-pathological variables and outcome is given in Table 17.2. A more in depth report of statistical methods has been previously published (Liu *et al.*, 2004).

Basic nonparametric tests fall into two main categories: tests to determine whether there is a difference between groups, and tests to determine whether there is a relationship between two variables. To test whether there is a difference between two independent groups (e.g., whether the protein expression in normal ductal

TABLE 17.1. Nonparametric statistical tools. Different nonparametric statistical used that have been used for TMA biomarker studies are shown, along with their input and function.

Question answered	Type of data	Statistical test
Are these two groups different?	Continuous	Mann-Whitney U
Is there a difference in these 3+ groups?	Continuous	Kruskal Wallis
Are these two dependent groups different?	Continuous	Wilcoxon
Are these two variables associated?	Continuous	Spearman Correlation
Are these two variables associated?	Categorical	Chi-Square or Fisher's Exact
Is there a survival difference in these two groups?	Categorical	Kaplan-Meier and Log-Rank
Is this variable significantly correlated with survival?	Continuous	Univariate Cox Model
Is this variable significantly correlated with survival after correction for other prognostic covariates?	Continuous or Categorical	Multivariate Cox Model

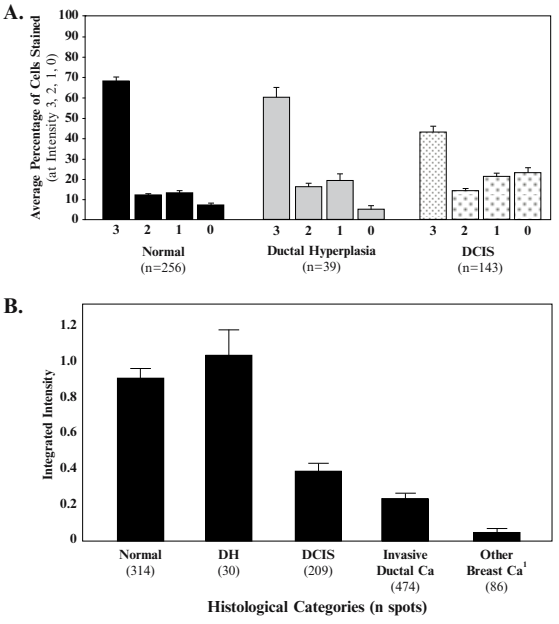

FIGURE 17.4. Annexin A1 expression using a breast TMA. (**A**) *Annexin A1 expression is highly skewed*. The histogram of the maximum intensity of Annexin A1 immunoreactivity is given for three ductal histologies: normal, hyperplasia without atypia, and carcinoma *in situ*. (**B**) *Annexin A1 expression in breast glandular epithelial cells*. The integrated intensity for different histopathologies is given. The bar graph indicates the mean, and the error bars represent one standard error of the mean.
[1]Other breast cancers include invasive lobular carcinoma, mucinous colloid carcinoma, mixed invasive (ductal-tubular or ductal-lobular) carcinoma, invasive tubular carcinoma, and medullary carcinoma)

epithelium is significantly different from that seen in invasive ductal carcinoma), the Mann-Whitney U-test is typically used. To extend the test to more than two groups (e.g., to test whether there is a difference among all ductal histologies), the Kruskal-Wallis test is used.

In order to test whether there is a relationship between two variables, there are three main tests that can be used, depending on the data type. For two continuous variables (e.g., "positivity", tumor size or age at diagnosis), a Spearman correlation can be determined. If the two variables are categorical (e.g., high vs. low biomarker expression, high or low stage, or ER positive or negative), the Chi-square test can be used. If the two categorical variables are both binary, the Fisher exact test is preferred. If one variable is continuous while the other is categorical, then the continuous

TABLE 17.2. Clinico-pathological associations. The statistical tools needed to calculate whether a biomarker is statistically associated with typical clinico-pathological variables are shown.

Variable	Biomarker is continuous	Biomarker is dichotomized
Age at diagnosis		
Years (continuous)	Spearman correlation	Mann-Whitney U
Stage		
I&II vs. III&IV (Categorical)	Mann-Whitney U	Fisher's Exact
I, II, III, IV (Categorical)	Kruskal Wallis	Chi-square
1, 2, 3, 4 (Continuous)	Spearman Correlation	Kruskal Wallis
Grade		
I&II vs. III (Categorical)	Mann-Whitney U	Fisher's Exact
I, II, III (Categorical)	Kruskal Wallis	Chi-square
1, 2, 3 (Continuous)	Spearman Correlation	Kruskal Wallis
Lymph node status		
Positive vs. Negative (Categorical)	Mann-Whitney U	Fisher's Exact
Tumor size		
Centimeters (Continuous)	Spearman Correlation	Mann-Whitney U
T1, T2, T3 (Categorical)	Kruskal Wallis	Chi-square
Lymphovascular Invasion		
Present vs. Absent (Categorical)	Mann-Whitney U	Fisher's Exact
Estrogen Receptor Status		
Positive vs. Negative (Categorical)	Mann-Whitney U	Fisher's Exact
Survival		
Time in years until event	Univariate Cox Model	Kaplan-Meier and Log-Rank
After correction for covariates	Multivariate Cox Model	Multivariate Cox Model

data from each categorical type can be considered independent groups, and the Mann-Whitney or Kruskal-Wallis test can be used.

With spot-level data, one can test whether biomarker expression is correlated with progression to disease by comparing the protein expression in each spot against the histology and nuclear grade of that spot. To compare the expression in normal tissues against invasive tissues, the Mann-Whitney U test can be used. To extend the test to all histopathologies, the Kruskal-Wallis test can be used. The protein expression can also be considered with respect to nuclear grade of each spot using the Kruskal-Wallis test (using protein level as a continuous variable and grade as a categorical variable).

After case-level pooling, biomarker expression can be compared with the clinico-pathological variables and outcome data of that case. To test whether biomarker expression is associated with a categorical clinico-pathological variable (e.g., estrogen receptor status, nodal status or TNM stage), the Mann-Whitney or Kruskal-Wallis test can be used. If the clinico-pathological variable is continuous (e.g., age at diagnosis or tumor size), the Spearman correlation can be used.

Sometimes it may be convenient to dichotomize the biomarker expression at some natural cutpoint such as the median or at a quartile, and convert protein expression into a categorical variable, as high or low in expression. The Chi-square or Fisher exact test can be used to compare the dichotomized protein expression with categorical clinico-pathological variables,

and the Mann-Whitney U test can be used if the variable being compared against is continuous.

Dichotomizing the biomarker expression at a cutpoint is especially useful in visualizing the survival differences in the two groups. This difference in survival (as defined as time to any defined event) of a dichotomized patient cohort can be visualized on a Kaplan-Meier curve. The statistical significance of the difference in survival between the two groups is evaluated using the log-rank test. To test whether biomarker expression as a continuous variable is correlated with survival, a Cox proportional hazard model may be used. By adding other known prognostic variables (such as stage, grade and nodal status) to the Cox model, one can evaluate whether the variable remains significant even after correcting for the prognostic information contained in the other variables.

Biomarkers can be scored and analyzed independently, or they can be analyzed together in a multivariate setting. A common method of multimarker analysis is hierarchical clustering. Hierarchical clustering works by calculating a similarity matrix, determined by calculating the Spearman correlation coefficient between all pairs of variables. The two most similar cases (with the highest correlation coefficient) are merged together to form one case, resulting in one less case. The similarity matrix is recalculated and the clustering process repeated, until all cases are clustered into a single cluster. This process can be visualized on a dendogram, with the most similar cases clustered most closely with each other. The dendogram may be divided into two or more natural groupings. The clusters of cases that are most similar to each other based on their protein expression can result in prognostically distinct groups, in terms of disease-free survival, recurrence, or response to treatment therapies. Moreover, by studying the clustering of markers (i.e., the biomarkers are the input variables instead of cases), multimarker analyses can reveal novel protein interactions and pathways in tumorigenesis, allowing better treatment strategies through tailored drug combinations.

DISEASE PROGRESSION STUDY

As discussed previously, there are three main ways biomarker TMA studies can be used to reveal novel associations in breast cancer, and an example of each is given below. Using spot-level data, studies can reveal whether a biomarker is correlated with cancer progression by comparing its expression against different histopathologies. Using case-level data, clinico-pathological variables, and outcome data, studies can reveal whether a biomarker is prognostically significant and if it can predict patient outcome. By using several biomarkers at once, multivariate analysis can reveal distinct patient groups based on their molecular expression patterns, which prove to be predictive of their survival.

Annexin A1 Study. Our study on annexin A1 (Shen *et al*., 2006) is one example of how TMA analysis can reveal how the distribution of a biomarker expression varies with cancer progression. Annexin A1 is a calcium- and phospholipid-binding protein that was found to be downregulated in many cancers. To study the expressional pattern of Annexin A1 in breast tissues, we

used a high-density TMA containing 242 surgical cases of 210 patients that underwent surgery at the UCLA Medical Center from 1995 to 2000.

The integrated value of annexin A1 expression was compared across various human breast histopathologies (Figure 17.4). To determine whether the level of expression in ductal carcinoma *in situ* (DCIS), invasive ductal carcinoma (IDC) or lymph-node metastasis were significantly lower than that of normal tissues, we used the Mann-Whitney test to compare each malignancy with normal tissues in a pairwise fashion. As shown, annexin A1 expression was significantly lower in DCIS and IDC cells as compared to normal ductal epithelial cells ($P < 0.0001$).

Furthermore, we found that the expression of tissues from lymph node metastasis was higher than in primary tumor cells, even after correction for nuclear grade. We used a pairwise comparison of primary against metastatic tumors of the same nuclear grade from the same patient. The data were graphed both on a bivarate scattergram as well as in a cell bar chart. The Wilcoxon signed rank test showed that there was a statistically significant difference in expression ($P = 0.043$).

In conclusion, a decrease in annexin A1 was found in DCIS and IDC cells, suggesting that loss of annexin A1 might be an early event in breast cancer development. Because we found that decreasing annexin A1 is correlated with tumor progression, our study is consistent with findings from other tissue models and suggests that annexin A1 may function as a tumor suppressor gene in breast.

GATA-3 Study. There are several studies that have investigated the value of expression data as a prognosticator for disease. One example of such a study is the TMA analysis of GATA-3 on breast tissues (Mehra *et al*., 2005). A TMA containing 139 cases of invasive breast carcinomas treated at the University of Michigan Medical Center was used for this study.

The disease-free survival of patients split by GATA-3 expression were visualized on a Kaplan-Meier plot and the difference between the survival curves was evaluated using the log-rank test ($P = 0.005$). Low GATA-3 levels were associated with a lower probability of survival, as well as larger tumor size (Wilcoxon test, $P = 0.03$), presence of lymph node metastasis (Kruskal-Wallis test, $P = 0.0002$), higher histologic grade (Kruskal-Wallis test, $P < 0.0001$), negative ER (Wilcoxon test, $P < 0.0001$), negative PR (Wilcoxon test, $P < 0.0001$), and HER-2/neu overexpresion (Wilcoxon test, $P = 0.03$).

To evaluate whether GATA-3 expression was an independent predictor of outcome, adding new prognostic information above and beyond that provided by conventional clinico-pathological variables, a multivariate Cox model of disease-specific survival was used. GATA-3 was found to be significant with a P-value of 0.05, a Hazard Ratio of 0.12 and a 95% confidence interval of 0.01–1.01. Low GATA-3 expression was predictive of disease-specific survival in lymph-node negative ($P = 0.02$, log-rank test) and ER-positive ($P = 0.04$, log-rank test) subgroups. By TMA analysis, Mehra *et al*. (2005) were able to show that GATA-3 is a prognostic biomarker in breast cancer that may be used to identify patients with a more aggressive phenotype, especially in ER-positive, node-negative patients.

MULTIMARKER STUDY

Beyond single-marker analysis, several biomarkers can be analyzed together in a multi-marker setting, analogous to cDNA gene expression profiling. The analysis of combinations of expression from multiple markers can sometimes prove more beneficial than single marker staining when predicting prognosis or determining a treatment regimen. For example, Rhodes *et al*. (2003) studied multiple biomarker candidates using a prostate cancer TMA, and Makretsov *et al*. (2004), examined numerous biomarkers on a breast TMA.

As one might expect, multimarker studies present an additional level of complexity over single marker studies. The description of current and future approaches to analyze such data is beyond the scope of this chapter. However, one currently used method is hierarchical clustering of biomarkers to identify distinct groups based on their association. An example of how hierarchical clustering can lead to a protein profile with significant prognostic power, is a TMA study of 438 cases of invasive breast cancer (Makretsov *et al*., 2004). By using immunoreactivity data on a set of 19 biomarkers that had a p-value < 0.2 in a univariate log-rank test, a simplified set of 11 biomarkers was created that could replicate the same clusters with high agreement. These clusters were highly significant in their overall and disease-specific survival, and were independent of nodal status and tumor size. By using clustering analysis of biomarker data, combinations of prognostic markers can be used together to yield better patient prognosis and treatment guidelines than with single markers or traditional clinicopathological variables.

In conclusion, by being able to array hundreds of different tissue samples on a single slide, TMA technology has created a high throughput technique to address the disadvantages of analyzing individual slides, while maintaining a high agreement with other conventional methods. Combining TMA protein expression data with patient demographics, clinico-pathological and outcome information has created an excellent platform for the analysis and validation of biomarkers. Moreover, multimarker analyses can lead to novel patient groups based on prognosis or treatment response, or can lead to the discovery of novel gene interactions and pathways in cancer progression. With the improvement of automated image analysis software, a TMA study may be completed in a rapid and consistent manner, while new methodologies in biostatistics and data mining may lead to exciting discoveries in cancer etiology.

REFERENCES

Abd El-Rehim, D.M., Ball, G., Pinder, S.E., Rakha, E., Paish, C., Robertson, J.F., Macmillan, D., Blamey, R.W., and Ellis, I.O. 2005. High-throughput protein expression analysis using tissue microarray technology of a large well-characterised series identifies biologically distinct classes of breast cancer confirming recent cDNA expression analyses. *Int. J. Cancer 116*: 340–350.

Bancroft, J.D., and Stevens, A. 1996. *Theory and Practice of Histological Techniques*, Churchill Livingstone.

Battifora, H. 1986. The multitumor (sausage) tissue block: novel method for immunohistochemical antibody testing. *Lab. Invest. 55*: 244–248.

Bertheau, P., Cazals-Hatem, D., Meignin, V., de Roquancourt, A., Verola, O., Lesourd, A., Sene, C., Brocheriou, C., and Janin, A. 1998. Variability of immunohistochemical reactivity

on stored paraffin slides. *J. Clin. Pathol. 51*: 370–374.

Callagy, G., Cattaneo, E., Daigo, Y., Happerfield, L., Bobrow, L.G., Pharoah, P.D., and Caldas, C. 2003. Molecular classification of breast carcinomas using tissue microarrays. *Diagn. Mol. Pathol. 12*: 27–34.

Camp, R.L., Charette, L.A., and Rimm, D.L. 2000. Validation of tissue microarray technology in breast carcinoma. *Lab. Invest. 80*: 1943–1949.

Camp, R.L., Chung, G.G, and Rimm, D.L. 2002. Automated subcellular localization and quantification of protein expression in tissue microarrays. *Nat. Med. 8*: 1323–1327.

Corzo, C., Corominas, J.M., Tusquets, I., Salido, M., Bellet, M., Fabregat, X., Serrano, S., and Sole, F. 2006. The MYC oncogene in breast cancer progression: from benign epithelium to invasive carcinoma. *Cancer Genet. Cytogenet. 165*: 151–156.

DeRisi, J., Penland, L., Brown, P.O., Bittner, M.L., Meltzer, P.S., Ray, M., Chen, Y., Su, Y.A., and Trent, J.M. 1996. Use of a cDNA microarray to analyse gene expression patterns in human cancer. *Nat. Genet. 14*: 457–460.

Dolled-Filhart, M., McCabe, A., Giltnane, J., Cregger, M., Camp, R.L., and Rimm, D.L. 2006. Quantitative in situ analysis of beta-catenin expression in breast cancer shows decreased expression is associated with poor outcome. *Cancer Res. 66*: 5487–5494.

Fergenbaum, J.H., Garcia-Closas, M., Hewitt, S.M., Lissowska, J., Sakoda, L.C., and Sherman, M.E. 2004. Loss of antigenicity in stored sections of breast cancer tissue microarrays. *Cancer Epidemiol. Biomark. Prev. 13*: 667–672.

Gillett, C.E., Springall, R.J., Barnes, D.M., and Hanby, A.M. 2000. Multiple tissue core arrays in histopathology research: a validation study. *J. Pathol. 192*: 549–553.

Giltnane, J.M., and Rimm, D.L. 2004. Technology insight: identification of biomarkers with tissue microarray technology. *Nat. Clin. Pract. Oncol. 1*: 104–111.

Giltnane, J.M., Murren, J.R., Rimm D.L., and King, B.L. 2006. AQUA and FISH analysis of HER-2/neu expression and amplification in a small cell lung carcinoma tissue microarray. *Histopathology 49*: 161–159.

Hoos, A., and Cordon-Cardo, C. 2001. Tissue microarray profiling of cancer specimens and cell lines: opportunities and limitations. *Lab. Invest. 81*: 1331–1338.

Hoos, A., Urist, M.J., Stojadinovic, A., Mastorides, S., Dudas, M.E., Leung, D.H., Kuo, D., Brennan, M.D., Lewis, J.J., and Cordon-Cardo, C. 2001. Validation of tissue microarrays for immunohistochemical profiling of cancer specimens using the example of human fibroblastic tumors. *Am. J. Pathol. 158*: 1245–1251.

Jacobs, T.W., Prioleau, J.E., Stillman, I.E., and Schnitt, S.J. 1996. Loss of tumor marker-immunostaining intensity on stored paraffin slides of breast cancer. *J. Natl. Cancer Inst. 88*: 1054–1059.

Kallioniemi, O.P., Kallioniemi, A., Kurisu, W., Thor, A., Chen, L.C., Smith, H.S., Waldman, F.M., Pinkel, D., and Gray, J.W. 1992. ERBB2 amplification in breast cancer analyzed by fluorescence in situ hybridization. *Proc. Natl. Acad. Sci. U S A 89*: 5321–5325.

Kononen, J., Bubendorf, L., Kallioniemi, A., Barlund, M., Schraml, P., Leighton, S., Torhorst, J., Mihatsch, M.J., Sauter, G., and Kallioniemi, O.P. 1998. Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat. Med. 4*: 844–847.

Krajewska, M., Olson, A.H., Mercola, D., Reed, J.C., and Krajewski, S. 2007. Claudin-1 immunohistochemistry for distinguishing malignant from benign epithelial lesions of prostate. *Prostate 67*: 907–910.

Liu, X., Minin, V., Huang, Y., Seligson, D.B., and Horvath, S. 2004. Statistical methods for analyzing tissue microarray data. *J. Biopharm. Stat. 14*: 671–685.

Makretsov, N. A., Huntsman, D.G., Nielsen, T.O., Yorida, E., Peacock, M., Cheang, M.C., Dunn, S.E., Hayes, M., van de Rijn, M., Bajdik, C., and Gilks, C.B. 2004. Hierarchical clustering analysis of tissue microarray immunostaining data identifies prognostically significant groups of breast carcinoma. *Clin. Cancer Res. 10*: 6143–6151.

Mehra, R., Varambally, S., Ding, L., Shen, R., Sabel, M.S., Ghosh, D., Chinnaiyan, A.M., and Kleer, C.G. 2005. Identification of GATA3 as a breast cancer prognostic marker by global gene expression meta-analysis. *Cancer Res. 65*: 11259–11264.

Nocito, A., Kononen, J., Kallioniemi, O.P., and Sauter, G. 2001. Tissue microarrays (TMAs) for high-throughput molecular pathology research. *Int. J. Cancer 94*: 1–5.

Rhodes, D.R., Sanda, M.G., Otte, A.P., Chinnaiyan, A.M., and Rubin, M.A. 2003. Multiplex biomarker approach for determining risk of prostate-specific antigen-defined recurrence of prostate cancer. *J. Natl. Cancer Inst. 95*: 661–668.

Rimm, D.L., Camp, R.L., Charette, L.A., Olsen, D.A., and Provost, E. 2001. Amplification of tissue by construction of tissue microarrays. *Exp. Mol. Pathol. 70*: 255–264.

Rubin, M., Dunn, A.R., Strawderman, M., and Pienta, K.J. 2002. Tissue microarray sampling strategy for prostate cancer biomarker analysis. *Am. J. Surg. Pathol. 26*: 312–319.

Schoenberg Fejzo, M., and Slamon, D.J. 2001. Frozen tumor tissue microarray technology for analysis of tumor RNA, DNA, and proteins. *Am. J. Pathol. 159*: 1645–1650.

Schraml, P., Kononen, J., Bubendorf, L., Moch, H., Bissig, H., Nocito, A., Mihatsch, M.J., Kallioniemi, O.P., and Sauter, G. 1999. Tissue microarrays for gene amplification surveys in many different tumor types. *Clin. Cancer. Res. 5*: 1966–1975.

Shen, D., Nooraie, F., Elshimali, Y., Lonsberry, V., He, J., Bose, S., Chia, D., Seligson, D., Chang, H.R., and Goodglick, L. 2006. Decreased expression of annexin A1 is correlated with breast cancer development and progression as determined by a tissue microarray analysis. *Hum. Pathol. 37*: 1583–1591.

Speel, E.J., Hopman, A.H., and Komminoth, P. 2006. Tyramide signal amplification for DNA and mRNA in situ hybridization. *Methods Mol. Biol. 326*: 33–60.

Stewart, J., Fleshner, N., Cole, H., and Sweet, J. 2007. Comparison of annexin II, p63 and alpha-methylacyl-CoA racemase immunoreactivity in prostatic tissue: a tissue microarray study. *J. Clin. Pathol. 60*: 773–780.

Subramanian, G., Adams, M.D., Venter, J.C., and Broder, S. 2001. Implications of the human genome for understanding human biology and medicine. *JAMA 286*: 2296–2307.

Torhorst, J., Bucher, C., Kononen, J., Haas, P., Zuber, M., Kochli, O.R., Mross, R., Dieterich, H., Moch, H., Mihatsch, M., Kallioniemi, O.P., and Sauter, G. 2001. Tissue microarrays for rapid linking of molecular changes to clinical endpoints. *Am. J. Pathol. 159*: 2249–2256.

Venter, J.C. *et al*. 2001. The sequence of the human genome. *Science 291*: 1304–1351.

Yang, X.R., Charette, L.A., Garcia-Closas, M., Lissowska, J., Paal, E., Sidawy, M., Hewitt, S.M., Rimm, D.L., and Sherman, M.E. 2006. Construction and validation of tissue microarrays of ductal carcinoma in situ and terminal duct lobular units associated with invasive breast carcinoma. *Diagn. Mol. Pathol. 15*: 157–161.

Yang, X.R., Sherman, M.E., Rimm, D.L., Lissowska, J., Brinton, L.A., Peplonska, B., Hewitt, S.M., Anderson, W.F., Szeszenia-Dabrowska, N., Bardin-Mikolajczak, A., Zatonski, W., Cartun, R., Mandich, D., Rymkiewicz, G., Ligaj, M., Lukaszek, S., Kordek, R., and Garcia-Closas, M. 2007. Differences in risk factors for breast cancer molecular subtypes in a population-based study. *Cancer Epidemiol. Biomark. Prev. 16*: 439–443.

18
Systematic Validation of Breast Cancer Biomarkers Using Tissue Microarrays: From Construction to Image Analysis

Catherine M.A. Kelly, Denise N. Ryan, Sarah A. Penny, and William M. Gallagher

INTRODUCTION

Fundamental changes are occurring in our approach to breast cancer research, including diagnosis and treatment. Omic-based discovery approaches, such as DNA-microarray gene expression profiling, are in part responsible for this shift (Brennan *et al*., 2005; McGee *et al*., 2006). These high-throughput technologies represent powerful tools capable of identifying breast cancer biomarkers. Promising biomarkers can then be studied using tissue microarrays (TMAs) that allow for omic-scale pathological screening and validation (Hoos and Cordon-Cardo, 2001). It is hoped that the incorporation of the tools and techniques of molecular biology into the routine management of breast cancer patients will result in: (1) better disease classification, (2) identification of patients who will derive most benefit from treatment, and (3) assist in the discovery of less toxic, targeted treatments. Such objectives should bring us closer to the ultimate goal of personalized management of cancer.

Validated Biomarkers

Breast cancer is a global health issue. It is the most common cause of cancer death in European women, accounting for 132,000 deaths in 2006. An estimated 429,900 women were diagnosed with breast cancer in the European Union in 2006, representing 28.9% of all female cancers diagnosed (Ferlay *et al*., 2007). At diagnosis, 90% of patients appear to have operable tumors that are confined to the breast and ipsilateral axilla; however, up to 50% of these women will develop metastatic disease (Rutgers, 2007).

The use of adjuvant chemotherapy has become the standard of care after locoregional treatment. Some treatment guidelines recommend adjuvant treatment for all those women whose tumor exceeds 1 cm, or women with smaller tumors associated with unfavorable histological features (Carlson, 2006).

Although adding chemotherapy reduces the risk of recurrence on average by ~ 25%, the absolute benefit for an individual patient is small, ranging from 1% to 5%.

The vast majority of estrogen receptor positive (ER+) breast cancer is overtreated with chemotherapy and would be cured by hormonal treatment alone (Fisher *et al.*, 2001). The challenges, therefore are, to identify the patients who clearly do not derive benefit from adjuvant chemotherapy from those who do. Secondly, one needs to improve treatment for those patients who will derive a benefit from chemotherapy both in the adjuvant and metastatic setting in terms of improved outcomes and minimal toxicity. To achieve these goals, we need more and better breast cancer biomarkers.

DEFINITION OF A BIOMARKER

There are many definitions of what constitutes a biomarker. On a basic level, a biomarker may be an analyzer in a clinical sample. A broader definition may include any measure that would predict a disease state or response to treatment. A biomarker can be a gene expression profile, a proteomic or metabolomic panel, a gene mutation, a cell surface receptor or it could represent a novel therapeutic target (Figure 18.1).

It is estimated that the revenue for biomarker-related products and services will expand more than sixfold, from an estimated $452 million in 2003 to $2.9 billion in 2008 (Rubenstein, 2003). Since the completion of the Human Genome Project, governmental agencies, academia, and industry have been investing massive amounts of time and resources into biomarker identification and their biomedical applications. Currently, we have very few clinically meaningful biomarkers in breast cancer, namely ER, the progesterone receptor (PR), and human epidermal growth factor-2 (HER-2). The bottleneck at this point lies not in identifying more biomarkers, but being able to dissect out the ones with direct disease or drug application. This is a complex problem and frequently we are working in a kind of retrospective manner from our predictor or biomarker to an outcome. In the early stages of the biomarker pipeline, we may know very little regarding the pathophysiological role of the biomarker being investigated or, indeed, if it actually has one. While gene signatures may predict for poor versus good prognosis, we commonly understand very little regarding the genes in the signature; indeed, some commentators refer to this situation as a "black box". Debate surrounds whether it is sufficient for them to remain as a signature of unknowns or their individual components need to be characterized (Baker, 2005).

Two large randomized clinical trials, MINDACT (Microarray in node negative disease may avoid chemotherapy) and TAILORx (Trial assigning individualized options for treatment), are presently using the 70-gene prognostic signature, Mammaprint (Agendia, Amsterdam, the Netherlands), and the 21-gene profile, Oncotype DX (Genomic health Inc, Redwood City, CA), respectively, to select patients for chemotherapy. It is expected that these gene signatures will complement clinicopathological information and assist in breast cancer decision-making and represent important omic-derived biomarkers in breast cancer management.

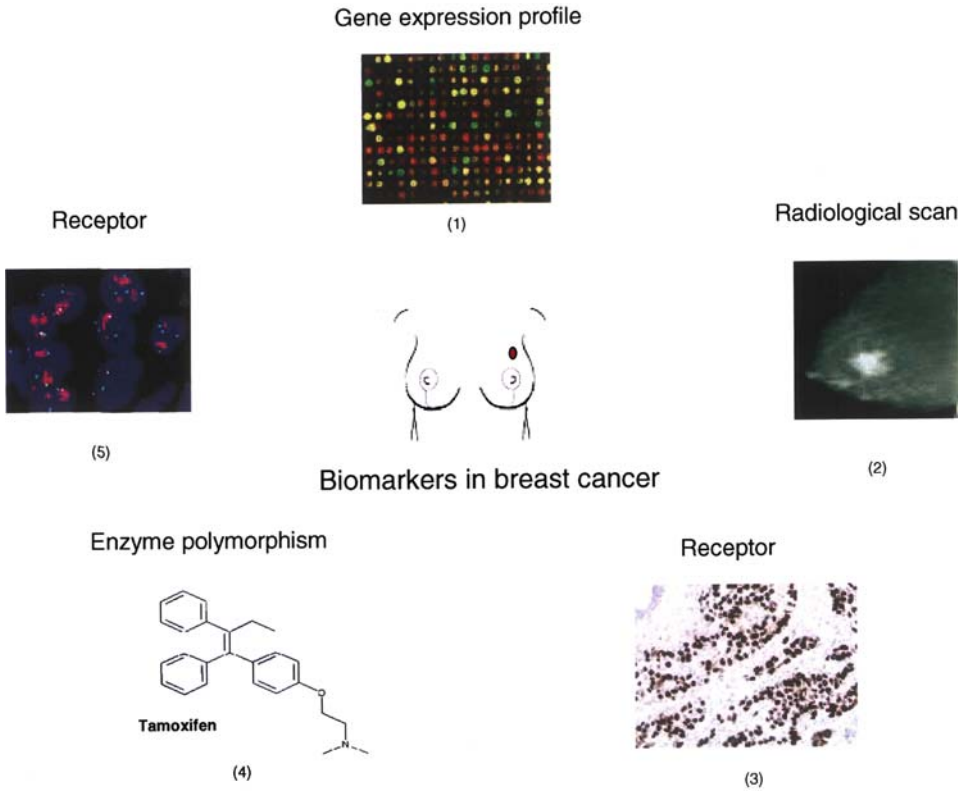

FIGURE 18.1. Examples of current and emerging biomarkers used in the oncological management of breast cancer. (1) Gene expression profiles can be used to predict outcome or treatment response. (2) Mammography is a frequently used breast cancer screening tool. (3) Estrogen receptor status is of fundamental importance as a predictive and prognostic marker in breast cancer. (4) CYP2D6 has an important role in the metabolic activation and prediction of response to tamoxifen. (5) HER-2 testing is used as a prognostic factor for outcome and a predictor of response to trastuzumab

CANDIDATE BIOMARKER IDENTIFICATION USING DNA MICROARRAYS

Omic-based discovery approaches allow us to probe biological systems at the level of the genome, proteome, and metabolome. In applying these technologies, vast amounts of data can be generated, some of which may contain clinically important information in the form of novel biomarker(s) (Figure 18.2).

In DNA microarray experiments, large datasets detailing the expression of many thousands of genes (some over-expressed, others the same or under-expressed) are generated. In order to determine which of these genes are potential biomarkers, novel bioinformatics approaches have been applied to them. O'Brien *et al*. (2007) applied between group analysis, a bioinformatics approach specifically tailored towards microarray data (Culhane *et al*., 2002), to the van't Veer dataset (van't Veer *et al*., 2002) and identified a cohort of putative biomarkers.

Centromere protein-F (CENPF), one of the biomarkers identified by this approach and validated on a breast cancer TMA, was shown to correlate with poor outcome and markers of aggressive

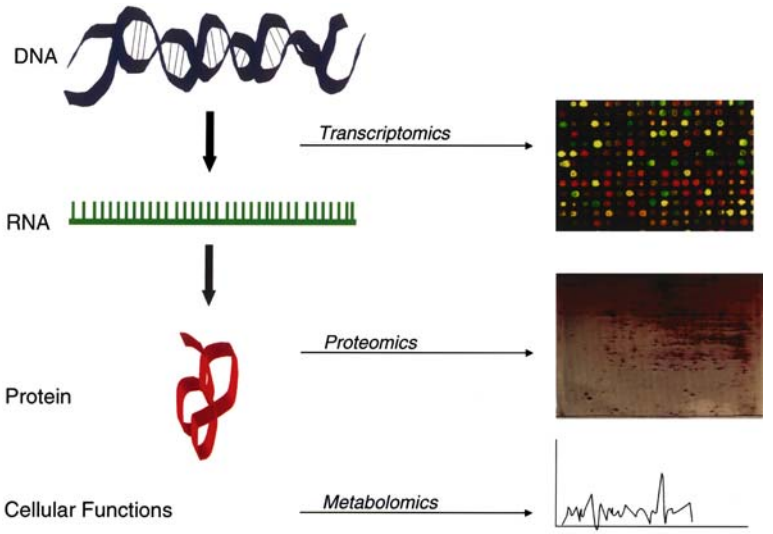

FIGURE 18.2. "Omics" – The global view. Omic-based discovery approaches allow us to probe biological systems at the level of the genome, proteome and metabolome

tumor behavior, i.e., ER negativity and high tumor grade (O'Brien *et al.*, 2007). CENPF also proved to be a marker of chromosomal instability. Carbonic anhydrase IX was also identified by this approach and its expression was found to correlate positively to tumor size, grade, hypoxia-inducible factor α, Ki-67, cyclin E, and cyclin A2 expression (Brennan *et al.*, 2006). It was associated with reduced relapse-free survival, overall survival, and breast cancer-specific survival.

SYSTEMATIC APPROACH TO BIOMARKER IDENTIFICATION AND VALIDATION

Once a biomarker or series of putative biomarkers have been identified via a high-throughput method, the next logical step is systematic validation. Rifai *et al.* (2006) describe a comprehensive biomarker pipeline as consisting of six components: candidate discovery, qualification, verification, research assay optimization, biomarker validation, and commercialization. A comprehensive biomarker pathway is illustrated in Figure 18.3 and discussed in the next section.

Candidate Identification

In our group, breast cancer biomarkers have been identified from a number of sources including in-house DNA microarray datasets generated from a novel breast cancer cell line model system with altered invasiveness (Hughes *et al.*, 2007), as well as directly from and reanalysis of publicly available DNA microarray datasets (van't Veer *et al.*, 2002; O'Brien *et al.*, 2007). We compiled a list of ranked candidate breast cancer biomarkers and submitted it to the Swedish Human Proteome Resource (HPR) for high-throughput antibody generation (Uhlen *et al.*, 2005). The HPR uses bioinformatics to design peptide fragments to be utilized as epitopes for the development of polyclonal antibodies. The resultant antibodies are then tested using Western blot analysis and reviewed using cell pellet arrays and tissue microarrays containing human normal and cancer tissue. The expression pattern for each antibody can be reviewed on the Human Protein Atlas (www.proteinatlas.org).

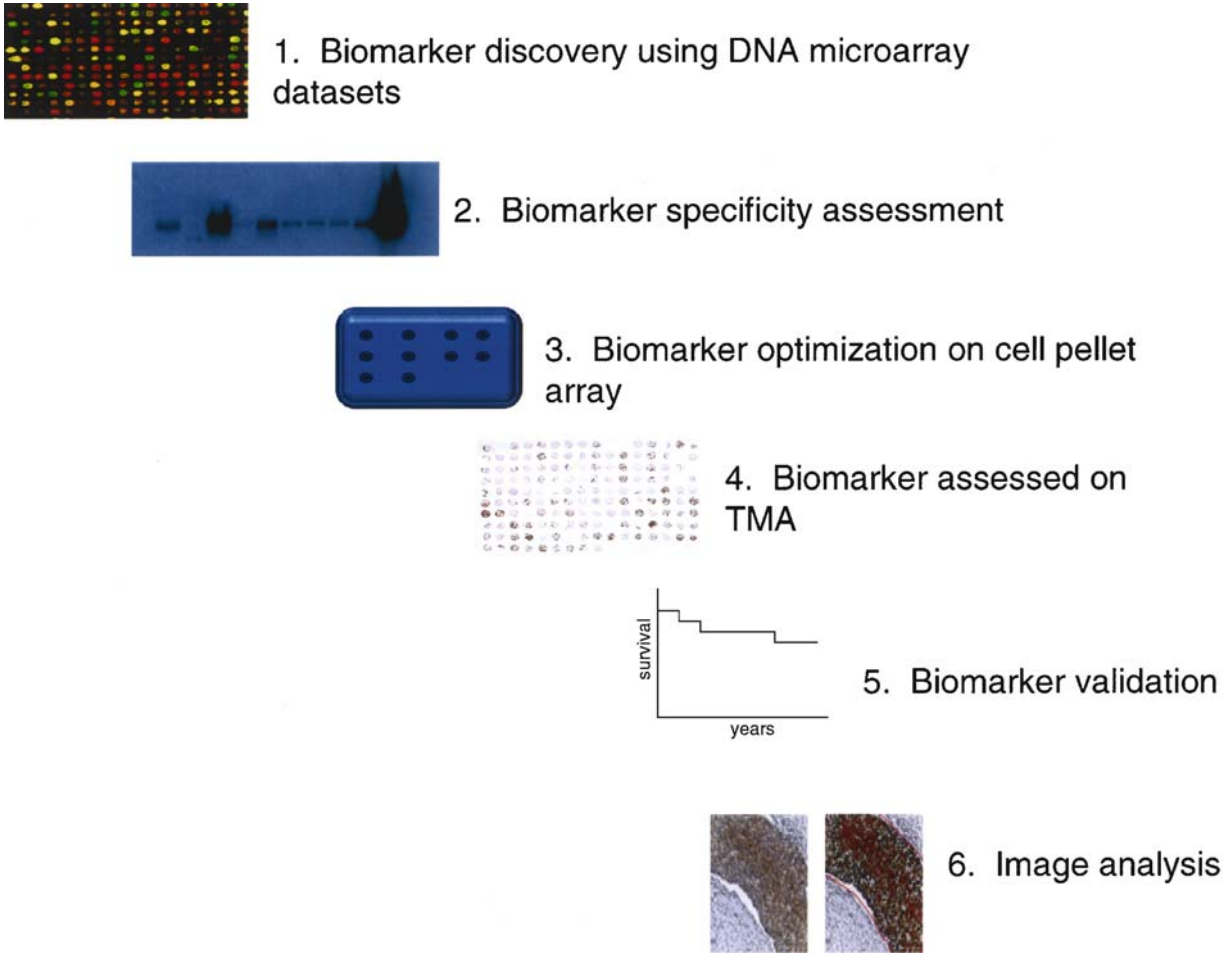

FIGURE 18.3. The biomarker pathway. (1) Bioinformatic approaches are used to mine DNA microarray datasets. (2) Antibodies generated to the putative biomarkers are assessed for specificity using Western blot analysis of cell line extracts. (3) Immunohistochemical optimisation of the biomarker on the same cell line as (2); the cells are formalin-fixed paraffin embedded and incorporated into a cell pellet array. (4) Once optimised on cheap, renewable cell pellet arrays, the candidate biomarkers are assessed on clinical material in a TMA. (5) Staining patterns are scored and correlated to clinical data pertaining to the TMA. (6) Image analysis using staining algorithms and commercial considerations for selected biomarkers

Biomarker Specificity Assessment

The specificity of incoming antibodies is examined *via* Western blot analysis using breast cancer cell line extracts, as well as *via* the use of protein arrays. Recombinant proteins derived from the cloned gene products used for antibody generation can act as positive controls in the process.

Biomarker Immunohistochemical Optimization

The immunohistochemical protocol for each antibody is optimized using a cell pellet array generated from the above cell lines (see protocol later in the text). Cross-comparisons can be made with prior optimization data from the Swedish HPR and by incorporating relevant cell lines into the cell pellet arrays.

Biomarker Assessment on Tissue Microarrays

Following the initial antibody optimization using Western blot and cell pellet array-based immunohistochemical analysis, the next platform in our biomarker pipeline is TMAs (see following section). Antibodies are assayed on TMAs containing breast cancer cores from hundreds of donors. Further comparisons relating to staining intensity and localization of the staining is made using the HPR data for each specific antibody.

Biomarker Validation and Clinical Correlation

Clinical information pertaining to each tissue core on the TMA enables evaluation of the clinical significance of identified biomarkers. Each core is linked to clinical information relating to tumor histological subtype, size, grade, lymph node status, hormone receptor status, and outcome data. This allows the identification of the genes associated with invasion and tumor progression at the RNA level, which are also implicated in patient prognosis at the protein level.

Automated Image Analysis

Automated systems are used for staining TMAs, which assist in the standardization of the staining process. Similarly, high-throughput image scanning instruments convert the stained slides into digital slides for scoring either manually via facile display on a computer or automatically using image analysis algorithms. Such algorithms enable staining quantification and generate continuous rather than ordinal data. We also use strategies which enable the exchange of TMA data in a user-friendly and web-accessible format.

TISSUE MICROARRAYS

Kononen *et al.* (1998) (Brennan *et al.*, 2008) developed TMAs to address the limitation of conventional molecular pathology and enable omic-scale molecular pathology screening (Kallioniemi *et al.*, 2001). TMA technology allows for the rapid visualization of molecular targets in thousands of tissue specimens at a time, either at the DNA, RNA, or protein level. Before the advent of TMAs and their adoption as a key component to biomarker validation, conventional pathological approaches struggled to keep up with increasing numbers of candidate biomarkers generated by omic-based discovery approaches.

TMAs are becoming an essential aspect of biomarker studies for a number of reasons (Brennan *et al.*, 2007). In a single

TMA experiment, hundreds of cores from different sources can be assessed simultaneously for one biomarker expression. Prior to the development of TMAs, this experiment would have required probing of hundreds of full-face tissue sections. A TMA map is linked to computer databases holding, clinico-pathological data, so results pertaining to predictive and prognostic properties of a biomarker can be generated instantly. TMAs are also relatively easy to construct; it is a standardized approach to what is often a much unstandardised area. Finally, TMAs are fabricated using tiny cores (usually 0.6–2 mm in diameter) of donor tissue; thus, conserving a precious resource.

Tissue Microarray Construction

To construct a TMA, an instrument known as a tissue arrayer is required. Most institutions use manual arrayers, but there are automated and semi-automated machines available. A tissue arrayer is a precision instrument that uses punches to create holes in a paraffin block at precise locations. The first punch creates a hole in a recipient block, the second punch removes a core of tissue from the donor paraffin block and deposits it into the recipient block. Up to 1,000 tissue cores can be arrayed into the recipient block. X–Y micrometer scaling systems define the co-ordinates of the array.

Donor Block

In most instances, the donor tissue is formalin-fixed, paraffin-embedded tissue (FFPE), although other material can be arrayed and will be discussed later in the text. An important aspect when preparing the donor block is to determine exactly which part of the block is to be arrayed. Usually a full-face tissue section is cut from the block, a H&E stain is then performed and the area of interest mapped by a pathologist. The corresponding area can be marked on the donor block and will represent the area to be sampled and inserted into the recipient block. The donor block should be thick enough to allow the removal of a core of a reasonably size; 3–4 mm thickness cores usually being required.

Recipient Block

To construct the recipient block, paraffin with melting temperature of 55–58 °C is poured into a mold. A cassette is placed on the top and it is then placed on a cool plate to solidify. Once the mold is removed, the block is examined for holes caused by bubbles during the preparation and it is pared to remove irregular edges. A section can be taken from the recipient block surface with a microtome to ensure that it is flat and parallel to the plastic cassette which forms the base of the block.

Designing the Tissue Microarray

As mentioned above, the tissue to be inserted into the TMA must be selected, reviewed by a pathologist and the appropriate area marked. A decision should be made regarding how large the arrray will be. A map illustrating the precise location of each core with an identification code (so they can be correlated back to associated clinical data) is obviously an essential part to TMA construction, TMAs containing tissue from patients with continuous follow-up will become more valuable as clinical events accumulate.

Although as many as 1,000 cores can be arrayed in a 40 × 25 mm block, this is often not practical. Errors may occur when inserting the correct core listed on the map and also such high-density blocks can crack

when cutting. Most arrays tend to hold between 250 to 500 cores. The spacing between cores is also important; if it is too large, the array can be slow and difficult to read, and can increase the chance of the pathologist getting lost when scoring it using a manual microscope. If the spacing is too short and too many cores are inserted into the recipient block, the middle part of the array can lift up in comparison to the edges of the array; this is due to an accumulation of residual paraffin caused by the slight size difference between the sample tissue and the holes in the array.

Size of the Punches

Punches are available in a variety of diameters, usually between 0.6 to 2 mm. The 0.6 mm core is used most commonly, with a 0.8 mm spacing in between cores. Larger diameter punches can be used, but these can cause damage to the donor block and it means less cores can be arrayed, e.g., 100–150 cores measuring 2 mm can be placed in single TMA block of 45 × 25 mm. Larger punches will also use up the tissue block more quickly.

Representivity of Tissue Microarrays

How representative is a TMA of the tumors the cores have been taken from? We know that tumors are heterogenous and, as such, when we take a 0.6 mm core from a tissue block, how can we be certain that we are getting an adequate representation of the tumor? The first point to be made on this subject and one frequently mentioned by researchers using TMAs is that the basic principal of TMA analysis is fundamentally different from conventional histological analysis. TMAs are commonly used in population-based research to determine the frequency of expression of biomarkers in cell populations, using large sample sets as opposed to making clinical diagnoses on individual cases (Kallioniemi *et al*., 2001).

Torhorst *et al*. (2001) compared the prognostic significance of ER, PR, and p53 in quadruplicate experiments for > 500 patients using TMA technology. Four independent TMAs were constructed by acquiring 0.6 mm biopsies from one central and from three peripheral regions of each of the FFPE tumor specimens. Immunostaining for ER, PR, and p53 expression was performed on both the conventional full-face sections and the TMA sections. Compared with conventional large section analysis, a single sample from each tumor identified approximately 95% of the information for ER, 75–81% for PR, and 70–74% for p53. It was concluded that tissue heterogeneity did not negatively influence the predictive power of the TMA results. The results demonstrated that 0.6 mm diameter samples in an array format were sufficently representative of their donor tumors, and could be used to establish associations between molecular alterations and clinical endpoints.

Sectioning the Array Block

The TMA can be sectioned using a microtome, as would be used when sectioning routine tissue blocks. Before the TMA is sectioned, it should be placed in an oven (60°C for 3–5 min or overnight at 37°C) with the array side face down on a glass slide. This softens up the paraffin and allows the tissue to adhere to the walls of the holes made in the recipient block. Once the array block is out of the oven, gentle pressure is applied over the cassette bottom to level off the surface of the array and ensure that all the cores are at the same level. The blocks can then be placed on ice

in preparation for cutting. The TMA block is sectioned between 3–6 μm (typically 5 μm) to generate TMA slides for molecular analyses. An adhesive-coated tape sectioning system helps to transfer the precise locations of the tissue spots in the TMA block on to the microscopic slides. Kallioniemi *et al*. (2001) suggest that because the morphology of the tissue changes as more sections are cut, the first and every 50th section of every TMA block should be stained with H&E to keep track of the morphology and representivity of the specimen.

Storage of the TMA slides

There is no consensus as to the appropriate long-term storage of TMA slides. Some researchers store them in dessicators, while others choose cold room storage. In a study by Fergenbaum *et al*. (2004), a single block TMA was constructed from 125 invasive breast carcinomas. Estrogen and progesterone receptor status, along with HER-2 expression, were determined in sections cut after 6 months of storage at room temperature and compared with those cut and stained on the same day. The percentage of positive cases for stored versus fresh sections was similar for ER status but higher in the fresh sections for PR and HER-2. The authors concluded that the loss of immunoreactivity is an important problem in TMAs and that improved methods of sectioning and storage are essential to minimize loss of immunoreactivity.

CELL PELLET ARRAYS

The specificity of putative biomarkers is generally first established via Western blot analysis; such experiments routinely use relevant cell line lysates. Once specificity is determined to be optimal, i.e., discrete band at the predicted molecular weight, and possibly some differential expression is demonstrated between the various cell lines examined, the biomarker can be studied using immunohistochemical methodologies on FFPE tissue. At this point, it is good practice to construct some cell pellets arrays from the cell lines used in Western blot analysis in order to gauge initial staining quality, determine cellular localization, test different antibody dilutions and antigen retrieval methods. This early antibody optimisation is a cheap, renewable platform that will act to save waste of often limited tissue sections.

Constructing a Cell Pellet

To make a cell pellet from a cell line, we use the following protocol. A flask of adherent cells is rinsed in phosphate buffered saline (PBS) with 1 mM EDTA. Trypsin is added and the flask is incubated for 3 min or until the cells have detached.

Once detached, cold culture media is added and the cells are centrifuged at 1,200–1,500 rpm for 10 min at 4°C. The supernatant is removed and the pellet is resuspended in cold PBS. The cells are centrifuged again at 1,200–1,500 rpm for 5 min at 4°C. The supernatant is removed and the pellet is resuspended in 10 ml of 4% formalin for approximately 1 h at room temperature. After fixation with formalin, the cells are centrifuged again and the supernatant is removed and 750 μl of formalin is added. A solution of 5% agarose in PBS is made and microwaved to dissolve fully; following this, it is placed in a waterbath at 65°C. The tube with the cells is placed into a waterbath and ~ 750 μl of agarose is added. The suspension is mixed thoroughly and transferred to a 24-well plate. It is allowed to

cool at 4°C for at least 30 min, but not more that 72 h. The agarose block is removed and placed in a specimen bag in a cassette and carried through standard tissue processing. Once tissue processing is complete, it can be embedded in paraffin and then used as a donor block for a cell pellet array.

FROZEN TUMOR TISSUE MICROARRAY

Most TMAs are composed of cores of FFPE tissue. During the fixation and embedding processes, changes in the tissue occur and may result in damage to or blocking of antigenic sites; this is often why good results achieved using an antibody in Western blot analysis does not necessarily translate to similar results immunohistochemically. In order to overcome this problem, Fejzo and Slamon (2001) constructed frozen tumor TMAs. A frozen TMA involves taking fresh tissue, frozen at −70°C and embedded in OCT compound. The frozen tissue is then arrayed into a recipient OCT block. The recipient block is sectioned and the array is evaluated without fixation. DNA and RNA hybridization and protein analysis can be performed and any other application previously used in routine immunohistochemical analysis. The drawback of this approach is the expertise required to construct the TMA, and the storage of frozen tissue can be a problem.

FROZEN PROTEIN ARRAY

Miyaji *et al.* (2002) developed an array platform that can use whole tissue or cells, cell lysates, *in vitro* transcribed or purified proteins, or laser capture microdissection-derived material. The method involves arraying the specimen into a preformed frozen recipient block which is then sectioned. The sections are placed on nitrocellulose (NC)-coated slides. Once bound to the NC-coated slide, the material forming the area loses any residual histomorphological features and now it is a homogenized and quantifiable platform for the detection of protein. Many NC-coated slides can be generated and a different antibody can probe each slide.

TISSUE IMMUNOBLOTTING

In order to make assaying protein in FFPE tissue more quantitative and to increase throughput of conventional TMAs, the technique of tissue immunoblotting was developed by Chung *et al.* (2006). This technique involves taking an unstained, deparaffinized TMA and stacking multilayered transfer membranes over it. The stack is packaged in a sealed pouch containing Tris-glycine transfer buffer. The pouch is then heated in a stepped approach i.e., 1 h at 55°C, 0.5 h at 65°C and for 2 h at 80°C. This results in the transfer of proteins from the tissue to the membranes. Once the transfer is complete, the membranes are separated and can be used for immunoblotting.

TISSUE MICROARRAY ASSAY

Any assay that has been used on FFPE whole tissue sections can also be applied to TMAs. The most frequently used technique is immunohistochemistry (IHC), involving the detection of protein antigens

using specific monoclonal or polyclonal antibodies. Before application of an antibody to a TMA, it must be optimized and the next section will discuss antibody optimization in more detail. The original tissue blocks will have been processed in different batches in the same laboratory or they will have been processed in a completely different laboratory. Variations in tissue processing and fixation times are very difficult to control and need to be considered when using TMAs. Control tissue and cell lines used in the optimization process can be incorporated onto the TMA for increased standardization.

Methods of detecting antibody binding primarily involve chromogenic techniques. Fluorochromes can also be used to allow for better quantification of the target protein and its precise localization. However, high magnification, transient nature of the stain and the need for counterstains are some of the drawbacks of using fluorescent techniques (Braunschweig *et al*., 2005). TMAs can also be used for *in situ* analysis such as mRNA *in situ* hybridization, TUNEL, *in situ* PCR and *in situ* RT-PCR.

WESTERN BLOT ANALYSIS AND ANTIBODY OPTIMIZATION

As discussed above, the most widely used assay performed on TMAs is IHC. In order to elucidate the importance of potential biomarkers on such large cohorts of clinical samples, antibodies must be optimized for their specific conditions on IHC. One technique employed is to confirm the specificity of such antibodies by Western blotting initially before proceeding to IHC on TMAs. Many factors including antibody dilution of primary and secondary antibodies, antigen retrieval and choice of detection systems must be thoroughly optimized before proceeding to large-scale TMA experiments.

WESTERN BLOTTING

Western blotting is a technique commonly used to positively identify a specific protein in a mixture of proteins and to obtain qualitative data regarding protein expression. In the case of preparation for TMA-based studies, Western blotting is employed to determine the specificity of the antibody chosen for optimization. Commonly, cell lines are used to act as controls, both positive and negative. The cell lines used as controls may be cells known to be positive or negative for expression of the protein of interest. These same cell lines can subsequently be used as controls in IHC experiments after fixation methods. Many considerations must be taken into account when optimizing Western blotting conditions for a specific antibody. Issues such as selection of lysis buffers, preparation of cell lysate from cells or tissues, and optimization of the Western blotting technique for each particular antibody must be taken into consideration. Western blot analysis is a labor intensive technique and, despite great care, the common problems encountered when the blot is developed include, no bands, multiple bands, high background staining, bands of interest being too high or too low; these problems have been addressed extensively by others (www.abcam.com/index.html?pageconfig=popular_protocols).

IMMUNOHISTOCHEMISTRY

At the early optimization stage, cell pellet arrays are very useful tools for validation of the antibody. Once such arrays have been used to standardize a staining protocol, one can move onto full-face sections and ultimately TMAs. The optimization of an antibody for IHC involves finding the optimal conditions for antigen retrieval, identifying the best antibody dilution, application of secondary antibody and choosing the best detection system (Hewitt, 2006; Chung *et al*., 2007). One of the important aspects of IHC optimization is selection of the correct method of antigen retrieval. To start with, an antibody should be investigated without the use of antigen retrieval and also using the following methods: pepsin, Tris/EDTA (pH 9.0), sodium citrate pH (6.0). Alternative buffers may also need to be tested to find the optimal result. Almost all FFPE tissue requires an antigen retrieval step before immunohistochemical staining can be performed, as methylene bridges formed during fixation form cross-linkages in proteins and, therefore, commonly mask antigenic sites.

The two methods of antigen retrieval are heat-mediated and enzymatic, both serve to break the methylene bridges and expose the antigenic sites in order to allow the antibodies to bind. Enzymatic retrieval can sometimes damage the morphology of the section, so the concentration and treatment time need to be optimized in this case. Several antigen retrieval methods must be tested, however, to find the approach that gives optimal staining.

Once the optimal antigen retrieval method is established, the antibody concentration can be adjusted to give the desired result. Another important factor in IHC optimization is the choice of primary antibody. After the specificity of the primary antibody has been determined using Western blotting and has also been tested on cell pellet arrays, it can be used on TMAs. Controls such as negative antibody control and isotype controls should be included in these experiments. The cell lines used in Western blotting and cell pellet arrays, as previously described, can also act as controls, both positive and negative.

The detection system used must also be optimized. Commonly used systems include Avidin-Biotin Complex (ABC) amplification with DAB (a substrate for the horseradish peroxidase enzyme) for detection of the target protein. Fixation of tissues must also be taken into consideration when considering TMA experimental success. 10% neutral buffered formalin (NBF) is the most commonly used fixative. Other fixatives include paraformaldehyde (PFA) or Bouin solution (formalin/picric acid).

The ideal fixation time will depend on the size of the tissue block and the type of tissue, but fixation between 18 to 24h seems to be suitable for most applications. The time of fixation of the tissue is important to consider as under-fixation can lead to only edge staining, with strong signal on the edges of the section and no signal in the middle, and over-fixation can cause masking of the epitope.

Many factors need to be taken into account when optimizing IHC conditions (Riera *et al*., 1999). IHC is a technically intensive process. Factors at every stage including tissue collection, fixation, processing, embedding, sectioning, storage of tissue, antigen retrieval and staining can impact on the final results achieved. Frequently encountered problems in IHC include no staining, nonspecific staining, and background staining. There are extensive publications

discussing the problems encountered and possible solutions to them (Shi *et al*., 2001). This subject has been extensively discussed by Hayat (2002, 2004–2006).

Tissue Microarrays

TMAs have many advantages in IHC experiments as they allow for the technique to be carried out on multiple specimens simultaneously leading to remarkable standardization between samples. Standardization is also seen in antigen retrieval, incubation of primary antibodies, washing, etc. TMAs, in effect, allow numerous IHC experiments to be carried out simultaneously, hence standardizing the protocol and allowing for accurate comparisons between samples and therefore effective analysis (Shergill *et al*., 2004).

MANUAL ANALYSIS

The interpretation and analysis of IHC experiments is a crucial step. The stained TMA needs to be assessed initially to determine the presence or absence and cellular localization of the biomarker in the tumor specimens. Manual analysis also involves determining the amount and intensity of staining in the tumors. Scoring models involve allocating a numerical value (e.g., 0, 1, 2, and 3) on the basis of intensity or staining and a percentage indicating the extent of positive tissue staining. Staining results can also be reported as positive or negative. When scoring a TMA where hundreds of cores are being scored, it is advisable to document staining in all compartments and percentage of cells staining during the first analysis, as this will prevent the need to come back again at a future date and repeat what can be a very tedious process. A scoring system is designed for the biomarker in question. The cores are then scored blind by two independent pathologists. If the scoring is discordant for a core, it is reviewed again and a consensus is reached and a score allocated. The TMAs are usually analyzed at ×20 by the percentage of stained tumor cells at each level of staining intensity. The scoring results are recorded in an Excel database and then transferred into the TMA database containing the clinical information on the TMA. When samples are arrayed in duplicate, the mean of scoring results is often taken for statistical analysis (Winter *et al*., 2007).

Problems of inter and intra-variability should be considered when manually scoring a TMA. Manual scoring produces only a limited amount of data and is difficult to duplicate due to its subjective nature. Hence, there is a need for automated analysis which can distinguish stain patterns and quantify staining as referred to earlier in the text.

DIGITAL SLIDE SCANNING

Digital slide scanning involves the use of a camera or scanner to produce a virtual microscope image (Steinberg and Ali, 2001). A virtual microscope image, as opposed to a simple, static image allows user interaction, including zooming into and scrolling across the image (Conway *et al*., 2006). The virtual image may be stored in an online database and can then be viewed and analysed remotely, by multiple users concurrently.

There are many digital slide scanning technologies now available, most with dedicated software for slide scanning, analysis and database management. Both the Aperio ScanScope system

(www.aperio.com/productsservices/prod-systems.asp) and the Bacus Labs Inc. BLISS system (www.olympusamerica.com/seg_section/product.asp?) are popular in the literature, with newer technologies appearing all the time, including the Nanozoomer from Hamamatsu (www.olympusamerica.com/seg_section/product.asp?product=1040). Each system provides efficient, automatic calibration, with one-step scanning and a simple user interface allowing a degree of user control, when required. This enables the efficient capture of many images that pathologists can then score and annotate with ease, from any networked location. Alternatively, the high resolution images may be used in automated analysis.

A large amount of data is associated with each TMA, in addition to the virtual microscope image, including the clinical data relating to the patients and the TMA construction, staining and assessment data (Conway *et al*., 2006). Following scoring and before analysis can begin, the TMA must be dearrayed – all the data must be assigned to its individual TMA spot. This requires a dedicated storage and integration program. There now exist sophisticated packages that integrate virtual microscopy and relational database technology to automatically dearray a TMA, thus efficiently turning the data into a useable format.

IMAGE STORAGE

The vast quantities of data involved in each potential biomarker investigation using TMAs require not only efficient analysis, but also a suitable storage device allowing easy retrieval. A large, multi-cached server is required to hold these volumes and may be privately owned or rented from a larger company. This server is then networked and the data available through a specially designed website. There are many projects online, such as the Irish Breast Cancer Portal (www.breastcancerportal.org), which uses the Distiller software from SlidePath (www.slidepath.com), that host clinical data linked to TMA assays in a fully searchable format. These projects are fully searchable in terms of antibody, or any aspect of clinical data, thus allowing patient stratification leading to more targeted therapies.

There have also been attempts to create a larger database of IHC markers in cancer and normal tissues, which could be likened to the Human Genome Project. The largest and most integrated of such projects, with collaborators from across Europe is the Swedish Human Proteome Resource (Uhlen *et al*., 2005), publicly available at www.proteinatlas.org, where antibodies are searchable by their gene name.

DIGITAL SLIDE SCANNING AND STORAGE

Digital slide scanning and storage provides numerous benefits, the data is collated in a digital format, with high magnification and good resolution images (useful for manual and automated analysis). It allows easy comparison of TMA tissues and staining, while slides can be viewed across the web concurrently by many scientists or pathologists. The process is efficient and time-effective and automatic dearraying allows for efficient complex analysis, facilitating accurate biomarker identification.

CONCLUSION

In conclusion, there is an urgent need to identify clinically relevant breast cancer biomarkers. Omic-based strategies have

the ability to generate large amounts of data, the challenge however lies in teasing out the important biomarkers which could be a gene signature, a novel metabolite or well characterized protein with applications in the clinic. TMAs are an important development and have enabled molecular pathology to keep pace with high-throughput techniques of the omic-era. It is anticipated that TMAs will continue to contribute to, and become even more important in the areas of biomarker validation, drug development, basic translational research and clinical epidemiology with routine incorporation of TMAs into randomized controlled trials. It is envisaged that improved analysis tools for TMAs will facilitate the study of pathological specimens in new and more informative ways in the future.

Acknowledgements. Funding is acknowledged from Cancer Research Ireland and the Health Research Board of Ireland, the latter under the auspices of the 'Breast Cancer Metastasis: Biomarkers and Functional Mediators' research programme. The UCD Conway Institute is funded by the Programme for Third Level Institutions (PRTLI), as administered by the Higher Education Authority (HEA) of Ireland.

REFERENCES

Baker, M. 2005. In biomarkers we trust? *Nat. Biotechnol. 23*: 297–304.

Becker, N. 1998. Cancer mortality and prevention in the European Union. *Eur. J. Surg. Oncol. 4*: 370–374.

Braunschweig, T., Chung, J.Y., and Hewitt, S.M. 2005. Tissue microarrays: Bridging the gap between research and the clinic. *Expert Rev. Proteom. 2*: 325–336.

Brennan, D.J., O'Brien, S.L., Fagan, A., Culhane, A.C., Higgins, D.G., Duffy, M.J., and Gallagher, W.M. 2005. Application of DNA microarray technology in determining breast cancer prognosis and therapeutic response. *Expert Opin. Biol. Therapy 5*: 1069–1083.

Brennan, D.J., Jirstrom, K., Kronblad, A., Millikan, R.C., Landberg, G., Duffy, M.J., Ryden, L., Gallagher, W.M., and O'Brien, S.L. 2006. CA IX is an independent prognostic marker in premenopausal breast cancer patients with one to three positive lymph nodes and a putative marker of radiation resistance. *Clin. Cancer Res. 12*: 6421–6431.

Brennan, D.J., Rexhapaj, E., O'Brien, S.L., McSherry, E., O'Connor, D.P., Fagan, A., Culhane, A.C., Higgins, D.G., Jirstrom, K., Millikan, R.C., Landberg, G., Duffy, M.J., Hewit, S.M., and Gallagher, W.M. 2008. Altered Cytoplasmic-to-Nuclear Ratio of Survivin Is a Prognostic Indicator in Breast Cancer. *Clin. Cancer Res. 14(9) In press.*

Carlson, R.W., Brown, E., Burstein, H.J., Gradishar, W.J., Hudis, C.A., Loprinzi, C., Mamounas, E.P., *et al*. 2006. NCCN task force report: Adjuvant therapy for breast cancer. *J. Nat. Comprehen. Cancer Network 4*: (SUPPL. 1): S1–S26.

Chung, J.Y., Braunschweig, T., Baibakov, G., Galperin, M., Ramesh, A., Skacel, M., Gannot, G., Knezevic, V., and Hewitt, S.M. 2006. Transfer and multiplex immunoblotting of a paraffin embedded tissue. *Proteomics 6*: 767–774.

Chung, J.Y., Braunschweig, T., Tuttle, K., and Hewitt, S.M. 2007. Tissue microarrays as a platform for proteomic investigation. *J. Mol. Histol. 38*: 123–128.

Conway, C.M., O'Shea, D., O'Brien, S., Lawler, D.K., Dodrill, G.D., O'Grady, A., and Barrett, H., *et al*. 2006. The development and validation of the virtual tissue matrix, a software application that facilitates the review of tissue microarrays on line. *BMC Bioinformat. 7*: 256.

Culhane, A.C., Perriere, G., Considine, E.C., Cotter, T.G., and Higgins, D.G. 2002. Between-group analysis of microarray data. *Bioinformatics 18*: 1600–1608.

Fejzo, M.S., and Slamon, D.J. 2001. Frozen tumor tissue microarray technology for analysis of tumor RNA, DNA, and proteins. *Am J. Pathol. 159*: 1645–1650.

Fergenbaum, J.H., Garcia-Closas, M., Hewitt, S.M., Lissowska, J., Sakoda, L.C., and Sherman, M.E. 2004. Loss of antigenicity in stored sections of breast cancer tissue microarrays. *Cancer Epidem. Biomark. Prevent. 13*: 667–672.

Ferlay, J., Autier, P., Boniol, M., Heanue, M., Colombet, M., and Boyle, P. 2007. Estimates of the cancer incidence and mortality in europe

in 2006. *Annals of Oncology 18, (3) (March 01, 2007)*: 581–592.

Fisher, B., Jeong, J.H., Dignam, J., Anderson, S., Mamounas, E., Wickerham, D.L., and Wolmark., N. 2001. Findings from recent national surgical adjuvant breast and bowel project adjuvant studies in stage I breast cancer. *J. Nat. Cancer Inst. Monog. 30*: 62–66.

Hayat, M.A. 2002. *Microscopy, Immunohistochemistry, and Antigen Retrieval Methods*. Kluwer/Academic/Plenum, New York.

Hayat, M.A. Ed. 2004–2006. *Immunohistochemistry and In-Situ Hybridization of Human Carcinomas*. Elsevier/Academic, San Diego, CA.

Hewitt, S.M. 2006. The application of tissue microarrays in the validation of microarray results. *Methods Enzymol. 410*: 400–415.

Hoos, A., and Cordon-Cardo, C. 2001. Tissue microarray profiling of cancer specimens and cell lines: Opportunities and limitations. *Lab. Invest. 81*: 1331–1338.

Hughes, L., O'Brien, S.L., Gallagher, W.M., and McDonnell, S. 2007. DNA microarray-based transcriptomic profiling of an isogenic cell culture model of breast tumour cell invasion. *Anticancer Res. 27*: 1353–1359.

Kallioniemi, O.P., Wagner, U., Kononen, J., and Sauter, G. 2001. Tissue microarray technology for high-throughput molecular profiling of cancer. *Human Mol. Genetics 10*: 657–662.

Kononen, J., Bubendorf, L., Kallionimeni, A., Barlund, M., Schraml, P., Leighton, S., Torhorst, J., Mihatsch, M., Sauter, G., and Kallionimeni, O.P. 1998. Tissue microarrays for high throughput molecular profiling of tumor specimens. *Nature Med. 4*: 844–847.

McGee, S.F., O'Connor, D.P., and Gallagher, W.M. 2006. Functional interrogation of breast cancer: From models to drugs. *Expert Opin. Drug Discov. 1*: 569–584.

Miyaji, T., Hewitt, S.M., Liotta, L.M., and Star, R.A. 2002. Frozen protein arrays: A new method for arraying and detecting recombinant and native tissue proteins. *Proteomics 2*: 1489–1493.

O'Brien, S.L., Fagan, A., Fox, E.J., Millikan, R.C., Culhane, A.C., Brennan, D.J., McCann, A.H., *et al*. 2007. CENP-F expression is associated with poor prognosis and chromosomal instability in patients with primary breast cancer. *Intern. J. Cancer 120*: 1434–1443.

Riera, J., Simpson, J.F., Tamayo, R., and Battifora, H. 1999. Use of cultured cells as a control for quantitative immunocytochemical analysis of estrogen receptor in breast cancer: The quicgel method. *Am. J. Clin. Pathol. 111*: 329–335.

Rifai, N., Gillette, M.A., and Carr, S.A. 2006. Protein biomarker discovery and validation: The long and uncertain path to clinical utility. *Nature Biotech. 24*: 971–983.

Rubenstein, K. 2003. *Post-genomic Biomarkers Revolutionizing Drug Development and Diagnostics*. Westborough, MA: D&MD Publications.

Rutgers, E.J. 2007. Breast international group I MINDACT trial: A prospective trial to validate the amsterdam signature. In: Govindan, R. Ed. *Amercian Society of Clinical Oncology, Education Book*.

Shergill, I.S., Freeman, A., and Mundy, A.R. 2004. Tissue microarrays in urology. *BJU Intl. 94*: 22–25.

Shi, S.R., Cote, R.J., and Taylor, C.R. 2001. Antigen retrieval techniques: Current perspectives. *J. Histochem. Cytochem. 49*: 931–937.

Steinberg, D.M., and Ali, S.Z. 2001. Application of virtual microscopy in clinical cytopathology. *Diagnost. Cytopathol. 25*: 389–396.

Torhorst, J., Bucher, C., Kononen, J., Haas, P., Zuber, M., Kochli, O.R. and Mross, F., *et al*. 2001. Tissue microarrays for rapid linking of molecular changes to clinical endpoints. *Am. J. Pathol. 159*: 2249–2256.

Uhlen, M., Bjorling, E., Agaton, C., Szigyarto, C.A., Amini, B., Andersen, E., and Andersson, A.C., *et al*. 2005. A human protein atlas for normal and cancer tissues based on antibody proteomics. *Mol. Cell. Proteomics 4*: 1920–1932.

Van't Veer, L.J., Dai, H., van de Vijver, M.J., He, Y.D., Hart, A.A., Mao, M., and Peterse, H.L., *et al*. 2002. Gene expression profiling predicts clinical outcome of breast cancer. *Nature 415*: 530–536.

Winter, J.L., Stackhouse, B.L., Russell, G.B., and Kute, T.E. 2007. Measurement of PTEN expression using tissue microarrays to determine a race-specific prognostic marker in breast cancer. *Arch. Pathol. Lab. Med. 131*: 767–772.

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Phyllodes Tumors of the Breast: The Role of Immunohistochemistry in Diagnosis

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INTRODUCTION

Phyllodes tumors of the breast are uncommon fibroepithelial neoplasms, typified by hypercellular stroma and elongated ducts with irregular leaf-like patterns due to stromal proliferation. Its numbers are much fewer than breast carcinomas, with a proportion of only 1.5% compared to breast carcinomas. Phyllodes tumors have a higher frequency in Asian women (Tan *et al*., 2005b). Classification into three categories of benign, borderline, and malignant is based on a spectrum of histological features such as stromal hypercellularity and cytologic atypia, stromal overgrowth, mitotic rate, and nature of microscopic margins. However, there are still difficulties in accurate categorization of phyllodes tumors and predicting their clinical outcome. Histologic features such as grade (Moffat *et al*., 1995; Reinfuss *et al*., 1996), stromal overgrowth (Chaney *et al*., 2000), tumor necrosis and heterologous stromal elements (Cohn-Cedermark *et al*., 1991), as well as a combination of morphologic parameters (Moffat *et al*., 1995), are reported to be predictive. However, specific parameters that are associated with recurrence are not agreed upon; with a substantial number of reports concluding that adequate margins are the most important (Barth, 1999; Kleer *et al*., 2001; Tan *et al*., 2005b), and that histologic factors have an inconsistent influence on biologic behavior.

Biological markers have been studied in phyllodes tumors using immunohistochemistry, with p53 perhaps being the most widely evaluated (Feakins *et al*., 1999; Kuenen-Boumeester *et al*., 1999; Tse *et al*., 2002; Millar *et al*., 1999). Whilst p53 expression in stromal cells has been associated with malignant histological features, it does not appear to predict likelihood of recurrence. c-kit (CD117) expression is also reported in the stroma of malignant phyllodes tumors (Sawyer *et al*., 2003; Chen *et al*., 2000), and CD117 stromal staining has been shown to correlate with disease recurrence (Tan *et al*., 2005d). Other studies have demonstrated that heparan sulfate expression (Koo *et al*., 2006), epidermal growth factor receptor

(EGFR) overexpression and mutations (Kersting *et al*., 2006), c-myc overexpression (Sawyer *et al*., 2003), Ki-67 (Kocova *et al*., 1998; Niezabitowski *et al*., 2001; el-Naggar *et al*., 1990), may also be associated with tumor growth and malignancy. In this discussion, the role of immunohistochemical detection of biological markers in phyllodes tumors is reviewed, highlighting their potential utility from the diagnostic, prognostic, and pathogenetic perspectives.

CLINICOPATHOLOGICAL FEATURES OF PHYLLODES TUMORS

Phyllodes tumor of the breast was first characterized as a distinct entity by Johannes Müller in 1838, under the term cystosarcoma phyllodes. The use of cystosarcoma to describe this lesion was not intended to connote malignancy, but rather, to reflect its cystic and fleshy appearance. In fact, a benign nature was implied in the original account. Since then there have been many reports on this fascinating entity, and apart from phyllodes tumor being the current universally adopted term that has replaced cystosarcoma phyllodes, multiple studies to identify histologic parameters that predict biologic behavior have yielded variable results.

The phyllodes tumor is encompassed in the category of fibroepithelial breast neoplasms, to which the much commoner fibroadenoma also belongs. Macroscopically, the phyllodes tumor is circumscribed and lobulated, sometimes reaching large proportions that can stretch and ulcerate the overlying skin. Microscopically, there is a biphasic proliferation of epithelial and stromal components with leaf-like fronds formed by an exaggerated intracanalicular growth pattern and variably cellular stroma protruding against elongated meandering clefted spaces lined by epithelium. Designation of benign, borderline, and malignant phyllodes tumors depends on a constellation of histologic parameters as described: with benign phyllodes tumors showing circumscribed borders, mild or moderate stromal hypercellularity and cytologic atypia, up to 4 mitoses per 10 high power fields, and absence of stromal overgrowth. A malignant tumor is defined by marked stromal hypercellularity and cytologic atypia, presence of stromal overgrowth, brisk mitotic activity of 10 or more mitoses per 10 high power fields, and permeative margins. Borderline phyllodes tumors show some but not all characteristics found in malignant lesions.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry is a method by which specific antigens in tissues and cells can be localized and identified with light microscopy using antigen-antibody recognition (Taylor *et al*., 2006). It was developed in 1940 by Coons who used an immunofluorescence technique to detect antigens on frozen tissues. The widespread application of immunohistochemistry in diagnostic surgical pathology occurred only in the 1990s when more sensitive detection systems like the peroxidase-antiperoxidase, avidin-biotin conjugate and biotin-streptavidin methods were established.

The ready availability of monoclonal antibodies and effective use of immunohis-

tochemistry on paraffin embedded tissues have resulted in this technique becoming firmly entrenched in the diagnostic surgical pathologist's armamentarium of ancillary tools when evaluating pathologic material (Hayat, 2004–2006).

Interpretation of immuohistochemical stains on tissues requires an assessment of its sensitivity and specificity of particular antibodies, familiarity with methods of antigen retrieval, and an awareness of potential pitfalls, which are not in the scope of this chapter.

ROLE OF IMMUNOHISTOCHEMISTRY IN DIFFERENTIAL DIAGNOSIS OF PHYLLODES TUMORS

Histologically, benign phyllodes tumor may be difficult to distinguish from fibroadenoma. At the other end of the spectrum, malignant phyllodes tumor with stromal overgrowth may present challenges in distinction from spindle cell metaplastic carcinoma and primary breast sarcoma. While the majority of fibroadenomas can be readily separated from phyllodes tumors using standard light microscopic criteria, in some instances, fibroadenomas with an intracanalicular growth pattern and stromal hypercellularity can resemble phyllodes tumors.

CD34

CD34 is the human hematopoietic progenitor cell antigen, comprising a 110 kDa protein that is expressed by hematopoietic embryonic cells and endothelial cells. It is a sensitive though not a specific marker for endothelial differentiation. In the normal breast, stromal fibroblasts express CD34, which becomes diminished in malignant disease. In fibroadenomas, CD34 reactivity is demonstrated in stromal cells (Chauhan *et al*., 2003), staining interlobular, intracanalicular, and pericanalicular fibroblasts (Silverman and Tamsen, 1996). There is more variable expression of CD34 by phyllodes tumors. Moore and Lee (2001) found that immunohistochemical expression of CD34 was greater in fibroadenomas than in phyllodes tumors especially the malignant variety. In their study, diffuse reactivity of the stromal component of fibroadenomas was present, with their spindled cells reflecting a median staining proportion of 100%, as compared to phyllodes tumors with a median value of 75%. Less staining for CD34 was encountered in malignant than in benign and borderline phyllodes tumors. The staining pattern of CD34 was also reported to be more patchy in phyllodes tumors as compared to fibroadenomas.

Proliferation Indices

Core biopsies of fibroepithelial neoplasms with cellular stroma pose both diagnostic and management dilemmas; i.e., fibroadenoma can lead to patient discharge while a suggestion of phyllodes tumor on core biopsy warrants complete excision. A study of 29 such cases by Jacobs *et al*. (2005) revealed that an assessment of proliferation indices on the core biopsies may be discriminatory, with both immunohistochemical detection of Ki-67 and topoisomerase IIα indices being significantly greater in core biopsies that subsequently turned out to be phyllodes tumor than those that were later confirmed as fibroadenoma. The authors also advocated using

mitotic activity and stromal cellularity as potential arbiters. In our own preliminary study of core biopsies of fibroepithelial neoplasms for which there was uncertainty with regard to a diagnosis of fibroadenoma versus phyllodes tumor, we discovered possible utility of bcl2, Ki-67 and CD34 immunostaining (Akhilesh *et al*., 2007). The role of Ki-67 in the grading of phyllodes tumors is further elaborated below.

Cytokeratins

Cytokeratins are generally believed to be the most fundamental markers of epithelial differentiation because their specific composition in epithelial cells reflects both cell type and differentiation status. Cytokeratins act as a cytoplasmic scaffold that endow epithelial cells with the ability to sustain mechanical and nonmechanical stresses. Other functions of cytokeratins include participation in the response to stress, cell signaling and apoptosis. The cytokeratin family is a highly complex multigene family of polypeptides, with molecular weights ranging from 10 to 68 kDa. To date, 20 cytokeratins have been discovered, of which 12 (CK9-CK20) belong to the acidic type-I and 8 (CK1–CK8), belonging to the neutral-basic type-II subfamily (Tan *et al*., 2005c).

Cytokeratins are particularly important in the diagnosis of metaplastic spindle cell carcinoma, and demonstrate immunohistochemical reactivity in their spindle cells which are of epithelial origin (Dunne *et al*., 2003). In monophasic spindle cell metaplastic breast carcinoma, histologic differentiation from malignant phyllodes tumor with stromal overgrowth as well as from primary breast sarcoma can be potentially challenging. Cytokeratin immunohistochemistry can be used to make the distinction, with the study by Dunne *et al*. (2003) disclosing no cytokeratin expression in stromal areas of phyllodes tumors. The authors also suggested that CD34 and to a lesser extent, bcl2, could be used in favoring phyllodes tumor from metaplastic spindle cell carcinoma, especially in small biopsies, due to the possible heterogeneity of cytokeratin staining in the latter. The cytokeratin that was most frequently expressed in the metaplastic spindle cell carcinomas was 34βE12. Distinction of malignant phyllodes tumor with stromal overgrowth from primary breast sarcoma is more problematic and may not be readily resolved with immunohistochemistry. Thorough sampling to identify a focal phyllodal pattern on histology is crucial.

ROLE OF IMMUNOHISTOCHEMICALLY IN GRADING OF PHYLLODES TUMORS

Biological markers that are reported to be immunohistochemistically useful for the purpose of grading phyllodes tumors include p53, Ki-67 (MIB1), c-kit (CD117), CD10, EGFR, endothelin, and heparan sulfate.

p53

p53 is a tumor suppressor gene that is widely studied in neoplastic processes. Located on chromosome 17p13.1, it encodes for a 53 kDa nuclear phosphoprotein that is expressed in all normal cells at low levels. The wild-type (normal) p53 gene is involved in cell cycle regulation as well as apoptosis.

As the half-life of wild-type p53 is short, immunohistochemistry is believed to highlight expression of mutant p53 protein that is more stable with a longer half-life. In breast phyllodes tumors, the role of p53 has been well investigated (Millar *et al*., 1999; Niezabitowski *et al*., 2001), with some authors suggesting a possible prognostic function, while its predictive utility has not been validated by others. It is believed that p53 immunohistochemical expression in stromal cells can be relied upon as an adjunctive tool in the diagnosis of malignancy in phyllodes tumors (Gatalica *et al*., 2001; Tse *et al*., 2003). A study of 278 tissue microarrays of breast phyllodes tumors disclosed a statistically significant association of immunohistochemical detection of stromal p53 with grade (Tan *et al*., 2005d), but no correlation was discovered with recurrent disease.

Ki-67 (MIB1)

The well-known MIB1 antibody recognizes Ki-67, a nuclear nonhistone protein that is strictly linked to cell proliferation. The expression of Ki-67 in active phases of the cell cycle (G_1, S, G_2, and M), and its absence in the G_0 resting phase makes it a common and useful marker for assessing the degree of cell proliferation (Tan *et al*., 2005a). Previous studies have shown a high degree of correlation of stromal Ki-67 expression with phyllodes tumor grade (Silverman and Tamsen, 1996), with reports of Ki-67 index in stromal cells of 10–20% in low grade, and 20–40% in high grade phyllodes tumors. In another study, immunohistochemical reactivity for MIB1 was documented as 5% and 15% for benign and malignant phyllodes tumors, respectively, in a series of 37 cases (Kocova *et al*., 1998). Similar findings were concluded by Umekita and Yoshida (1999) when they reviewed 46 phyllodes tumors, with malignant phyllodes tumors showing higher MIB1 expression than borderline and benign ones (Figure 19.1A).

c-kit (CD117)

CD117, also known as c-kit, is a membrane bound tyrosine kinase receptor. Dimerization and autophosphorylation of CD117 is known to inhibit apoptosis *via* the phosphatidylinositol-3 kinase/Akt system and potentiate cell proliferation *via* the Ras/MAP kinase pathway and JAK/STA signalling (Kitamura and Hirotab, 2004). As CD117 overexpression is characteristically observed in gastrointestinal stromal tumors, it has served as a therapeutic target by drugs (Glivec) used to manage patients with these tumors. More recently, CD117 has also been found in the stromal cells of malignant phyllodes tumors, and it was postulated that its overexpression may be instrumental in the growth of these tumors (Sawyer *et al*., 2003). Although gain-of-function mutations of CD117 have been reported in gastrointestinal stromal tumors, mast cell neoplasms and germ cell tumors (Kitamura and Hirotab, 2004), no activating mutations of CD117 have been described in breast phyllodes tumors (Sawyer *et al*., 2003; Carvalho *et al*., 2004).

In the study by Tan *et al*. (2005d), CD117 protein expression within stromal cells was significantly associated with myriad morphologic parameters, including grade and recurrent disease. The association with phyllodes tumor grade has been corroborated by other authors (Sawyer *et al*., 2003; Chen *et al*., 2000). The validity of the correlation with recurrence needs

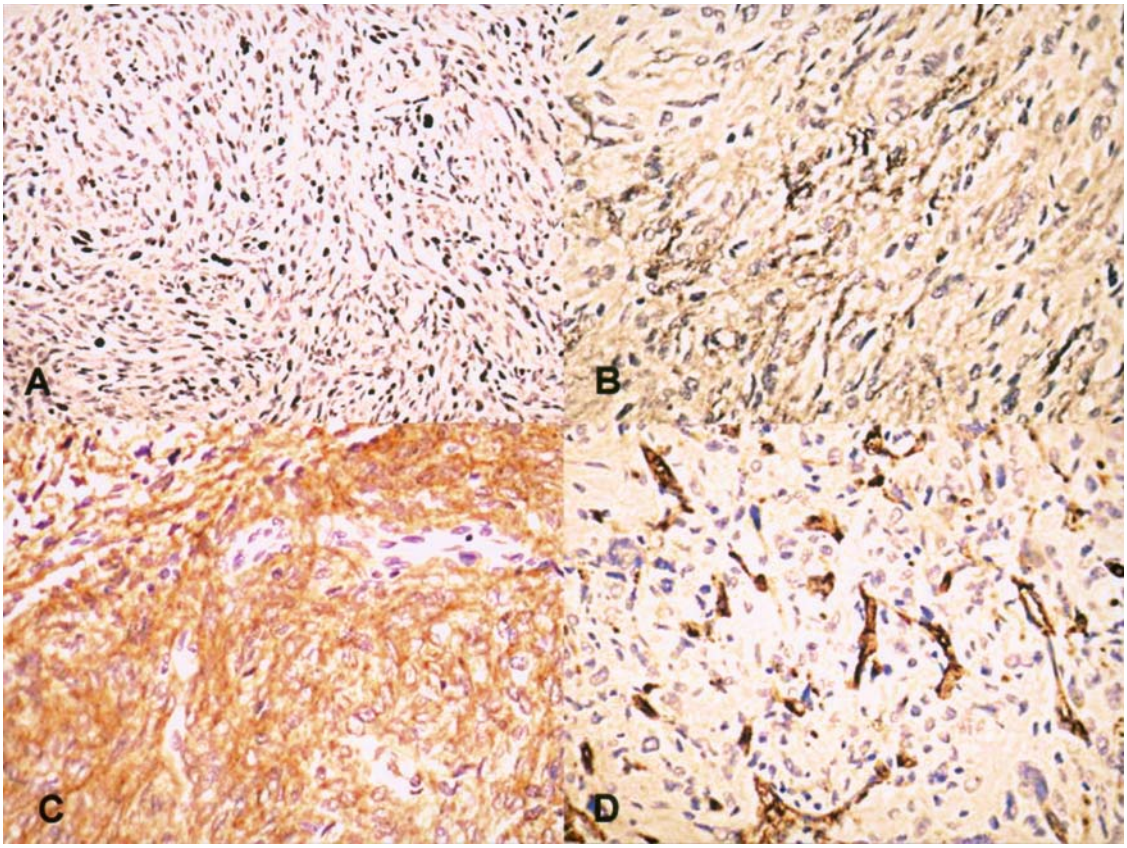

FIGURE 19.1. Immunohistochemistry performed on a malignant phyllodes tumor showing Ki-67 (MIB1) staining of nuclei (A); CD10 decoration of the cytoplasm of stromal cells (B); EGFR delineation of cytoplasm of stromal cells (C); CD34 accentuating arciform delicate microvessels, but not the stromal cells of the tumor (D)

further corroboration as there were only 17 cases that were CD117 stromal positive on immunohistochemistry in that study. If the role of CD117 in malignancy and recurrence of phyllodes tumors is affirmed, its expression can be advantageously harnessed for therapeutic purposes.

CD10

CD10, or the common acute lymphoblastic leukaemia antigen (CALLA), is a cell surface neutral endopeptidase expressed by lymphoid precursor cells and some B lymphoid cells. It is used in the immunohistochemical workup of hematolymphoid neoplasms. CD10 expression in the breast is not well-documented, with only a few reports on its expression in myoepithelial cells, and its use as a diagnostic myoepithelial marker (Moritani *et al.*, 2002). CD10 has recently been reported to be expressed in spindle cell neoplasia, and has been used to differentiate endometrial stromal sarcoma from leiomyoma and leiomyosarcoma. There are few studies of the expression of CD10 in mammary fibroepithelial lesions. In phyllodes tumors, immunohistochemical expression of CD10 increased with grade, from a low 3% to 6% expression in benign phyllodes tumors to a high 32–50% in

borderline and frankly malignant tumors (Mechtersheimer *et al*., 1990; Tse *et al*., 2005), with the difference being statistically significant (Figure 19.1B). Strong staining was seen in subepithelial areas of phyllodes tumors with higher stromal cellularity and mitotic activity, and it was concluded that CD10 may be a useful adjunct in the assessment of phyllodes tumor grade (Tse *et al*., 2005).

Epidermal growth factor receptor

The epidermal growth factor receptor (EGFR) is the cell-surface receptor for members of the epidermal growth factor family of extracellular protein ligands. EGFR belongs to the family of four closely related tyrosine receptor kinases: EGFR (ErbB-1), HER-2 (ErbB-2), HER-3 (ErbB-3) and HER-4 (ErbB-4). Mutations affecting EGFR are frequently encountered in cancers, and the EGFR signaling pathway is one of the most important pathways that regulates growth, survival, proliferation, and differentiation in mammalian cells. EGFR expression in phyllodes tumors is described as being expressed in 19% of all tumors, with a progressive increase from benign to malignant phyllodes, reaching a high 63% in frankly malignant phyllodes tumors, with reactivity predominantly in stromal cells (Figure 19.1C). This expression was also reported to be correlated with p53, p16, c-kit, cyclin A, and cyclin E (Kersting *et al*., 2006). Further analysis of EGFR positive overexpressed cases showed whole gene and intron 1 amplification in the majority of the cases, indicating that gene amplification is the main mechanism. This observation has significant implications, raising the possibility of using a tyrosine kinase inhibitor as a potential therapeutic agent in malignant cases.

Endothelin

Endothelins are potent vasoactive peptides that were originally isolated from vascular endothelial cells. They consist of ET1, ET2, and ET3, with ET2 and ET3 differing from ET1 by two and six amino acid residues, respectively. ET1 is expressed primarily by endothelial cells, ET2 by epithelial cells of the kidney and intestine, and ET3 is found in the brain. ET1 is an important member of the so-called endothelin axis, which includes the three endothelins, two receptors (ET-receptor A, and ET-receptor B), and two endothelin converting enzymes (ECE-1, ECE-2). While this axis is active in many cancers including breast cancer, there is also a relative low level of synthesis of endothelins by inflammatory cells such as macrophages and monocytes. Within the endothelin axis, ET1 preferentially binds to ETR-A compared to other ETs (ET2, ET3), as well as ETR-B. The binding of ET to the receptors will lead to activation of phospholipase C and MAPK pathways, an increase in intracellular calcium, and the induction of immediate early genes. Furthermore, endothelin may also lead to expression of vascular endothelial growth factor. In phyllodes tumors, both epithelial and stromal elements are decorated with ET1. Tse *et al*. (2007) showed variable immunohistochemical expression of ET1 in stromal cells of phyllodes tumors, with a progressive increase in parallel with tumor grade.

Heparan sulfate

Heparan sulfate is an important biomolecule that is essential in maintaining cell-cell and cell-extracellular matrix adhesion, mediating receptor-ligand binding, and regulating the activities of growth and

motility factors (Yip *et al*., 2006). Its primary structure is characterized by repeats of disaccharide units of a uronic acid and a derivative of glucosamine. The biosynthetic process of heparan sulfate is highly complicated whereby it can undergo modifications such as sulfation, epimerization, and acetylation to generate a great structural diversity of heparan sulfate chains. These chains are covalently attached to core proteins to form heparan sulfate proteoglycans. There is accumulating evidence highlighting the influence of heparan sulfate in modulating many physiological processes and diseases such as cancer. Studies have indicated that this molecule may be altered structurally during malignant transformation of colon cancer cells and indirectly promote growth factor signalling, leading to tumor cell proliferation. Differential heparan sulfation patterns in breast cancer cells have been demonstrated by various groups. Safaiyan *et al*. (1998) have shown that there is selective reduction of 6-O sulfation in heparan sulfate from transformed breast epithelial cells. Several reports have also shown that syndecan 1 is an important heparan sulfate proteoglycan in cell signalling and tumor cell progression in breast cancer (Yip *et al*., 2006).

In phyllodes tumors, there is higher stromal expression of heparan sulfate in higher histologic grades (Koo *et al*., 2006). Categorization of such tumors into their respective grades can be potentially aided by using a combination of 10E4 antibody together with antibodies against other well-known biomarkers MIB1 and p53. The relevance of heparan sulfate expression in breast phyllodes tumors in routine surgical pathology practice may lie in not only more accurate assignment of tumor grade, but also in the prediction of biologic behavior.

Microvessel density

Assessment of microvessel density has been shown to correlate with phyllodes tumor grade, with a progressive increase in microvessels from benign to borderline to malignant phyllodes tumors. This association is independent of mitotic count, nuclear pleomorphism, and stromal overgrowth, suggesting that angiogenesis may be an important independent predictor of malignancy (Tse *et al*., 2001, 2003). Microvessel density also disclosed correlation with stromal cellularity and margin status of phyllodes tumors; and the stromal microvessel density showed a statistically significant difference between benign and borderline phyllodes tumors. Identification of microvessels can be enhanced by immunohistochemical staining for endothelial cells using factor 8, CD31, and CD34. For CD34 immunohistochemistry, it is important to distinguish delineation of microvessels from decoration of the stromal spindle cells (Figure 19.1D).

Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is a potent angiogenic peptide that stimulates angiogenesis, and its immunohistochemical detection can therefore reflect the grade of phyllodes tumor. A retrospective review of 185 phyllodes tumors (105 benign, 51 borderline, 29 malignant) using VEGF immunohistochemistry showed expression in the epithelium in 29% and in the stromal cells in 31% of cases (Tse *et al*., 2004). There was a significant increase of VEGF in the stromal cells with increasing degree of

malignancy, but there was no correlation with VEGF expression in the epithelium. Statistical overlap of stromal VEGF and microvessel density in predicting malignancy suggested that angiogenesis may be an effector mechanism for VEGF. Assessment of stromal VEGF may therefore be useful as an adjunctive diagnostic criterion in the histologic assessment of malignancy in phyllodes tumors.

ROLE OF IMMUNOHISTOCHEMISTRY IN PROGNOSIS OF PHYLLODES TUMORS

Few biological markers are consistently demonstrated to have independent prognostic value in breast phyllodes tumors; examples are CD117 (Tan *et al*., 2005d), p53, and Ki-67 (Yonemori *et al*., 2006). In the latter study which involved 41 patients with 20 benign, 5 borderline, and 16 malignant phyllodes tumors, it was found that both p53 and MIB1 indices on immunohistochemistry were correlated with recurrence-free and overall survival. Feakins *et al*. (2000) discovered that stromal platelet derived growth factor (PDGF) receptor β positivity, and epithelial PDGR/stromal PDGFR β copositivity were significantly associated with disease related deaths in a series of 41 women with breast phyllodes tumors, and suggested that PDGF-dependent paracrine and autocrine pathways may be operative in the pathogenesis and prognosis of these tumors. However, the numbers were small, and it remains to be seen if these findings are borne out in larger prospective studies.

ROLE OF IMMUNOHISTOCHEMISTRY IN UNDERSTANDING THE PATHOGENESIS OF PHYLLODES TUMORS

Fibroepithelial neoplasms, including the phyllodes tumor, consist of a proliferation of both stromal and epithelial components. It seems that neoplastic activity occurs dominantly in the stromal rather than epithelial component of phyllodes tumors, and that the epithelium is a passive element within the tumor. Expression of various immunohistochemical markers in phyllodes tumors has also been described predominantly within the stromal region, lending weight to this perspective. Challenging this traditional concept, however, has been an investigation of the spatial relationship between stromal mitoses and the epithelial component that showed mitoses to occur more frequently close to rather than remote from the epithelial component, supporting the notion that stromal growth in these tumors is depended on the epithelial component and affirming the close interaction between these two compartments (Sawhney *et al*., 1992). Further corroboration of this interplay is afforded by findings of immunohistochemical expression of p53 that is accentuated in the perithelial region of the tumor (Millar *et al*., 1999; Kleer *et al*., 2001; Tse *et al*., 2002), as well as reports of p53 reactivity found in breast epithelium in conjunction with stromal staining (Dacic *et al*., 2002; Witte *et al*., 1999). Similarly, Sawyer *et al*. (2000) found distinct molecular alterations in both epithelial and stromal components of phyllodes tumors, indicating that they dually participated in

the neoplastic process. There was further evidence of a relationship lent by an association between stromal nuclear β-catenin with epithelial Wnt5a expression (Sawyer *et al*., 2002). Endothelin (ET1) staining in both epithelial cells and stromal cells also increased in a parallel manner with histologic grade of the tumor, confirming an intimate interaction between the epithelium and the stroma. ET1 can act in a paracrine fashion, suggesting that the ET axis may be involved in the pathogenesis of phyllodes tumors, and that the benign epithelial component may be an active participant of the pathogenetic process. As malignant progression occurs in phyllodes tumors, the stromal component loses the dependence on the epithelial element, and stromal overgrowth ensues (Tan *et al*., 2005b).

In conclusion, the biological markers examined in this review, they may have import in breast phyllodes tumors, they are by no means exhaustive. Our purpose was to describe those that have been either more frequently or recently discussed, and which can be evaluated on routine paraffin-embedded material using immunohistochemistry. While the list of light microscopic morphologic criteria for categorizing phyllodes tumors is well established is are unlikely to be supplanted, immunohistochemistry, which is readily available in surgical pathology laboratories, may be of potential use in corroborating histologic grade, determining prognosis or distinguishing it from mimics. More work needs to be done to ascertain which markers stand the test of time and scrutiny of larger studies in order to become part of the regular armamentarium of the process of grading and prognostication of phyllodes tumors (see Esposito and Dabbs in this volume).

REFERENCES

Akhilesh, M., Somani, A., and Tan, P.H. 2007. Core biopsies of fibroepithelial neoplasms: are there predictors of phyllodes tumors? *Pathol. Int. 57S*: A5–A6.

Barth, R.J. Jr., 1999. Histologic features predict local recurrence after breast conserving therapy of phyllodes tumors. *Breast Cancer Res. Treat. 57*: 291–295.

Carvalho, S., Silva, A.O., Milanezi, F., Ricardo, S., Leitão, D., Amendoeira, I., and Schmitt, F.C. 2004. c-KIT and PDGFRA in breast phyllodes tumours: overexpression without mutations? *J. Clin. Pathol.57*: 1075–1079.

Chaney, A.W., Pollack, A., Mcneese, M.D., Chaney, A.W., Pollack, A., McNeese, M.D., Zagars, G.K., Pisters, P.W., Pollock, R.E., and Hunt, K.K. 2000. Primary treatment of cystosarcoma phyllodes of the breast. *Cancer 89*: 1502–1511.

Chauhan, H., Abraham, A., Phillips, J.R., Pringle, J.H., Walker, R.A., and Jones, J.L. 2003. There is more than one kind of myofibroblast: analysis of CD34 expression in benign, in situ, and invasive breast lesions. *J. Clin. Pathol. 56*(4): 271–276.

Chen, C.M., Chen, C.J., Chang, C.L., Shyu, J.S., Hsieh, H.F., and Harn, H.J. 2000. CD34, CD117, and actin expression in phyllodes tumor of the breast. *J. Surg. Res. 94*: 84–91.

Cohn-Cedermark, G., Rutqvist, L.E., Rosendahl, I., and Silfverswärd, C. 1991. Prognostic factors in cystosarcoma phyllodes. A clinicopathologic study of 77 patients. *Cancer 68*: 2017–2022.

Dacic, S., Kounelis, S., Kouri, E., and Jones, M.W. 2002. Immunohistochemical profile of cystosarcoma phyllodes of the breast: a study of 23 cases. *Breast J.8*: 376–381.

Dunne, B., Lee, A.H.S., Pinder, S.E., Bell, J.A., and Ellis, I.O. 2003. An immunohistochemical study of metaplastic spindle cell carcinoma, phyllodes tumor and fibromatosis of the breast. *Hum. Pathol. 34*: 1009–1015.

el-Naggar, A.K., Ro, J.Y., McLemore, D., and Garnsy, L. 1990. DNA content and proliferative activity of cystosarcoma phyllodes of the breast. Potential prognostic significance. *Am. J. Clin. Pathol. 93*: 480–485.

Feakins, R.M., Mulcahy, H.E., Nickols, C.D., and Wells, C.A. 1999. p53 expression in phyllodes

tumours is associated with histological features of malignancy but does not predict outcome. *Histopathology 35*: 162–169.

Feakins, R.M., Wells, C.A., Young, K.A., and Sheaff, M.T. 2000. Platelet-derived growth factor expression in PT and fibroadenomas of the breast. *Hum. Pathol. 31*: 1214–1222.

Gatalica, Z., Finkelstein, S., Lucio, E., Tawfik, O., Palazzo, J., Hightower, B., and Eyzaguirre, E. 2001. p53 protein expression and gene mutation in phyllodes tumors of the breast. *Pathol. Res. Pract. 197*: 183–187.

Hayat, M.A. Ed. 2004–2006. *Immunohistochemistry and In situ Hybridization of Human Cracinomas,* Vols. 1–4. Elsevier/Academic, San Diego, CA.

Jacobs, T.W., Chen, Y.Y., Guinee, D.G., Holden, J.A., Cha, I., Bauermeister, D.E., Hashimoto, B., Wolverton, D., and Hartzog, G. 2005. Fibroepithelial lesions with cellular stroma on breast core needle biopsy. Are there predictors of outcome on surgical excision? *Am. J. Clin. Pathol. 124*: 342–354.

Kersting, C., Kuijper, A., Schmidt, H., Packeisen, J., Liedtke, C., Tidow, N., Gustmann, C., Hinrichs B., Wulfing, P., Tio, J., Boecker, W., van Diest, P., Brandt, B., and Buerger, H. 2006. Amplifications of the epidermal growth factor receptor gene (egfr) are common in phyllodes tumors of the breast and are associated with tumor progression. *Lab. Invest. 86*: 54–61.

Kitamura, Y., and Hirotab, S. 2004. Kit as a human oncogenic tyrosine kinase. *Cell Mol. Life Sci. 61*: 2924–2931.

Kleer, C.G., Giordano, T.J., Braun, T., and Oberman, H.A. 2001. Pathologic, immunohistochemical, and molecular features of benign and malignant phyllodes tumors of the breast. *Modern Pathol.14*: 185–190.

Kocova, L., Skalova, A., Fakan, F., and Rousarova, M. 1998. Phyllodes tumor of the breast: immunohistochemical study of 37 tumours using MIB1 antibody. *Pathol Res. Pract. 194*: 97–104.

Koo, C.Y., Bay, B.H., Lui, P.C.Y., Tse, G.M.K., Tan, P.H., and Yip, G.W.C. 2006. Immunohistochemical expression of heparan sulfate correlates with stromal cell proliferation in breast phyllodes tumors. *Modern Pathol. 19*(10): 1344–1350.

Kuenen-Boumeester, V., Henzen-Logmans, S.C., Timmermans, M.M., van Staveren, I.L., van Geel, A., Peeterse, H.J., Bonnema, J., and Berns, E.M. 1999. Altered expression of p53 and its regulated proteins in phyllodes tumours of the breast. *J. Pathol. 189*: 169–175.

Mechtersheimer, G., Kruger, K.H., Born, I.A., and Moller, P. 1990. Antigenic profile of mammary fibroadenoma and cystosarcoma phyllodes. A study using antibodies to estrogen- and progesterone receptors and to a panel of cell surface molecules. *Pathol. Res. Pract. 186*: 427–438.

Millar, E.K., Beretov, J., Marr, P., Sarris, M., Clarke, R.A., Kearsley, J.H., and Lee, C.S. 1999. Malignant phyllodes tumours of the breast display increased stromal p53 protein expression. *Histopathology 34*: 491–496.

Moffat, C.J., Pinder, S.E., Dixon, A.R., Elston, C.W., Blamey, R.W., and Ellis, I.O. 1995. Phyllodes tumours of the breast: a clinicopathological review of thirty-two cases. *Histopathology 27*: 205–218.

Moore, T., and Lee, A.H.S. 2001. Expression of CD34 and bcl-2 in phyllodes tumors, fibroadenomas and spindle cell lesions of the breast. *Histopathology 38*: 62–67.

Moritani, S., Kushima, R., Sugihara, H., Bamba, M., Kobayashi, T.K., and Hattori, T. 2002. Availability of CD10 immunohistochemistry as a marker of breast myoepithelial cells on paraffin sections. *Modern Pathol. 15*: 397–405.

Niezabitowski, A., Lackowska, B., Rys, J., Kruczak, A., Kowalska, T., Mitus, J., Reinfuss, M., and Markiewicz, D. 2001. Prognostic evaluation of proliferative activity and DNA content in the phyllodes tumor of the breast: immunohistochemical and flow cytometric study of 118 cases. *Breast Cancer Res. Treat. 65*: 77–85.

Reinfuss, M., Mitus, J., Duda, K., Stelmach, A., Ryś, J., and Smolak, K. 1996. The treatment and prognosis of patients with PT of the breast. An analysis of 170 cases. *Cancer 77*: 910–916.

Safaiyan, F., Lindahl, U., and Salmivirta, M. 1998. Selective reduction of 6-O-sulfation in heparan sulfate from transformed mammary epithelial cells. *Eur. J. Biochem. 252*: 576–582.

Sawhney, N., Garrahan, N., Douglas-Jones, A.G., and Williams, E.D. 1992. Epithelial-stromal interactions in tumors. A morphologic study of fibroepithelial tumors of the breast. *Cancer 70*: 2115–2120.

Sawyer, E.J., Hanby, A.M., Ellis, P., Lakhani, S.R., Ellis, I.O., Boyle, S., and Tomlinson, I.P. 2000.

Molecular analysis of phyllodes tumors reveals distinct changes in the epithelial and stromal components. *Am. J. Pathol. 156*: 1093–1098.

Sawyer, E.J., Hanby, A.M., Rowan, A.J., Gillett, C.E., Thomas, R.E., Poulsom, R., Lakhani, S.R., Ellis, I.O., Ellis, P., and Tomlinson, I.P. 2002. The Wnt pathway, epithelial-stromal interactions, and malignant progression in phyllodes tumours. *J. Pathol. 196*: 437–444.

Sawyer, E.J., Poulsom, R., Hunt, F.T., Jeffery, R., Elia, G., Ellis, I.O., Ellis, P., Tomlinson, I.P., and Hanby, A.M. 2003. Malignant phyllodes tumors show stromal overexpression of c-myc and c-kit. *J. Pathol. 200*: 59–64.

Silverman, J.S., and Tamsen, A. 1996. Mammary fibroadenoma and some phyllodes tumour stroma are composed of CD34+ fibroblasts and factor XIIIa+ dendrophages. *Histopathology 29*: 411–419.

Tan, P.H., Bay, B.H., Yip, G., Selvarajan, S., Tan, P., Wu, J., Lee, C.H., and Li, K.B. 2005a. Immunohistochemical detection of Ki-67 in breast cancer correlates with transcriptional regulation of genes related to apoptosis and cell death. *Modern Pathol. 18*: 374–381.

Tan, P.H., Jayabaskar, T., Chuah, K.L., Lee, H.Y., Tan, Y., Hilmy, M., Hung, H., Selvarajan, S., and Bay, B.H. 2005b. Phyllodes tumors of the breast: the role of pathologic parameters. *Am. J. Clin. Pathol. 123*(4): 1–12.

Tan, P.H., Aw, M.Y., Yip, G., Bay, B.H., Sii, L.H., Murugaya, S., and Tse, G.M. 2005c. Cytokeratins in papillary lesions of the breast: is there a role in distinguishing intraductal papilloma from papillary ductal carcinoma in situ? *Am. J. Surg. Pathol. 29*: 625–632.

Tan, P.H., Jayabaskar, T., Yip, G., Tan, Y., Hilmy, M., Selvarajan, S., and Bay, B.H. 2005d. p53 and c-kit (CD117) protein expression as prognostic indicators in breast phyllodes tumors: a tissue microarray study. *Modern Pathol. 18*(12): 1527–1534.

Taylor, C.R., Shi, S.R., Barr, N.J., and Wu, N. 2006. *Techniques of immunohistochemistry: principles, pitfalls, standardization*. In: Dabbs D, editor. *Diagnostic Immunohistochemistry*. 2nd edition. Churchill Livingstone Elsevier, Philadelpia, PA, 1–42.

Tse, G.M., Ma, T.K., Chan, K.F., Law, B.K., Chen, M.H., Li, K.H., Chan, E.C., and Mak, M.K. 2001. Increased microvessel density in malignant and borderline mammary phyllodes tumours. *Histopathology 38*: 567–570.

Tse, G.M., Putti, T.C., Kung, F.Y., Scolyer, R.A., Law, B.K., Lau, T.S., and Lee, C.S. 2002. Increased p53 protein expression in malignant mammary phyllodes tumors. *Modern Pathol. 15*: 734–740.

Tse, G.M., Lui, P.C., Scolyer, R.A., Putti, T.C., Kung, F.Y., Law, B.K., Lau, T.S., and Lee, C.S. 2003. Tumour angiogenesis and p53 protein expression in mammary phyllodes tumors. *Modern Pathol. 16:* 1007–1013.

Tse, G.M., Lui, P.C., Lee, C.S., Kung, F.Y., Scolyer, R.A., Law, B.K., Lau, T.S., Karim, R., and Putti, T.C. 2004. Stromal expression of vascular endothelial growth factor correlates with tumor grade and microvessel density in mammary phyllodes tumors: a multicenter study of 185 cases. *Hum. Pathol. 35*: 1053–1057.

Tse, G.M., Tsang, A.K., Putti, T.C., Scolyer, R.A., Lui, P.C., Law, B.K., Karim, R.Z., and Lee, C.S. 2005. Stromal CD10 expression in mammary fibroadenomas and phyllodes tumours. *J. Clin. Pathol. 58*: 185–189.

Tse, G.M., Chaiwun, B., Lau, K.M., Scolyer, R., Lee, C.S., Karim, R.Z., Putti, T.C., Law, B.K., Lui, P.C., and Tan, P.H. 2007. Endothelin I expression correlates with atypical histological features in mammary phyllodes tumours. *J. Clin. Pathol. 60*: 1051–1056.

Umekita, Y., and Yoshida, H. 1998. Immunohistochemical study of hormone receptor and hormone-regulated protein expression in phyllodes tumour: comparison with fibroadenoma. *Virchows Arch. 433*: 311–314.

Witte, F., Honig, A., Mirecka, J., and Schauer, A. 1999. Cystosarcoma phyllodes of the breast: prognostic significance of proliferation and apoptosis associated genes. *Anticancer Res. 19*: 3355–3359.

Yip, G.W., Smollich, M., and Gotte, M. 2006. Therapeutic value of glycosaminoglycans in cancer. *Mol. Cancer Ther. 5*: 2139–2148.

Yonemori, K., Hasegawa, T., Shimizu, C., Shibata, T., Matsumoto, K., Kouno, T., Ando, M., Katsumata, N., and Fujiwara, Y. 2006. Correlation of p53 and MIB-1 expression with both the systemic recurrence and survival in cases of phyllodes tumors of the breast. *Pathol. Res. Pract. 202*(10): 705–12. Epub 2006 Aug 2.

20
Phyllodes Tumor of the Breast: Prognostic Assessment Using Immunohistochemistry

Nicole Nicosia Esposito and David J. Dabbs

INTRODUCTION

Fibroepithelial tumors of the breast represent a heterogeneous group of biphasic neoplasms that range from benign to malignant and include fibroadenomas and phyllodes tumors. Phyllodes tumors, first fully characterized by Johannes Müller in 1838, constitute 0.3–1% of all breast neoplasms and were considered benign until the first reported case of a metastatic phyllodes tumor in 1931 by Lee and Pack. The term "cystosarcoma phyllodes" was initially described by Müller based on the tumor's "leaflike" projections into cystic spaces, sarcomatous stroma, and "fleshy" gross appearance. This term has since been discouraged as > 70% of these lesions follow a benign course and only rarely exhibit cystic degeneration; "phyllodes tumor", coined by the World Health Organization in 1981, is now the preferred term.

Clinically, phyllodes tumors resemble fibroadenomas but tend to occur in older women. As described by Lifshitz *et al.* (2003), they appear mammographically as lobulated, round, or oval masses with well-circumscribed borders and rarely contain calcifications. Histologically, phyllodes tumors are characterized by a double-layered epithelial component arranged in cleft-like ducts surrounded by a variably cellular spindled-cell stroma. The stromal component is usually prominent and can show morphologic patterns that range from fibroadenoma-like to frankly sarcomatous. Though comprised of both epithelium and stroma, it has been established through clonal analysis that the stroma alone is the neoplastic compartment in these tumors. Standard therapy includes mastectomy or wide local excision, depending on the degree of malignity and tumor size. Axillary lymph node dissection is currently not routinely performed, as the rate of lymph node metastases is < 1%. Rather, like sarcomas in general, metastases are *via* the hematogenous route and are strictly composed of the sarcomatous element of the primary breast tumor. The most common metastatic sites are lung and bone. Overall recurrent and visceral metastatic rates range from 8% to

40% and 1% to 21%, respectively. Unlike infiltrating ductal and lobular carcinomas, in which the utility of adjuvant treatment is well-known, the role of postoperative radiotherapy and chemotherapy remains to be fully established in the treatment of phyllodes tumors.

Currently, phyllodes tumors are generally classified as benign, borderline, or malignant based on gross and microscopic features, including stromal cellularity, cellular pleomorphism, mitotic activity, margin appearance, and stromal distribution (Figure 20.1). Prognostic assessments based solely on histologic classification, however, can be problematic. For example, histologically benign phyllodes tumors have reportedly metastasized and many histologically malignant phyllodes tumors neither recur nor metastasize.

Various ancillary tools have thus been examined in efforts to resolve the disparity between histologic classification and the behavior of phyllodes tumors. Immunohistochemistry, the process of localizing specific proteins in tissues or cells based on antigen-antibody recognition, has been the most widely used method. Additional studies based on morphologic examination and, more recently, chromosomal and molecular analysis of phyllodes tumors have been performed. This chapter summarizes these investigations, with a special emphasis on immunohistochemical assessment of phyllodes tumors. For a comprehensive discussion of the application of immunohistochemistry to detect antigens, the reader is referred to Hayat (2004–2006).

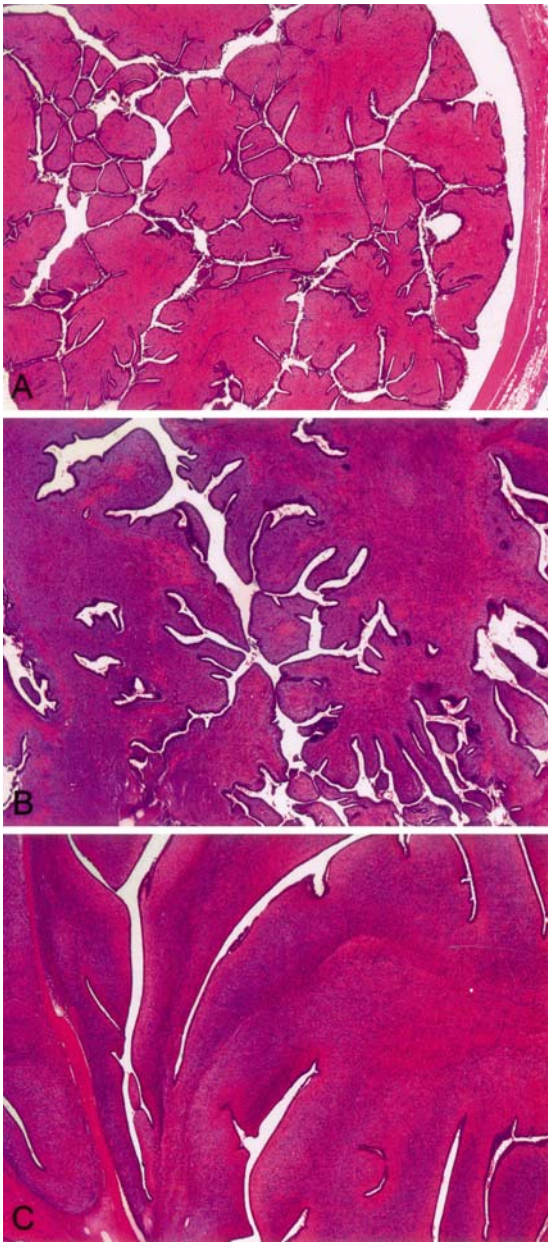

FIGURE 20.1. Phyllodes tumors. Phyllodes tumors are currently classified as benign (A), borderline (B), or malignant (C) based on degree of stromal cellularity, cellular atypia, mitotic rate, and the presence of pushing versus infiltrative borders (H&E, 20x)

IMMUNOHISTOCHEMICAL ANALYSIS AS A PREDICTOR OF PROGNOSIS IN PHYLLODES TUMORS

The most widely studied ancillary tool in recent years for the determination of prognosis in phyllodes tumors has been detection of proteins using immunohistochemistry. The proteins targeted are many, and can be grouped into markers of proliferation, tumor-suppressor genes, hormone receptors, and proteins with targeted therapy implications.

MARKER OF PROLIFERATION: KI-67 (MIB-1)

MIB-1 is a monoclonal antibody that reacts with Ki-67, a nuclear antigen expressed in non-G_0 proliferating cells. Ki-67 has been shown to be a useful predictor of prognosis in some tumors, including endometrial carcinomas, as reported by Oreskovic *et al*. (2004), and breast carcinomas, as reported by Molino *et al*. (1997). Several studies have examined the proliferative rate of phyllodes tumors using Ki-67 as a surrogate marker. Selected reports are summarized in Table 20.1. In addition to adequate resection margins, Niezabitowski *et al*. (2001) reported an inverse relationship between Ki-67 expression and overall survival in multivariate analysis. This finding has not been corroborated by studies by Kuijper *et al*. (2005), Kleer *et al*. (2001), or Esposito *et al*. (2006). Most reports do demonstrate increased Ki-67 expression with increased tumor grade. Nevertheless, the lack of a consistently reported relationship between Ki-67 expression and patient outcome suggests Ki-67 immunostaining cannot be used reliably to predict patient prognosis.

TABLE 20.1. Studies of Ki-67 in phyllodes tumors.

		Benign		*Borderline*		*Malignant*	
Reference	Year	n	LI^M (%)	n	LI^M (%)	n	LI^M (%)
Kuenen-Boumeester *et al*.	1999	10	5–6[a]	8	14–15[a]	1	33[a]
Kaya *et al*.	2001	31	5	–	–	–	–
Nieabitowski *et al*.	2001	52	1	23	6	42	12[a]
Kleer *et al*.	2001	7	4	7	16	6	50
Shpitz *et al*.	2002	16	5	4	14	3	25

LI^M = Mean labeling index.
[a] Calculated from data provided.

TUMOR-SUPPRESSOR GENE: P53

Mutations of the *p53* tumor-suppressor gene are one of the most common genetic abnormalities in cancer and are associated with increased expression in tumor cells by immunohistochemistry, apparently reflective of accumulation of mutant p53 molecules bound to heat-shock proteins. Evidence for *p53* mutations in phyllodes tumors is limited to a report by Kuenen-Boumeester *et al*. (1999), who reported a sequence alteration in codon 273 of the *p53* gene in one case of a malignant phyllodes that ultimately lead to the patient's death. As reported by Birch *et al*. (2001), phyllodes tumors are, however, found at an increased frequency relative to general population rates in patients with Li-Fraumeni syndrome, which results from a *p53* germline mutation. Most investigators have studied p53 at the protein level in phyllodes tumors *via* immunohistochemistry. p53 stromal expression in phyllodes tumors appears to be correlated

TABLE 20.2. Studies of p53 expression in phyllodes tumors.

		Benign	*Borderline*	*Malignant*
Reference	Year	+/n (%)	+/n (%)	+/n (%)
Millar *et al.*	1999	4/9 (44%)	–	6/6 (100%)
Suo and Nesland	2000	5/11 (45%)	7/7 (100%)	4/4 (100%)
Shpitz *et al.*	2002	1/16 (6%)	1/4 (25%)	2/3 (67%)
Tse *et al.*	2002	46/87 (53%)	21/37 (57%)	16/19 (84%)
Dacic *et al.*	2002	–	7/14 (50%)	6/9 (67%)

with increasing tumor grade, as evident in Table 20.2. In a study by Tse *et al.* (2002), p53 expression did not predict occurrence of tumor recurrence. Similarly, Shpitz *et al.* (2002) noted p53 correlated with malignant histologic features of phyllodes tumors, but not to predisposition of these tumors for local recurrence. Finally, Feakins *et al.* (1999) concluded p53 is not a useful determinant of tumor recurrence or long-term survival. Thus, it appears that p53 expression, similar to Ki-67, correlates with tumor grade but not necessarily to patient outcome. The incidence of specific p53 mutations in these tumors, and their potential prognostic value, however, remains to be elucidated.

STEROID RECEPTORS: ESTROGEN AND PROGESTERONE RECEPTORS

Estrogen receptor (ER) and progesterone receptor (PR) expression in breast carcinomas is a well-established predictive factor of response to adjuvant endocrine therapy. The epithelium in fibroadenomas of the breast has also been shown to express ER and PR. Similarly, ER and PR expression in phyllodes tumors is limited to the epithelium in most reports. Umekita and Yoshida (1998) found phyllodes tumors and fibroadenomas have similar hormone receptor status: ER and PR were detected in 32% and 96% of phyllodes tumors and 28% and 96% of fibroadenomas, respectively. It was not until a report in 2006 by Sapino *et al.* that steroid hormone receptor expression was demonstrated in the stroma, the neoplastic component of phyllodes tumors. In this report, ER-beta was the only hormone receptor expressed by the stroma of phyllodes tumors, as well as fibroadenomas, both at the protein and mRNA levels. Interestingly, fibroadenomas that were ER-beta positive were more common in younger women, while phyllodes tumors that were ER-beta positive were more common in older women. The authors thus concluded that a hormone-receptor mechanism may regulate the growth of fibroadenomas, and that mechanisms independent from estrogen stimulation may be involved in the growth of phyllodes tumors. Because of this finding, in addition to (1) the lack of reports that have correlated hormone receptor expression with patient outcome and (2) the restriction of the majority of hormone receptor expression to the non-neoplastic epithelial component, assessment of hormone receptor expression has no current predictive or prognostic importance in phyllodes tumors.

PROTEINS WITH TARGETED THERAPY IMPLICATIONS

C-KIT (CD117)

Overexpression of the *c-kit* oncogene, which encodes a tyrosine-kinase transmembrane receptor protein, characterizes

gastrointestinal stromal tumors (GISTs) that, like phyllodes tumors, show a spectrum of behavior from benign to malignant. The finding of c-kit overexpression in GISTs led to the development of targeted therapy with the KIT-receptor tyrosine-kinase inhibitor, imatinib mesylate (STI-571). C-kit expression in phyllodes tumors has only relatively recently been described (Table 20.3). Chen *et al*. first reported c-kit expression in the stroma of phyllodes tumors in 2000, and found c-kit expression to be preferentially expressed in histologically malignant phyllodes tumors, though they did not report patient outcome. Since then, several additional studies have reported increased c-kit expression in malignant phyllodes tumors compared to benign and/or borderline tumors. None, however, were able to establish a relationship between c-kit expression and patient outcome, with the exception of Tan *et al*.'s report in 2005 of a direct relationship between c-kit expression and recurrent disease. C-kit expression also correlated with stromal overgrowth in the same report, and multivariate analysis was not performed. Furthermore, using GISTs as a model, any predictive response of phyllodes tumors to imatinib mesylate predicates on the presence of activating mutations in the *c-kit* oncogene. To date, despite c-kit expression in a subset of phyllodes tumors, the vast majority have not been found to harbor *c-kit* mutations.

TABLE 20.3. Studies of c-kit (CD117) expression in phyllodes tumors.

		Benign	*Borderline*	*Malignant*
Reference	Year	+/n (%)	+/n (%)	+/n (%)
Chen et al.	2000	1/7 (14%)	–	9/12 (75%)
Sawyer *et al.*	2003	1/20 (5%)	0/2 (0%)	5/8 (62%)
Tse *et al.*	2004	17/101 (16%)	12/50 (24%)	13/28 (46%)
Esposito *et al.*	2006	1/16 (6.3%)	5/8 (63%)	4/6 (66%)

EPIDERMAL GROWTH FACTOR RECEPTOR

Epidermal growth factor receptor (EGFR) is overexpressed in a subset of lung cancers, leading to inappropriate activation of the anti-apoptotic Ras signal pathway. Pao *et al*. (2004) demonstrated epidermal growth factor receptor overexpression results from mutations in the tyrosine kinase domain of the gene. Treatment with tyrosine kinase inhibitors, such as gefitinib and erlotinib, has thus proved successful in the treatment of lung adenocarcinomas with EGFR overexpression, and leads to tumor regression.

Epidermal growth factor receptor expression has only recently been explored in phyllodes tumors. Kersting *et al*. (2006) reported EGFR expression in stromal tumor cells in 19% of phyllodes tumors overall, and in 75% of histologically malignant tumors. Furthermore, they found all eight malignant phyllodes tumors included in the study bore either whole gene or intron 1 amplification. Any potential relationship between EGFR expression and/or amplification and patient prognosis could not be analyzed, because none of the eight malignant tumors recurred or metastasized. This finding nonetheless suggests a subset of phyllodes tumors have EGFR mutations; whether such mutations infer susceptibility to tyrosine kinase inhibitors, and whether EGFR stromal expression is prognostically significant, remains to be investigated.

HER-2/NEU (ERBB2)

HER-2/neu, a member of the epidermal growth factor receptor gene family, is overexpressed in ~25% of invasive ductal carcinomas of the breast. Though associated with more aggressive tumor behavior, the development of the drug trastuzumab, which binds to the extracellular domain of HER-2 and inhibits cell proliferation in HER-2-overexpressed tumors, has lead to significant improvements in patient survival. HER-2/neu expression in phyllodes tumors does not appear to bear any predictive or prognostic significance. For example, Shpitz *et al*. (2002) reported positive HER-2/neu staining in only the ductal epithelium in 61% of phyllodes tumors, and none of 41 phyllodes tumors studied by Yonemori *et al*. (2006) were positive for HER-2/neu.

CHROMOSOMAL AND MOLECULAR ANALYSIS AS A PREDICTOR OF PROGNOSIS IN PHYLLODES TUMORS

The lack of consistent, prognostically significant markers by immunohistochemistry has led some investigators to study phyllodes tumors at the chromosomal and molecular levels. The most consistently reported chromosomal abnormality in phyllodes tumor to date is gains of 1q. Lu *et al*. (1997) reported gains of 1q in seven of 18 (39%) phyllodes tumors examined. Interestingly, all cases with gain of 1q recurred, and gain of 1q was significantly associated with stromal overgrowth, though all seven cases were classified as "benign" histologically. Polito *et al*. (1998) reported gains of 1q in 50% of 10 "low-grade" phyllodes tumors. A later study by Lae *et al*. (2007) also reported gains of 1q as the most common chromosomal abnormality, being present in 12 of 30 (40%) tumors examined. This study, however, demonstrated 1q gains in only one of nine benign tumors, while present in 11 of 21 (51%) borderline or malignant tumors. Outcome was not reported in this study, and thus any conclusions regarding 1q gains and patient prognosis could not be rendered.

Finally, analysis of loss of heterozygosity (LOH) as a surrogate marker of genomic instability has been examined in a wide variety of tumors, and has been shown to be prognostically significant in follicular thyroid tumors, some breast carcinomas, as well as other tumors in these volumes. Loss of heterozygosity analysis of phyllodes tumors, however, is limited to a single report by Wang *et al*. (2006). In this study, the fractional allelic loss (FAL), derived from loss of heterozygosity studies using single nucleotide polymorphism (SNP) arrays, was significantly higher in malignant phyllodes tumors compared to low- and intermediate-grade tumors. Fractional allelic loss was also linearly correlated with tumor mitotic rate. Patient follow-up data was not reported, however, and thus any relationship between FAL and patient outcome could not be established.

HISTOPATHOLOGIC ANALYSIS AS A PREDICTOR OF PROGNOSIS IN PHYLLODES TUMORS

The gold standard of determination of tumor behavior, i.e., whether a tumor is benign or malignant, is histopathologic

examination at the light-microscopic level by pathologists in the vast majority of cases. Histologic criteria for diagnosing phyllodes tumors as benign or malignant were first clearly defined by Norris and Taylor in 1967. In their report, malignant phyllodes tumors were characterized by size ≥ 4 cm, infiltrative margins, moderate to severe stromal cell atypia, and/or a high stromal mitotic rate (usually > 10 mitoses per 10 high-power-field). However, no one feature was reliable in separating tumors that recurred or metastasized from those that did not recur or metastasize. Similarly, Pietruszka and Barnes (1978) stratified 42 cases into benign, borderline, and malignant categories based on tumor contour, degree of stromal atypia, and mitotic activity. In their study, "local recurrences were experienced by six patients and occurred in all three categories; however, neoplasms classified as "malignant" were the only ones that developed metastases.

More recent reports examining long-term outcome in relationship to histologic and clinical characteristics have included larger sample sizes. Barrio *et al*. (2007) analyzed 293 phyllodes tumors with a mean follow-up of 7.9 years. Tumor necrosis and fibroproliferation, defined as the presence of "fibroadenomatoid" nodules, in the surrounding breast, and positive surgical margins of resection were associated with a higher actuarial local recurrence rate in univariate analysis. However, only fibroproliferation and necrosis remained important predictors of local recurrence in multivariate analysis. In contrast, Tan *et al*. (2005) found negative margin status reduced recurrence hazards by 51.7% in multivariate analysis in their study of 335 phyllodes tumors. Similarly, Niezabitowski *et al*. (2001) reported adequate resection margins to independently influence overall survival in their multivariate analysis of 118 phyllodes tumors. The importance of "adequate" resection margins, usually defined as > 1 cm, as a predictor of tumor recurrence has also been shown in numerous additional albeit smaller studies by Esposito *et al*. (2006), Kapiris *et al*. (2001), Chen *et al*. (2005), Asoglu *et al*. (2004), Cheng *et al*. (2006), Kleer *et al*. (2001), and Ben Hassouna *et al*. (2006).

Besides adequate resection margins, stromal overgrowth in phyllodes tumors has been associated with increased recurrent rates as well as increased risk of metastatic disease. Stromal overgrowth is typically defined as an increased stromal to epithelial ratio such that stroma entirely occupies one 40X field under the light microscope. In frankly malignant phyllodes tumors with stromal overgrowth, heterologous differentiation, such as osteosarcomatous, chondrosarcomatous, or liposarcomatous elements, may be found (Figure 20.2). In a study by Kario *et al*. (1990) that included 34 cases of phyllodes tumors, stromal overgrowth correlated with other aggressive clinical and histologic features, such as rapid growth and increased mitotic rate, and with local recurrence. Similarly, Hawkins *et al*. (1992) found stromal overgrowth to be one of the most reliable predictors of metastases in their study of 33 phyllodes tumors, eight of which metastasized. In a larger study of 101 phyllodes tumors with an almost 7-year mean follow-up time by Chaney *et al*. (2000), patients with stromal overgrowth had significantly lower 5-year and 10-year survival rates than patients without stromal overgrowth. It is important to note, however, that almost all

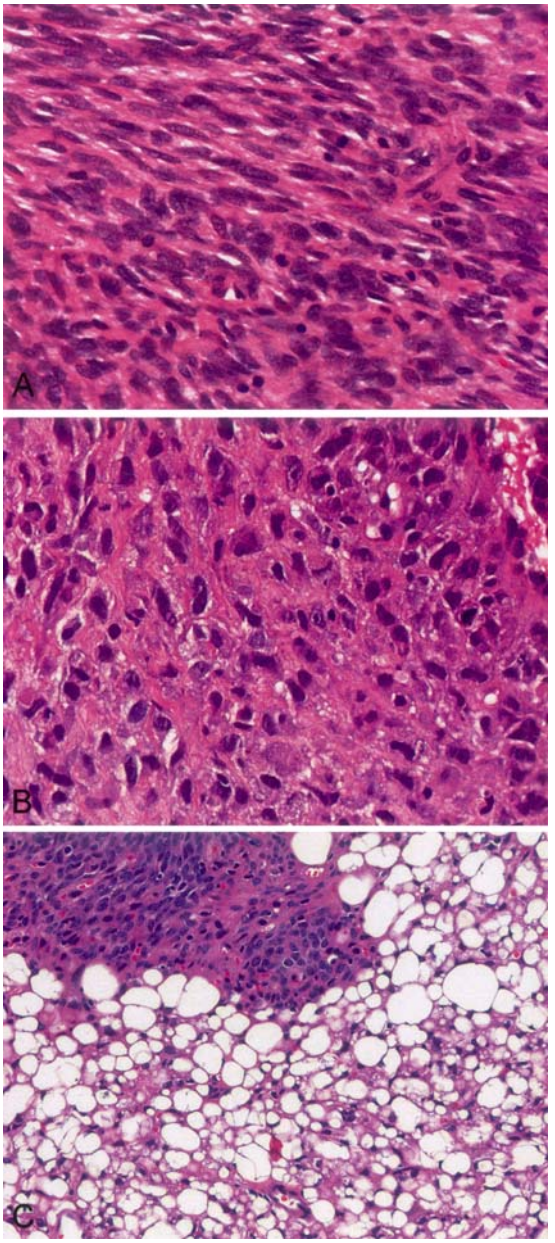

FIGURE 20.2. Stromal overgrowth in malignant phyllodes tumors. Defined as the presence of a hypercellular stroma without epithelial elements in one 40X field, stromal overgrowth is a key histologic finding in malignant phyllodes tumors. Depicted here are different patterns of stromal overgrowth seen in three malignant phyllodes tumors: fibrosarcoma-like stroma (A), pleomorphic stromal cells (B), and heterologous liposarcomatous stroma (C) (H&E, 400x)

of the cases in this study that had stromal overgrowth were classified as "malignant" histologically. This underscores the association of stromal overgrowth with malignancy, and cautions against the designation of tumors as "benign" or "borderline" if stromal overgrowth is present (see Tan *et al.*, in this volume).

CONCLUSION

Though numerous, studies aimed at developing better prognostic markers in phyllodes tumors have suffered from low sample size, owing to the rarity of this lesion, lack of patient follow-up data, lack of reproducibility, and/or lack of correlation between variables and patient outcome. Nevertheless, the most significant consistently reported variables in predicting recurrence are adequate resection margins, defined as a tumor to resection margin distance of > 1 cm, and the presence of stromal overgrowth. Studies aimed at detecting proteins by immunohistochemistry have failed to produce a single reliable biomarker for prognostic assessment, though the potential significance of some proteins, such as EGFR, are yet to be determined. Likewise, chromosomal and/or molecular analysis may prove useful in predicting the behavior of phyllodes tumors, but data are limited to date. Currently, prognostic assessment in phyllodes tumors is thus best made by combining the multiple clinical and pathologic factors examined in this chapter, such as tumor size, growth rate, histologic parameters such as stromal overgrowth, and the adequacy of resection margins.

REFERENCES

Asoglu, O., Ugurlu, M. M., Blanchard, K., Grant, C. S., Reynolds, C., Cha, S. S., and Donohue, J. H. 2004. Risk factors for recurrence and death after primary surgical treatment of malignant phyllodes tumors. *Ann. Surg. Oncol. 11*: 1011–1017.

Barrio, A. V., Clark, B. D., Goldberg, J. I., Hoque, L. W., Bernik, S. F., Flynn, L. W., Susnik, B., Giri, D., Polo, K., Patil, S., and Van Zee, K. J. 2007. Clinicopathologic features and long-term outcomes of 293 phyllodes tumors of the breast. *Ann. Surg. Oncol* [Epub ahead of print].

Ben Hassouna, J., Damak, T., Gamoudi, A., Chargui, R., Khomsi, F., Mahjoub, S., Slimene, M., Ben Dhiab, T., Hechiche, M., Boussen, H., and Rahal, K. 2006. Phyllodes tumors of the breast: a case series of 106 patients. *Am. J. Surg. 192*: 141–147.

Birch, J. M., Alston, R. D., McNally, R. J., Evans, D. G., Kelsey, A. M., Harris, M., Eden, O. B., and Varley, J. M. 2001. Relative frequency and morphology of cancers in carriers of germline TP53 mutations. *Oncogene 20*: 4621–4628.

Chaney, A. W., Pollack, A., McNeese, M. D., Zagars, G. K., Pisters, P. W., Pollock, R. E., and Hunt, K. K. 2000. Primary treatment of cystosarcoma phyllodes of the breast. *Cancer 89*: 1502–1511.

Chen, C. M., Chen, C. J., Chang, C. L., Shyu, J. S., Hsieh, H. F., and Harn, H. J. 2000. CD34, CD117, and actin expression in phyllodes tumor of the breast. *J. Surg. Res. 94*: 84–91.

Chen, W. H., Cheng, S. P., Tzen, C. Y., Yang, T. L., Jeng, K. S., Liu, C. L., and Liu, T. P. 2005. Surgical treatment of phyllodes tumors of the breast: retrospective review of 172 cases. *J. Surg. Oncol. 91*: 185–194.

Cheng, S. P., Chang, Y. C., Liu, T. P., Lee, J. J., Tzen, C. Y., and Liu, C. L. 2006. Phyllodes tumor of the breast: the challenge persists. *World J. Surg. 30*: 1414–1421.

Dacic, S., Kounelis, S., Kouri, E., and Jones, M. W. 2002. Immunohistochemical profile of cystosarcoma phyllodes of the breast: a study of 23 cases. *Breast J. 8*: 376–381.

Esposito, N. N., Mohan, D., Brufsky, A., Lin, Y., Kapali, M., and Dabbs, D. J. 2006. Phyllodes tumor: a clinicopathologic and immunohistochemical study of 30 cases. *Arch. Pathol. Lab. Med. 130*: 1516–1521.

Feakins, R. M., Mulcahy, H. E., Nickols, C. D., and Wells, C. A. 1999. p53 expression in phyllodes tumours is associated with histological features of malignancy but does not predict outcome. *Histopathology 35*: 162–169.

Hawkins, R. E., Schofield, J. B., Fisher, C., Wiltshaw, E., and McKinna, J.A. 1992. The clinical and histologic criteria that predict metastases from cystosarcoma phyllodes. *Cancer 69*: 141–147.

Hayat, M. A. Ed. 2004–2006. Immunohistochemistry and *In Situ* Hybridization of Human Carcinomas. Vols. 1–4. Elsevier/Academic, San Diego, CA.

Kapiris, I., Nasiri, N., A'Hern, R., Healy, V., and Gui, G. P. 2001. Outcome and predictive factors of local recurrence and distant metastases following primary surgical treatment of high-grade malignant phyllodes tumours of the breast. *Eur. J. Surg. Oncol. 27*: 723–730.

Kario, K., Maeda, S., Mizuno, Y., Makino, Y., Tankawa, H., and Kitazawa, S. 1990. Phyllodes tumor of the breast: a clinicopathologic study of 34 cases. *J. Surg. Oncol. 45*: 46–51.

Kaya, R., Pestereli, H. E., Erdogan, G., Gulkesen, K. H., and Karaveli, S. 2001. Proliferating activity in differential diagnosis of benign phyllodes tumor and cellular fibroadenomas: is it helpful? *Pathol. Oncol. Res. 7*: 213–216.

Kersting, C., Kuijper, A., Schmidt, H., Packeisen, J., Liedtke, C., Tidow, N., Gustmann, C., Hinrichs, B., Wulfing, P., Tio, J., Boecker, W., van Diest, P., Brandt, B., and Buerger, H. 2006. Amplifications of the epidermal growth factor receptor gene (egfr) are common in phyllodes tumors of the breast and are associated with tumor progression. *Lab. Invest. 86*: 54–61.

Kleer, C. G., Giordano, T. J., Braun, T., and Oberman, H. A. 2001. Pathologic, immunohistochemical, and molecular features of benign and malignant phyllodes tumors of the breast. *Mod. Pathol. 14*: 185–190.

Kuenen-Boumeester, V., Henzen-Logmans, S. C., Timmermans, M. M., van Staveren, I. L., van Geel, A., Peeterse, H. J., Bonnema, J., and Berns, E. M. 1999. Altered expression of p53 and its regulated proteins in phyllodes tumours of the breast. *J. Pathol. 189*: 169–175.

Kuijper, A., van der Groep, P., van der Wall, E., and van Diest, P. J. 2005. Expression of hypoxia-inducible factor 1 alpha and its downstream targets in fibroepithelial tumors of the breast. *Breast Cancer Res. 7*: R808–R818.

Lae, M., Vincent-Salomon, A., Savignoni, A., Huon, I., Freneaux, P., Sigal-Zafrani, B., Aurias, A., Sastre-Garau, X., and Couturier, J. 2007. Phyllodes tumors of the breast segregate in two groups according to genetic criteria. *Mod. Pathol. 20*: 435–444.

Lee, B. J., and Pack, G. T. 1931. Giant intracanalicular fibroadenomyxoma of the breast. The so-called cystosarcoma phylloides mammae of Johannes Muller. *Am. J. Cancer 15*: 2583–2609.

Lifshitz, O. H., Whitman, G. J., Sahin, A. A., and Yang, W. T. 2003. Radiologic-pathologic conferences of the University of Texas M.D. Anderson Cancer Center. Phyllodes tumor of the breast. *AJR Am. J. Roentgenol. 180*: 332.

Lu, Y. J., Birdsall, S., Osin, P., Gusterson, B., and Shipley, J. 1997. Phyllodes tumors of the breast analyzed by comparative genomic hybridization and association of increased 1q copy number with stromal overgrowth and recurrence. *Genes Chromos. Cancer 20*: 275–281.

Millar, E. K., Beretov, J., Marr, P., Sarris, M., Clarke, R. A., Kearsley, J. H., and Lee, C. S. 1999. Malignant phyllodes tumours of the breast display increased stromal p53 protein expression. *Histopathology 34*: 491–496.

Molino, A., Micciolo, R., Turazza, M., Bonetti, F., Piubello, Q., Bonetti, A., Nortilli, R., Pelosi, G., and Cetto, G. L. 1997. Ki-67 immunostaining in 322 primary breast cancers: associations with clinical and pathological variables and prognosis. *Int. J. Cancer 74*: 433–437.

Müller, J. 1838. Ueber den feinern Bau und die Formen der krankhaften Geschwülste. *1*: 54–57.

Niezabitowski, A., Lackowska, B., Rys, J., Kruczak, A., Kowalska, T., Mitus, J., Reinfuss, M., and Markiewicz, D. 2001. Prognostic evaluation of proliferative activity and DNA content in the phyllodes tumor of the breast: immunohistochemical and flow cytometric study of 118 cases. *Breast Cancer Res. Treat. 65*: 77–85.

Norris, H. J., and Taylor, H. B. 1967. Relationship of histologic features to behavior of cystosarcoma phyllodes. Analysis of ninety-four cases. *Cancer 20*: 2090–2099.

Oreskovic, S., Babic, D., Kalafatic, D., Barisic, D., and Beketic-Oreskovic, L. 2004. A significance of immunohistochemical determination of steroid receptors, cell proliferation factor Ki-67 and protein p53 in endometrial carcinoma. *Gynecol. Oncol. 93*: 34–40.

Organization, W. H. 1981. *Histologic Typing of Breast Tumors*. Vol. *2*, 2nd ed. Geneva, Switzerland: World Health Organization.

Pao, W., Miller, V., Zakowski, M., Doherty, J., Politi, K., Sarkaria, I., Singh, B., Heelan, R., Rusch, V., Fulton, L., Mardis, E., Kupfer, D., Wilson, R., Kris, M., and Varmus, H. 2004. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc. Natl. Acad. Sci. USA 101*: 13306–13311.

Pietruszka, M., and Barnes, L. 1978. Cystosarcoma phyllodes: a clinicopathologic analysis of 42 cases. *Cancer 41*: 1974–1983.

Polito, P., Cin, P. D., Pauwels, P., Christiaens, M., Van den Berghe, I., Moerman, P., Vrints, L., and Van den Berghe, H. 1998. An important subgroup of phyllodes tumors of the breast is characterized by rearrangements of chromosomes 1q and 10q. *Oncol Rep. 5*: 1099–1102.

Sapino, A., Bosco, M., Cassoni, P., Castellano, I., Arisio, R., Cserni, G., Tos, A. P., Fortunati, N., Catalano, M. G., and Bussolati, G. 2006. Estrogen receptor-beta is expressed in stromal cells of fibroadenoma and phyllodes tumors of the breast. *Mod. Pathol. 19*: 599–606.

Sawyer, E. J., Poulsom, R., Hunt, F. T., Jeffery, R., Elia, G., Ellis, I. O., Ellis, P., Tomlinson, I. P., and Hanby, A. M. 2003. Malignant phyllodes tumours show stromal overexpression of c-myc and c-kit. *J. Pathol. 200*: 59–64.

Shpitz, B., Bomstein, Y., Sternberg, A., Klein, E., Tiomkin, V., Kaufman, A., Groisman, G., and Bernheim, J. 2002. Immunoreactivity of p53, Ki-67, and c-erbB-2 in phyllodes tumors of the breast in correlation with clinical and morphologic features. *J. Surg. Oncol. 79*: 86–92.

Suo, Z., and Nesland, J. M. 2000. Phyllodes tumor of the breast: EGFR family expression and relation to clinicopathological features. *Ultrastruct. Pathol. 24*: 371–381.

Tan, P. H., Jayabaskar, T., Chuah, K. L., Lee, H. Y., Tan, Y., Hilmy, M., Hung, H., Selvarajan, S., and Bay, B. H. 2005. Phyllodes tumors of the breast: the role of pathologic parameters. *Am. J. Clin. Pathol. 123*: 529–540.

Tse, G. M., Putti, T. C., Kung, F. Y., Scolyer, R. A., Law, B. K., Lau, T. S., and Lee, C. S. 2002. Increased p53 protein expression in malignant mammary phyllodes tumors. *Mod. Pathol. 15*: 734–740.

Tse, G. M., Putti, T. C., Lui, P. C., Lo, A. W., Scolyer, R. A., Law, B. K., Karim, R., and Lee, C. S. 2004. Increased c-kit (CD117) expression in malignant mammary phyllodes tumors. *Mod. Pathol. 17*: 827–831.

Umekita, Y., and Yoshida, H. 1998. Immunohistochemical study of hormone receptor and hormone-regulated protein expression in phyllodes tumour: comparison with fibroadenoma. *Virchows Arch. 433*: 311–314.

Wang, Z. C., Buraimoh, A., Iglehart, J. D., and Richardson, A. L. 2006. Genome-wide analysis for loss of heterozygosity in primary and recurrent phyllodes tumor and fibroadenoma of breast using single nucleotide polymorphism arrays. *Breast Cancer Res. Treat. 97*: 301–309.

Yonemori, K., Hasegawa, T., Shimizu, C., Shibata, T., Matsumoto, K., Jouno, T., Ando, M., Katsumata, N., and Fujiwara, Y. 2006. Correlation of p53 and MIB-1 expression with both the systemic recurrence and survival in cases of phyllodes tumors of the breast. *Pathol. Res. Pract. 202*: 705–712.

21
Metaplastic Breast Carcinoma: Detection Using Histology and Immunohistochemistry

Tibor Tot

INTRODUCTION

Metaplastic breast carcinoma represents a group of rare diseases deviating from each other not only in their morphological appearance, but also in their prognosis. In addition to the routine histological analysis, immunohistochemistry plays a crucial role in delineating these tumors from usual breast carcinomas and sarcomas, in subtyping of these tumors into different prognostic categories and in the prediction of their response to individual therapy. Despite the wide variety of microscopic morphology of the lesions that are actually classified as metaplastic breast carcinoma, histological subtyping and grading separate properly patients with low-risk tumors adequately treated with wide surgical excision only and high-risk tumors requiring adjuvant therapy. Immunohistochemical receptor analysis shows presence or absence of therapeutic target molecules, and therefore is essential for adequate therapy planning.

Definition

Metaplasia means a pathological process during which a mature epithelial or mesenchymal tissue becomes transformed into another mature tissue. For example, simple glandular epithelium may become multilayered and squamous as a result of chronic irritation. Correspondingly, a metaplastic process in malignant tumors should mean a transformation of one type of malignant tissue to another: carcinoma to sarcoma, adenocarcinoma to squamous cancer. This process may affect different proportions of the tumor tissue and, in extreme, the entire tumor. As breast is a glandular organ, most of its malignant tumors are expected to be, and are, adenocarcinomas (epithelial malignant tumors showing glandular differentiation); thus metaplastic breast carcinoma is a primary breast tumor which is epithelial in origin, malignant and exhibits partly or entirely non-glandular differentiation.

According to the above definition, metaplastic carcinoma is a tumor initially

developed as adenocarcinoma but shifted its phenotype during progression. Another possibility is divergent differentiation of the tumor cells at the same time. Genetic analysis of certain type of metaplastic carcinomas supports the latter possibility (Zhuang *et al*., 1997). Metaplasia, neoplasia, and differentiation represent pathological processes which cannot be visualized; the morphologist can see only their end-result: a tumor showing partly glandular, partly non-glandular differentiation. In other words, a carcinoma partly consisting of structures of adenocarcinoma and partly of structures of another malignant tumor fills the criteria of metaplastic cancer. Adenosquamous carcinoma showing partly glandular and partly squamous differentiation, mucoepidermoid tumors, showing partly mucin-producing, partly squamous structures as well as tumors showing partly glandular and partly mesenchymal differentiation (the category termed "carcinosarcoma") belong to this tumor group. On the other hand, biphasic lesions with one or several benign components, such as phylloides tumor, adenomyoepithelioma etc., are excluded from this category. Regressive changes in the stroma of otherwise typical adenocarcinoma (e.g., ossification or cellular desmoplasia) do not qualify the lesion for metaplastic cancer. Tumors with non-neoplastic, epithelial-like cells in the stroma (like carcinomas with osteoclastic stromal giant cells) are not included into this category.

The definition raises the question of the minimal transformed proportion of the tumor required for establishing the diagnosis of metaplastic breast carcinoma. Whether a tumor with only minor foci of metaplastic changes in an otherwise ordinary ductal carcinoma should be classified as metaplastic cancer is unclear. Carter *et al*. (2006) suggest a kind of cut-off value, at least 20% of the tumor to be metaplastic for making this diagnosis. The second question raised by this definition is whether an adenocarcinoma entirely transformed into a non-glandular malignant tumor still represents a metaplastic carcinoma. Such a possibility theoretically exists and, in practice, points out the importance of generous sampling of the breast tumors for histological assessment to reach the proper diagnosis. However, the alternative of primary squamous carcinomas and primary sarcomas of the breast is a valid differential diagnostic option. Primary squamous carcinomas and primary sarcomas of the breast, on the other hand, seem to be much more infrequent than metaplastic carcinomas (Davis *et al*., 2005).

The next important issue for the definition of metaplastic carcinomas is establishing the evidence of primary epithelial (glandular) tumor differentiation. Is the presence of glandular spaces formed by atypical cells of epithelial character the only acceptable diagnostic feature; or is the epithelial immunophenotype of the cells morphologically not resembling their epithelial counterparts sufficient? Especially important in cases of spindle cell tumors, the issue is not yet solved: cytokeratin expression by the tumor cells seems to be the basis for classifying lesions as metaplastic carcinomas and not sarcomas (Adem *et al*., 2002; Carter *et al*., 2006).

In summary, tumors should be classified according to their histogenesis. Metaplastic breast carcinomas are epithelial in their origin but demonstrate altered, non-glandular tissue differentiation. The evidence for divergent cellular and/or histological differentiation

may be microscopic (glandular versus squamous, glandular versus mesenchymal tissue) or immunohistoichemical (cytokeratin expression versus absence of cytokeratin expression). Breast tumors showing histological features of adenocarcinoma and another malignant tumor(s) at the same time are clear examples of metaplastic breast carcinomas. Some other breast tumors of epithelial origin, but with non-glandular morphology, are currently also classified as metaplastic carcinomas (see below).

Current classification

The WHO Classification of Breast Tumors (Tavassoli and Devilii, 2003) delineates metaplastic carcinoma as a special tumor group with two subgroups within it. The first subgroup is characterized by the absence of mesenchymal component, the second one with the presence of it. The first subgroup includes purely squamous carcinoma without evidence of glandular differentiation (with its three variants: large cell keratinizing, spindle cell, and acantholytic), adenocarcinoma with spindle cell differentiation and adenosquamous carcinoma (including mucoepidermoid cancer). The second subgroup includes carcinoma with chondroid and with osseous metaplasia as well as carcinosarcoma.

According to the definition of metaplastic carcinoma discussed above, purely squamous carcinomas could represent a separate tumor group which does not belong to metaplastic category as neither initial glandular differentiation nor presence of at least two different malignant tissue types or cell types can be evidenced in these tumors. Similarly, grouping together carcinosarcoma and carcinomas with benign mesenchymal components can also be questioned. On the other hand, some carcinoma subtypes containing malignant non-glandular cells (e.g., carcinoma with choriocarcinomatous features or carcinoma with melanocytic differentiation), grouped in the actual WHO classification together with ductal carcinomas, would have better classified as metaplastic cancers. However, defining and classifying breast tumors is always very difficult, if not impossible task as the intention to categorize tumors showing wide individual variation in their morphology is problematic. All the definitions and the existing classification can be criticized with objective arguments. However, in the present chapter, we shall follow the categories of the current WHO classification.

HISTOLOGY

Modern radiological methods give overview images of the normal breast tissue and the lesions within it at low resolution. As such, they are similar to the morphologic method of whole organ sectioning and differ considerably from the conventional small-block method used in diagnostic pathology laboratories today. The conventional pathology method is based on sampling of small representative tissue pieces chosen by the unaided eye of the pathologists. These pieces represent selected fragments from the lesion with destroyed interrelation of the details within it. Although these fragments are fully sufficient to make the diagnosis, and to type and grade the lesion, to assess the hormone receptor status, and to carry out many molecular analyses, the fragments do not correlate with the radiological overview

image. These fragments are insufficient to make proper radio-pathologic correlation and to demonstrate the diseased part of the tissue in relation to the rest of the breast for the surgeon.

To correctly visualize all the lesions within the breast or within the excised part of it, the pathologists need to examine the available breast tissue in greater width, length, and depth. They need to analyze a contiguous piece of breast tissue as large as possible, preferably representing a cross-section over the whole specimen. Examining a sufficiently large contiguous piece of breast tissue, without fragmenting it into small tissue blocks, effectively bridges the gap between the low-resolution summation image of mammography and the high-resolution details under the microscope. Large section histopathology represents the currently most adequate diagnostic histology method in modern breast pathology. As modified and used in our laboratory, it retains the advantages of whole organ sectioning, but with substantially reduced turn-around time and cost, and also retains all the advantages of the conventional small block technique. Thus, it is fully sufficient for the needs of the oncologic therapy planning and also provides ideal background for proper radio-pathologic correlation and for multidisciplinary evaluation of cases in daily practice. Our department in Falun, Sweden, has used large sections in routine diagnostic breast pathology since the early 1980s and created a unique collection of more than 6,000 consecutive cases documented this way and worked-up with detailed mammographic pathologic correlation. The method used in our laboratory, adapted for the needs of modern breast radiology and pathology, has been published in detail (Tot *et al.*, 2002; Tot, 2005).

The cut-up of the specimen varies according to the type of the specimen (mastectomy or sector-resection/quadrantectomy or diagnostic open surgical biopsy) and according to the type of the lesion (microcalcifications, solitary or multiple tumors). Preferably, the specimen is received for cut-up in fresh state (unfixed) together with the specimen radiograph proving the presence of the preoperatively diagnosed radiological abnormality in the specimen. Cutting up formalin-fixed specimen may be technically easier; however, the central portions of such specimen are usually not properly fixed leading to histological artifacts. The specimen radiograph helps the pathologist to plan the cut-up on a way to include the entire lesion in a single cross-section, but a thorough macroscopic examination of the whole specimen is also important. One must register the macroscopic size of the specimen, preferably in three dimensions, the number and type of the marking sutures or other markers as well as the number and the position of the guide wires, needles, etc.

Metaplastic breast carcinomas, being often large tumors, are usually easily located by unaided eye examination and palpation. Smaller tumors are more difficult to detect, but are often palpable and easily seen on the specimen radiograph. It is advantageous to slice the specimen horizontally in the plane of the specimen radiogram, which means parallel with the pectoralis muscle, and parallel with the skin. Multiple tumors are more difficult to demonstrate in a single large section. In this case the plane of the slicing must be chosen on the basis of the palpation and the whole specimen radiogram. Mastectomy specimens are cut sagittally into 3–4 mm thick slices to properly demonstrate the pectoralis margin.

The thickness of the tissue slices is very important for the quality of the histology sections. For adequate slicing of a fresh breast specimen, special knife with a very sharp disposable blade is needed. The blade must be changed after every two or three breast specimens, in the case of very hard or calcified tissue even more often. The produced slices have to be evenly 3–4 mm thick. Variations of the thickness within the same slice or among the slices can remarkably reduce the technical quality of the sections. The slices must be thoroughly examined macroscopically. The well-formed tumor masses are described and measured in millimeters. The relation of the tumor(s) to the resection margins is described. At this step, one can take cytological material (touch imprints) for immuncytochemistry or image analysis (ploidy determination) in cases with macroscopically clearly defined tumors. Other tissue sampling (taking small tissue blocks) is delayed until after the second specimen radiography. Inks are used to mark the position of the surgical marking sutures and to retain the orientation of the specimen. The produced and marked slices are placed on a film in sequence to undergo the second specimen radiography. The radiologist compares the mammographic findings and the findings on the whole specimen radiogram with the radiological images of the slices and marks the slices with radiological abnormalities. The pathologist must correlate the macroscopical findings in the marked slices to the radiological abnormalities. The most representative slices are selected for embedding and processing: the slice with the largest tumor diameter, the slice containing the largest number of the tumor foci in the case of multifocal tumors, the slice containing macroscopically and/or radiologically discernible nonmalignant lesions, and the slice containing microcalcifications. It is worth mentioning that the pathologist is fully responsible for tissue sampling irrespective of the radiological leading. Therefore, he is encouraged to sample all the suspicious macroscopic abnormalities even if they were not marked out by the radiologist. The recommended average number of selected slices per case is 2–4. After this step, one may take small tissue blocks for immunohistochemistry, image analysis, flow cytometric analysis, molecular biological examination, for tumor bank. But one must be very careful to leave the most representative slices intact. Special examination of identical technical quality as in conventional small blocks can also be carried out on histology sections from the large tissue blocks.

Selected tissue slices are stretched on a cork plate and pinned with the cutting surface facing down. The slices are immersed in dishes containing the usual formalin solution for tissue fixation. By fixation of a stretched slide one can achieve a plane section surface. Thorough fixation of the slices is essential in this technique; suboptimal fixation causes difficulties on sectioning. After fixation the slices are removed from the cork plate and placed into a container of an appropriate size to be automatically processed. Processing (dehydration) of the large tissue slices follows the steps of the conventional processing procedure, and can be carried out in commercially available automatic processors. Metal brackets are used to prepare paraffin blocks as by changing the distance between the brackets one can adjust the size of the paraffin block to the size of the tissue slice. Although it is possible to section large blocks with usual conventional microtomes, specially designed

automated macrotomes are more suitable for this procedure. Sections of 3–4 µm thickness are recommended. Staining is carried out in adapted modified holders, but in the same automatic stainer as for the small-block sections. The recipe for H&E staining is the same as for small blocks. Special staining methods are also applicable. A properly prepared large histological section is of the same quality as that of the conventional small sections.

Using commercially available object glasses of standard size (12 × 9 cm) has the advantage of easy archiving, but also a limitation, as tissue slices > 8.5 cm in one dimension are difficult to include into these preparations. These slices may be properly visualized if bisected centrally and embedded into two large paraffin blocks.

Large histological section may measure 70–90 cm^2 representing a contiguous surface up to 40 times larger than that in the conventional small histological sections. Taking 2–4 large blocks from a case corresponds to a very thorough sampling of up to 160 conventional tissue blocks. A large section may include a nonfragmented crossection of an individual invasive tumor face up to 8 cm in diameter. Taken in the plane of the largest tumor diameter, a large histological section represents a permanent documentation over the size of the tumor allowing correct measurement, which can be repeated any time and serves as the basis for objective retrospective studies. Inclusion of the main tumor mass in one central plane also allows assessing intratumoral heterogeneity. As the tumor is included into the large section together with the peritumoral tissue, presence or absence of invasive satellite tumor foci, peritumoral *in situ* component or vascular invasion are easy to assess. The large contiguous surface area of the large section provides the opportunity of including all the *in situ*, invasive, and intravascular tumor structures in one plane and provides the opportunity of assessing the distribution (unifocal tumor, multifocality or diffuse growth) of the tumor structures as well as the real extent (the area including all the malignant structures) of the disease in breast cancer cases. Inclusion of a continuous cross section of the entire circumferential resection margin allows direct documentation of the circumferential surgical margin. The relation of the invasive and *in situ* tumor foci and their distance to this margin are also documented.

The large section technique is especially advantageous in cases of metaplastic carcinomas, as by inclusion of an entire cut surface of the tumor, the technique demonstrates intratumoral heterogeneity, one of the most important diagnostic features in these cases, and allows proper assessment of the proportion of different components of a tumor. Metaplastic breast carcinoma documented in a large histological section is illustrated in Figure 21.1.

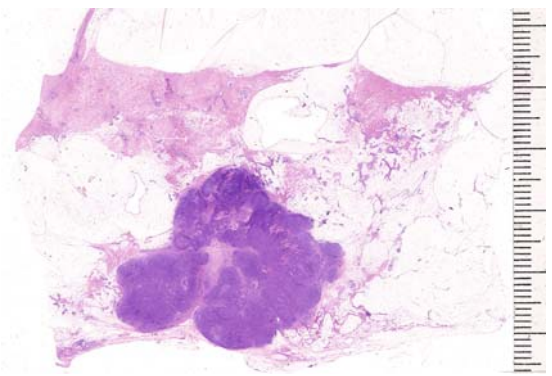

FIGURE 21.1. Large-format histology slide including the entire cross-section of a metaplastic breast carcinoma

In a study comparing the method of conventional sampling of small tissue blocks with the method of large block macrosectioning, Jackson *et al*. (1994) demonstrated that the large block method was far superior; it gave reliable tumor size measurement in 100% of the analyzed cases, compared to 63% of the cases documented by the conventional, small block method. Ductal carcinoma *in situ* was found more frequently (80% versus 64%) and its extent was significantly larger in large section cases. Concurrent carcinomas (multiple tumor foci) were seen significantly more often in large sections and their size was significantly smaller compared to that in the conventional method.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry has become a routine additional diagnostic procedure in most pathology laboratories (Hayat, 2004–2006). Evidencing the presence of certain protein molecules in the cells or in the stroma of a tumor represents a substantial support in diagnostic judgments in many cases. Antibodies can be produced practically against all the proteins of the human body and all of them can be visualized by modern immunohistochemistry techniques. This results in plethora of commercially available immunhistochemical markers. Automation and external quality assurance programs represent important attributes of high technical quality immunostaining but other parameters as fixation time, antigen retrieval method and the type of the used primary antibody may still influence the staining results. The importance of using internal and external positive and negative tissue controls cannot be overemphasized.

Assessment of the results of an immunostaining may be problematic even on a slide of technically superb quality. The pathologist has to be familiar with the type (granular or diffuse) and location (nuclear, cytoplasmatic, membranous or extracellular) of the expected reaction and with the pattern of expression of the analyzed antibody in the control tissue. The assessment includes analysis of the quality (intensity) of the staining (a weak reaction is most often ignored) and its quantity (e.g., the percentage of the stained tumor cells). The cut-off values for the interpretation of a staining as "positive" varies considerably in the literature; studies using a 0 cut-off (any staining) or 5%, 10%, 50% or 100% of the cells can be found addressing the same antibody and the same tumor type (Tot, 2003). Unfortunately, such variations also characterize the interpretation of the "basal-like" phenotype of breast carcinoma.

The most important antibodies in relation to metaplastic breast carcinomas belong to the family of cytokeratins. Like most human cells, cells of breast carcinomas contain cellular skeletal proteins called filaments in their cytoplasm. The filaments of the human cells are complex protein structures that can be divided into three groups according to their diameters: small (6 nm), intermediate (7–11 nm), and large (25 nm). A well-known example of small filaments is alpha smooth-muscle actin, which is present in smooth muscle cells, myofibroblasts, and in the myoepithelial cells of the breast as well as in the tumors originating from these cells. Microtubules are typical example of large filaments. The intermediate filaments rep-

resent a large group of molecules divided into five classes: desmin, vimentin, glial fibrillary acidic protein, neurofilaments, and cytokeratins.

The last class of the intermediate filaments, cytokeratins, represents the largest family of these polypeptides with 24 different members. They can be separated by two-dimensional electrophoresis on the basis of their different individual molecular weights (range 40–70 kD) and their isoelectric pH values. Twenty individual cytokeratins have been catalogued by Moll *et al*. (1982); they were first divided into type I (acidic) and type II (neutral – basic) based on isoelectric characteristics and then the individual type I and type II molecules were separated based on their molecular weight and numbered in order of their first description. Nine individual (1–9) cytokeratins belong to type I category and 11 (10–20) to type II. The natural molecular configuration of the cytokeratins within the cells is heterotetramerous with complexes of two identical type I and two identical type II cytokeratin molecules. The most constantly found heterotetramer in simple and glandular epithelia contains two molecules of cytokeratin 8 and two molecules of cytokeratin 18, thus the pair of cytokeratins 8–18 is a typical marker of glandular differentiation in both normal tissue and tumors. Likewise, the pair of cytokeratins 5–14 is typical of stratified epithelia and epithelia with squamous differentiation.

The expression of individual cytokeratins in the normal breast tissues is dependent on the stage of embryonic development. The luminal epithelial cells of the mature breast in the acini and ducts express mainly glandular type cytokeratins 8, 18, 7, and (variably) 19. The mature myoepithelial cells almost exclusively express the cytokeratins of stratified epithelia, 5, 6, 14, 15, and 17 (Moll *et al*., 1982). In epithelial hyperplasia the cells simultaneously express glandular and stratified type cytokeratins, while in low–grade neoplasia, like atypical ductal hyperplasia or ductal carcinoma *in situ* grade I, the cells exclusively express glandular cytokeratins. The vast majority of invasive breast carcinomas also retain their glandular cytokeratin phenotype; the exceptions are the "basal-like" carcinomas where the metaplastic breast carcinomas also belong (Reis-Filho *et al*., 2006).

In relation to metaplastic carcinomas of the breast, probably the most important diagnostic application of cytokeratins is evidencing the epithelial origin of the tumor. This is a minor problem if the histological appearance of the tumor indicates clearly focal glandular differentiation. However, in the tumors which are entirely or almost entirely metaplastic and of spindle cell character, expression of cytokeratins is the only proof of the epithelial origin of the tumor allowing proper categorization of the case as well as proper delineation of them from sarcomas. For this purpose, cocktails of different cytokeratin antibodies (pan-cytokeratin, wide-spectrum cytokeratin or multi-cytokeratin) are needed as they with their wide spectrum can better trace cytokeratin expression in the tumors than monotypic antibodies (Adem *et al*., 2002). Metaplastic breast carcinomas regularly react with these cytokeratin cocktails, at least focally. On the other hand, cytokeratin expression is not restricted to epithelial tumors and may appear in some sarcomas. Accepting cytokeratin expression as evidence for epithelial origin of a tumor is rather a matter of consensus and probability than the absolute truth.

Antibodies against cytokeratins 8 and/or 18 represent basic markers of glandular differentiation and are useful for evidencing such a differentiation in a metaplastic carcinoma, especially if large proportions of the tumor are nonglandular. As these cytokeratins are typically diffusely expressed by the vast majority of usual type breast carcinomas, absence of cytokeratin 8–18 expression in a breast tumor (even if focal) may *per se* indicate the diagnosis of metaplastic cancer. Cytokeratins 7 and 19 are also markers of glandular differentiation but their expression in breast tumors is somewhat more variable, making these antibodies less suitable for the same purpose. On rare occasion, gland-like spaces formed by cells not expressing glandular cytokeratins are seen in some metaplastic carcinomas indicating eccrin ductal differentiation (Tot, 2006).

Evidence for squamous differentiation, apart from the histology image of routinely stained slides, depends on the possibility of demonstrating expression of "basal-type" cytokeratins by tumor cells. There is some variation in defining the basal–like immunophenotype in the literature, the most widely accepted indicators being cytokeratins 5/6, 14 and 17 (Rakha *et al*., 2007). Positive staining for these molecules either with monospecific antibodies, or with a cocktail of them (e.g., cytokeratin 34 beta 12) indicate squamous differentiation even in the absence of typical histological features, such as keratinization or intercellular bridges. Figure 21.2 illustrates expression of cytokeratin 5/6 in a metaplastic breast carcinoma.

Antibodies other than cytokeratins are also in diagnostic use for demonstrating the metaplastic nature of these breast carcinomas. One of the most recently established markers for this purpose is maspin, a molecule originally isolated from normal breast tissue. The cells of the normal breast epithelium show only faint and focal cytoplasmic maspin expression, but the normal myoepithelial cells are consistently stained both in cytoplasm and nuclei. This pattern of maspin expression is identical to that in the acinar portion of the normal eccrine skin glands. The ductal portion of the eccrine skin glands, however, regularly demonstrates intense nuclear and cytoplasmic maspin positivity in both cell layers. The same maspin expression pattern was observed by us in the normal breast tissue in the vicinity of some metaplastic carcinomas and termed "eccrine duct lobules" (Tot, 2006). As demonstrated by Reis-Filho *et al*. (2003), maspin is regularly expressed by the cells of metaplastic breast carcinomas. Figure 21.3 demonstrates a metaplastic breast carcinoma with maspin expression.

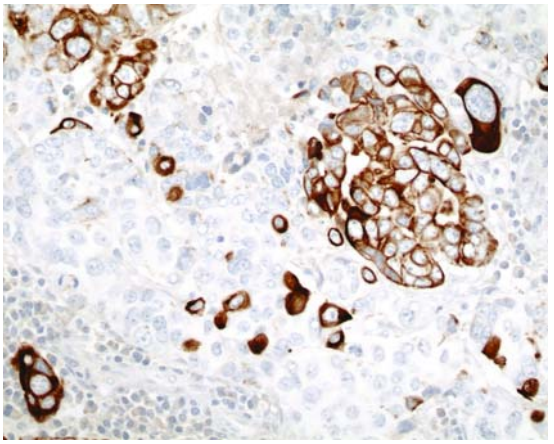

FIGURE 21.2. Metaplastic breast carcinoma with focal expression of cytokeratin 5/6

According to Koker and Kleer (2004), p63 expression is a highly sensitive and specific marker of metaplastic breast carcinomas. This molecule is involved in

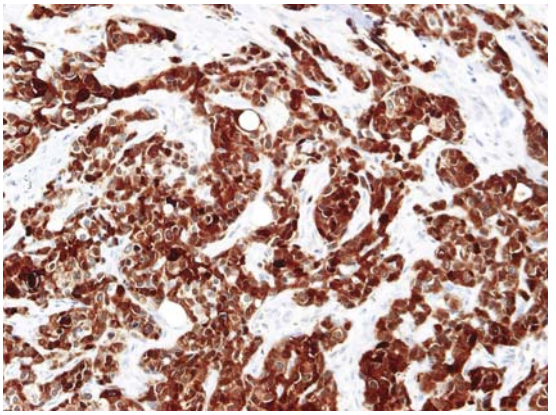

FIGURE 21.3. Metaplastic breast carcinoma showing intense diffuse maspin positivity

cellular differentiation and is expressed in the nuclei of the normal myoepithelial cells in the breast. In the study of these authors, 86.7% (13/15) metaplastic breast carcinomas expressed p63 in contrast to 0.6% (1/174) nonmetaplastic cancers. As breast sarcomas are consistently negative for p63, this antibody is very useful in separating spindle cell breast carcinomas from sarcomas.

Caveolin 1 is a newly detected antigen which is overexpressed in a subset of basal-like and metaplastic breast carcinomas. In the study of Savage *et al*. (2007), it was expressed in 90% of 39 metaplastic breast carcinomas and only in 9.4% of 245 nonmetaplastic breast carcinomas. Similarly to other markers of basal-like phenotype, this molecule is also expressed by the myoepithelium of the normal breast (as well as in endothelium and some fibroblasts) but not by the normal epithelium.

THE CONCEPT OF BASAL – LIKE TUMORS

The vast majority of breast carcinomas show glandular epithelial phenotype and, similarly to normal ductal and acinar epithelium, expresses glandular cytokeratins 8 and 18. Most of these tumors also contain estrogen- and progesterone receptors. However, a minority of breast carcinomas expresses markers that are typical of myoepithelium (presently erroneously called "basal cell layer"), most often cytokeratins 5, 6, 14 and 17. Such tumors are designated as "basal-like" breast carcinomas usually lacking estrogen and progesterone receptors. The myoepithelial (basal type) cytokeratins are also regularly present in squamous epithelium and squamous carcinomas; thus, it is not surprising that most of metaplastic carcinomas (especially those showing some degree of squamous differentiation) belong to the category of "basal-like tumors" (Reis-Filho *et al*., 2006).

This concept raises the question of origin of metaplastic breast tumors. According to the hypothesis of Boecker and Burger (2003) breast cancer develops from progenitor cells that are present as individual cells in normal breast tissue and can be identified as cells expressing cytokeratin 5 in the epithelial layer. In this context, it is more surprising that most of breast carcinomas loose cytokeratin 5 expression during their development and differentiation, but some of them retain it. Retaining the expression of this molecule qualifies the tumors for "basal-like" breast cancer.

Breast as a glandular organ develops in the embryo from the ectoderm. There are very few studies addressing the changes in cytokeratin expression of embryonic breast cells. The first cells of the solid embryonic buds formed by proliferation of certain ectodermal cells initiating a breast lobe express cytokeratins 14 and 5. Forming the breast epithelial layer, luminalization and branch-

ing of the ducts are associated with a switch in cytokeratin expression from cytokeratin 5 and 14 to cytokeratin 19 and at the end to cytokeratins 8 and 18 (Bartek *et al*., 1990; Jolicoeur *et al*., 2003). This evidence also suggests that tumors expressing cytokeratins 5 and/or 14 are on a lower level of tissue differentiation, more similar to the early embryonic tissue. If breast cancer develops from mature epithelial cells (in contrast to the Boecker concept referred above), "basal-like" tumors could be characterized by a kind of "switching back" to the early cytokeratin profile, which, once again, means a lower level of tumor differentiation.

Only few publications discuss the possibility of developing metaplastic breast carcinoma from benign precursor lesions. Already in 1940, Oliver suggested that squamous metaplasia in normal breast tissue may represent the origin of squamous breast carcinoma. Tot (2006) described eccrine duct lobules in the vicinity of a syringomatous squamous tumor in the breast. In contrast to the normal breast lobules, the acini of eccrine duct lobules lack cytokeratins 8 and 18 as well as smooth-muscle actin and express cytokeratins 5 and 6 as well as maspin. With this phenotype they show striking similarity to the ductal portion of dermal eccrine sweat glands.

In situ carcinoma is sometimes present in metaplastic tumors and may represent the initial phase of their development. Ductal carcinoma *in situ* with basal–like phenotype has been described (Bryan *et al*., 2006) and regarded for a possible precursor of invasive basal-like breast carcinomas. We have described *in situ* lesions similar to skin basaliomas showing eccrine differentiation in association with a high-grade metaplastic cancer (Tot, 2006).

CLINICOPATHOLOGICAL FEATURES

The clinical presentation of metaplastic breast carcinomas is not different from that of usual ductal carcinomas. The patients usually present with a palpable mass of several centimeter in size. On mammography, metaplastic carcinomas are usually well-delineated lobulated mass lesions. Calcification is present on rare occasions as a result of osseous metaplasia in the tumor (Gunham-Bilgen *et al*., 2002). Lack of diagnostic clinical and radiological signs in metaplastic carcinoma further emphasizes the importance of histological and immunohistological examination in diagnosing these tumors.

Squamous cell carcinomas of the breast are rare and often aggressive neoplasms. They clearly deviate in their immunophenotype from ductal carcinomas matched for age, tumor size, and nodal status (Grenier *et al*., 2007). This subgroup of metaplastic carcinomas is morphologically heterogeneous as the tumor may or may not keratinize. There are two variants which need particular attention during the diagnostic procedure, the acantholytic and the spindle cell variant.

These tumors are composed entirely of squamous tumor cells that deviate from glandular cells in their morphology allowing often proper diagnosis at microscopic examination of routinely stained histology sections. The larger and more vesicular nuclei and the abundant eosinophilic cytoplasm of the cells together with presence of intercellular bridges (desmosomes) are typical features. If in addition, signs of keratinization appear, the diagnosis is easy. Figure 21.4 illustrates a low-grade adenosquamous carcinoma.

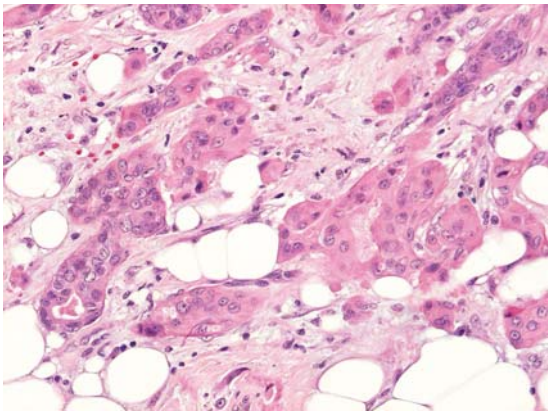

FIGURE 21.4. Metaplastic breast carcinoma showing histologically glandular and squamous differentiation at the same time. Note the gland-like structure containing secretion within the lumen (left lower corner of the image)

Squamous breast carcinomas may be macroscopically cystic and in this case the differential diagnosis may be problematic. Other breast tumors rarely presenting as cystic lesions are ductal carcinoma, medullary carcinoma, ductal carcinoma *in situ*, and cystic hypersecretory carcinoma. The acantholytic variant of squamous breast carcinoma contains microscopic cystic spaces lined by elongated cells producing a histologic picture resembling angiosarcoma. For proper diagnosis it is essential to confirm the epithelial nature of the tumor with CK5 or a broad-spectrum of cytokeratin cocktail (e.g., CK34 beta E12).

The spindle cell variant of squamous carcinoma, according to definition, expresses squamous (basal) cytokeratins and does not contain glandular structures. In practice, it is often grouped together with the next category in WHO classification, adenocarcinomas with spindle cell differentiation. In these cases, adenocarcinoma represents a (minor) proportion of the tumor regularly evident in well-sampled cases. All these tumors, if of low histology grade, resemble benign mesenchymal proliferations like fibromatosis; if high–grade they resemble pleomorphic sarcomas. Therefore, they are often designated as "sarcomatoid breast carcinoma" or "spindle cell carcinoma of the breast" Importantly, these tumors, especially if of large size, have a potential of hematogeneous dissemination, rather than to metastasize to the axillary lymph nodes. In other words, metaplastic sarcomatoid carcinomas with only minimal overt carcinomatous component have a biological behavior similar to that of sarcomas. Therefore, proper diagnosis is essential in these cases. The most often applied diagnostic criteria (although questioned by Davis *et al*., 2005) are presence of overt carcinomatous (invasive and/or *in situ*) differentiation in addition to the mesenchymal-like component, and/or cytokeratin expression in the spindle cells (Carter *et al*., 2006).

Adenosquamous carcinomas are characterized by the mixture of tumor tissue showing glandular differentiation and tissue showing squamous differentiation. The proportion of these components varies. Most of these tumors are well-differentiated and show diagnostic features at routine histological examination: presence of gland-like structures and keratinizing solid sheets of tumor cells at the same time. These tumors may possess a peripheral cell layer staining intensively with myoepithelial markers around the epithelial component. Thus, although infiltrative at histological examination, immunohistochemically they resemble an "in situ lesion". One of the variants of these tumors called "syringomatous squamous tumor" or "infiltrating syringomatous adenoma" exhibits, in addition to well-differentiated squamos

and glandular cells, a large number of fibroblast-like, nonatypical stromal cells forming a fibromatosis-like stroma. The prognosis of these tumors is excellent, although local recurrences may occur. These characteristics, together with the excellent prognosis of these lesions, motivated some authors not to use the word "carcinoma" but words such as "tumor" or "infiltrating adenoma" when designating these lesions (Tavassoli and Devilee, 2003). Some adenosquamous carcinomas have mucin-producing properties and with their morphology correspond to low-grade mucoepidermoid carcinomas of the salivary glands (Tavassoli and Devilee, 2003). These tumors are rare.

Some adenosquamous carcinomas are not well-differentiated but exhibit obvious cellular and nuclear atypia and high mitotic activity. Reaching proper diagnosis in these can be problematic as keratinization is usually absent and glandular spaces may be few. These tumors often exhibit a mosaic of glandular and quamous cytokeratins in their immunophenotype. The prognosis in these cases is far more unfavorable compared to low-grade adenosquamous carcinomas.

As discussed above, a whole category of metaplastic carcinomas is reserved in WHO classification for tumors having mesenchymal component. This category represents a group of tumors with varying morphology and prognosis. Presence of glandular epithelial component together with one or more mesenchymal components is the hallmark of these tumors. Their subcategorization depends on the characteristics of the mesenchymal component. The mesenchymal elements are sometimes histologically "bland", showing similarity with their normal counterparts: a chondroid component in the metaplastic carcinoma resembling normal cartilage or a osseous component resembling normal bone structures. These tumors are designated carcinoma with osseous metaplasia or carcinoma with chondroid metaplasia. In some of these tumors, the stromal component cannot be characterized this way as it only deviates from the normal tumor stroma by the presence of homogeneous background substance. These are called "matrix producing" breast carcinomas. Figure 21.5 demonstrated a metaplastic breast carcinoma with osseous metaplasia in the stromal component.

When the mesenchymal component exhibits histological signs of malignancy (e.g., cellularity, atypia, mitotic activity) the tumors are designated as "carcinosarcomas". All the types of sarcomas may be present, such as tissue of fibrosarcoma, osteosarcoma, chondrosarcoma, rhabdomyosarcoma or liposarcoma. These elements are regularly recognized in routine histological examination of the tumor tissue, but immunohistochemistry (markers S-100 protein, or markers of muscle differentiation) may

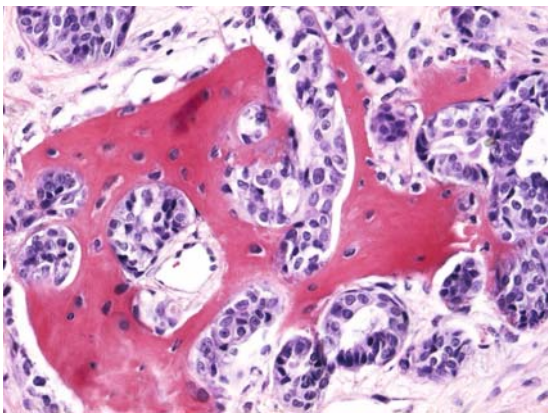

FIGURE 21.5. Metaplastic breast carcinoma with osseous metaplasia

support the diagnosis. The sarcomatous component of these tumors lack expression of epithelial markers, like cytokeratins. This is an important feature in differentiating carcinosarcomas from spindle cell carcinomas. Carcinosarcomas are aggressive tumors with poor prognosis. The prognosis of the tumor with metaplastic mesenchymal elements is more favorable.

PROGNOSTIC AND PREDICTIVE PARAMETERS

Despite some variation in the reported survival data in the literature, metaplastic breast carcinomas seem to deviate from usual ductal breast carcinomas in their prognostic features. For example, although metaplastic breast carcinomas are often several centimeters in size at the time of detection, lymph node metastases are relatively uncommon. According to Gibson *et al.* (2005), metaplastic breast carcinomas tend to present at a more advanced stage compared to other breast carcinomas, but when stratified for stage, survival seems to be similar to that of usual type breast cancers. The statistical results are, however, clearly influenced by the structure of the analyzed group. The standard prognostic parameters, such as tumor size, tumor grade, and lymph node status (as defined for the usual breast cancers) are not fully valid in metaplastic carcinomas.

On the other hand, proper typing of a metaplastic carcinoma is a very powerful and accurate prognostic procedure. As metaplastic breast carcinomas represent a group of diseases with substantially different prognosis, proper histopathological and immunohistological diagnosis can stratify these tumors into categories with excellent, intermediate, and poor prognosis. Those with excellent prognosis are low-grade adenosquamous carcinomas especially syringomatous squamous tumors which are locally aggressive neoplasias with some potential to locally recur, but without a potential of giving metastasis (Tavassoli and Devilee, 2003). Similar is the prognosis of low-grade "fibromatosis-like" spindle cell breast carcinomas. According to the classic study of Huvos *et al.* (1973), tumors with squamous differentiation (with exception of acantholytic variant) have an intermediate prognosis with 5 year survival rate of 63%. The acantholythic variant of squamous carcinomas belongs to the category of metaplastic carcinomas with poor prognosis, as well as high-grade adenosquamous carcinomas, sarcoma – like spindle cell carcinomas and carcinosarcomas, which are highly aggressive neoplasias (Tse *et al.*, 2006).

Not much information is available regarding the efficacy of the current therapy modalities in the management of metaplastic carcinomas. The predictive features, such as hormone receptor status, are of limited value in these cases; the metaplastic component of the tumors regularly lack estrogen receptors or progesterone receptors, while expression of these receptors by the glandular component varies dependent on the grade of its differentiation. Most of the metaplastic carcinomas are also HER-2 negative (Barnes *et al.*, 2005). Squamous cell breast carcinomas, for example, are always negative for hormone receptors and HER-2 (Grenier *et al.*, 2007). This fact also indicates that neither usual antihormonal therapy, nor herceptin therapy are effective in most metaplastic carcinomas. Nevertheless, according to the

findings of Pezzi *et al*. (2007), patients with metaplastic carcinomas present with large tumor size, less nodal involvement but higher tumor stage compared to their ductal counterparts and are treated more aggressively according to the current principles (more often with mastectomy and chemotherapy).

Expression of epidermal growth factor receptor (EGFR) by the cells of metaplastic carcinoma was recently evidenced. In nonselected series of metaplastic carcinomas, 60–76% expressed EGFR immunohistochemically although much less of them on molecular level analyzed with *in situ* hybridization (Leibl and Moinfar, 2005; Reis-Filho *et al*., 2005). Squamous breast carcinomas are EGFR-positive as frequently as in 85% of the cases (Grenier *et al*., 2007). Figure 21.6 demonstrates a metaplastic carcinoma with EGFR overexpression. As tumors with EGFR amplification are reported to be sensitive to protein kinase inhibitors, treatment with inhibitors, such as gefitinib, may be beneficial in some cases of metaplastic breast carcinoma.

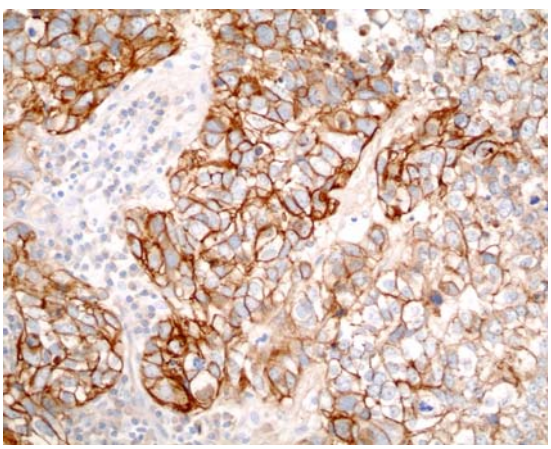

FIGURE 21.6. Metaplastic breast carcinoma with epithelial growth factor (EGFR) overexpression

REFERENCES

Adem, C., Reynolds, C., Adlakha, H., Roche, P.C., and Nascimento, A.G. 2002. Wide spectrum screening keratin as a marker of metaplastic spindle cell carcinoma of the breast. An immunohistochemical study of 24 patients. *Histopathology 40*: 556–562.

Barnes, P.J., Boutilier, R., Chiasson, D., R., and Rayson, D. 2005. Metaplastic breast carcinoma: clinical-pathologic characteristics and HER2/neu expression. *Breast Cancer Res. Treat. 91*: 173–178.

Bartek, J., Bartkova, J., and Taylor-Papadimitriu, J. 1990. Keratin 19 expression in the adult and developing human mammary gland. *Histochem. J. 22*: 537–544.

Boecker, W., and Burger, H. 2003. Evidence of progenitor cells of glandular and myoepithelial cell lineages in the human adult female breast epithelium: a new progenitor (adult stem) cell concept. *Cell Prolif. 1*. (suppl 36): 73–84.

Bryan, B.B., Schnitt, S.J., and Collins, L.C. 2006. Ductal carcinoma in situ with basal-like phenotype: a possible precursor to invasive basal-like breast cancer. *Mod. Pathol. 19*: 617–621.

Carter, M.R., Hornick, J.L., Lester, S., and Fletcher C.D.M. 2006. Spindle cell (sarcomatoid) carcinoma of the breast. A clinicopathologic and immunohistochemical analysis of 29 cases. *Am. J. Surg. Pathol. 30*: 300–309.

Davis, W.G., Hennessy, B., Babiera, G. Hunt, K., Valero, V., Buchholz, T.A., Sniege, N., and Gilcrease, M.Z. 2005. Metaplastic sarcomatoid carcinoma of the breast with absent or minimal overt invasive carcinomatous component. *Am. J. Surg. Pathol. 29*: 1456–1463.

Gibson, G.R., Qian, D., Ku, J.K., and Lai, L.L. 2005. Metaplastic breast cancer: clinical features and outcomes. *Am. Surg. 71*: 725–730.

Grenier, J., Soria, J.C., Mathieu, M.C., Andre, F., Abdelmoula, S., Velasco, V., Morat, L., Besse, B., Dunant, A., Spielmann, M., and Delaloge, S. 2007. Differential immunohistochemical and biological profile of squamous cell carcinoma of the breast. *Anticancer Res. 27*: 547–555.

Gunham-Bilgen, I., Memis, A., Ustun, E.E., Zekioglu, O., and Ozdemir, N. 2002. Metaplastic carcinoma of the breast: clinical, mammographic,

and sonographic findings with histological correlation. *Am. J. Roentgenol. 178*: 1421–1425.

Hayat, M.A. (Ed.). 2004–2006. *Immunohistochemistry and In Situ Hybridization of Human Carcinomas*, Vols 1–4. Elsevier, San Diego, CA.

Huvos, A.G., Lucas, J.C., and Foot, F.W. Jr. 1973. Metaplastic breast carcinoma. Rare form of mammary cancer. *N. Y. State J. Med. 73*: 1078–1082.

Jackson, P.A., Merchant, W., McCormick, C.J., and Cook, M.G. 1994. A comparison of large block macrosectioning and conventional techniques in breast pathology. *Virchows Arch. 425*: 243–248.

Jolicoeur, F., Gaboury, L.A., and Oligny, L.L. 2003. Basal cells of second trimester fetal breasts: immunohistochemical study of myoepithelial precursors. *Pediatr. Dev. Pathol. 6*: 398–413.

Koker, N.M., and Kleer, C.,G. 2004. p63 expression in breast cancer: a highly sensitive and specific marker of metaplastic carcinoma. *Am. J. Surg. Pathol. 28*: 1506–1512.

Leibl, S., and Moinfar, F. 2005. Metaplastic breast carcinomas are negative for Her-2 but frequently express EGFR (Her-1): potential relevance to adjuvant treatment with EGFR tyrosine kinase inhibitors? *J. Clin. Pathol. 58*: 700–704.

Moll, R., Franke, W.W., Schiller, D.L. Geiger, B., and Krepler, R. 1982. The catalogue of Human cytokeratins: patterns of expression in human epithelia, tumors and cultured cells. *Cell 31*: 11–24.

Oliver, R.L. 1940. Metaplasia in the breast. *Arch. Surg. 41*: 714–722.

Pezzi, C.M., Patel-Parekh, L., Cole, K., Franko, J., Klimberg, V.S., and Bland K. 2007. Characteristics and treatment of metaplastic breast cancer: analysis of 892 cases from the National Cancer Data Base. *Ann. Surg. Oncol. 14*: 166–173.

Rakha, E.A., El-Sayed, M.E., Green, A.R., Paish, E.C., Lee, A.H., and Ellis, E.O. 2007. Breast carcinoma with basal differentiation: a proposal for pathology definition based on basal cytokeratin expression. *Histopathology 50*: 434–438.

Reis-Filho, J.S., Milanezi. F., Paredes, J., Silva, P., Pereira, E.M., Maeda, S.A., de Carvalho, L.V., and Schmitt, F.C. 2003. Novel and classic myoepithelial/stem cell markers in metaplastic carcinokmas of the breast. *Appl. Immunohistochem. Mol. Morphol. 11*: 1–8.

Reis-Filho, J.S., Milanezi, F., Carvalho, S., Simpson, P.T., Steele, D., Savage, K., Lambros, M.B., Pereira, E.M., Nesland, J.M., Lakhani, S.R., and Schmitt, F.C. 2005. Metaplastic breast carcinomas exhibit EGFR, but not HER2, gene amplification and overexpression: immunohistochemical and chromogenic in situ hybridization analysis. *Breast Cancer Res. 7*: R1028–R1035.

Reis-Filho, J.S., Milanezi, F., Steele, D., Savage, K., Simpson, P.T., Nesland, J.M, Pereira, E.M., Lakhani, S.R., and Schmitt, F.C. 2006. Mataplastic breast carcinomas are basal-like tumors. *Histopathology 49*: 10–21.

Savage, K., Lambros, M.B., Robertson, D., Jones, R.L., Jones, C., Mackay, A., James M., Hornick, J.L., Pereira, E.M., Milanezi, F., Fletcher, C.D., Schmitt, F.C., Ashworth, A., and Resi-Filho, J.S. 2007. Caveolin 1 is overexpressed and amplified in a subset of basal-like and metaplastic breast carcinomas: a morphologic, ultrastructural, immunohistochemical, and in situ hybridization analysis. *Clin. Cancer Res. 13*: 90–101.

Tavassoli, F.A., and Devilee, P. (Eds.). 2003. *World Health Organization Classification of Tumours. Pathology & Genetics. Tumors of the Breast and Female Genital Organs*. IARC Press, Lyon, France. pp. 37–41.

Tot, T. 2002. Cytokeratins 20 and 7 as biomarkers: usefulness in discriminating primary from metastatic adenocarcinomas. *Eur. J. Cancer. 38*: 758–763.

Tot, T. 2005. *Colorectal Tumors. Atlas of Large Section Histopathology*. Thieme, Stuttgart – New York. pp. 129–144.

Tot, T. 2006. Eccrine ductal and acrosyringeal differentiation of the breast epithelium – a lesion associated with some metaplastic breast carcinomas. *Virchows Arch. 449*: 565–51.

Tot, T., Tabár, L., and Dean, P.B. 2002. *Practical Breast Pathology*. Thieme, Stuttgart/New York. pp. 115–123.

Tse, G.M., Tam, P.H., Putti, T.C., Lui, P.C., Chaiwun, B., and Law, B.K. 2006. Metaplastic carcinoma of the breast: a clinicopathological review. *J. Clin. Pathol. 59*: 1079–1083.

Zhuang, Z., Lininger, R.A., Man, Y.G., Albuquerque, A., Merino, M.J., and Tavassoli, F.A. 1997. Identical clonality of both components in mammary carcinosarcomas with differential loss of heterozygosity. *Mod. Pathol. 10*: 354–362.

22
Invasive Breast Cancer: Overexpression of HER-2 Determined by Immunohistochemistry and Multiplex Ligation-Dependent Probe Amplification

Paul J. van Diest, C.B. Moelans, D. Purnomosari, G. Pals, and R.A. de Weger

INTRODUCTION

The HER-2/*neu* proto-oncogene located on chromosome 17 encodes a 185-kD transmembrane tyrosine kinase growth factor receptor belonging to the epidermal growth factor receptor family (Bargmann *et al*., 1986) which is involved in cell growth and development (Popescu *et al*., 1989). HER-2 is amplified in 20–30% of breast carcinomas. This amplification of HER-2/*neu* is now known to confer a poor prognosis (Slamon *et al*., 1987; Baak *et al*. 1991) and may also predict a worse response to hormonal therapy (Wright *et al*., 1992) and standard chemotherapy regimens (Konecny *et al*., 2004). HER-2/*neu* proto-oncogene amplification is usually accompanied by overexpression of its protein (Slamon *et al*., 1989) as determined by immunohistochemistry (IHC).

The recent development of trastuzumab (Herceptin), a humanized monoclonal antibody to the extracellular domain of HER-2/*neu*, offers a new therapeutic approach for women with HER-2/*neu* positive breast cancer. In addition, HER-2/*neu* positive breast cancer patients respond better to taxane chemotherapy. However, the significant costs and toxicity of trastuzumab (Sparano, 2001) and taxanes have raised attention with regard to accurate determination of HER-2/*neu* status.

Currently, HER-2/*neu* status is determined using two methods: those that reveal gene amplification, and those aimed at detecting the overexpressed HER-2/*neu* protein (Di Leo *et al*., 2002; Hanna, 2001; Tubbs *et al*., 2001; Press *et al*., 2002). Some studies claim that gene amplification status better predicts response to therapy than protein overexpression does (Mass *et al*., 2001).

Immunohistochemistry (IHC) is the most commonly used method to assess protein overexpression. It is a rather easy morphological method which has many advantages but it may be hampered by

technical problems and requires strict quality control and standardization (Ginestier *et al*., 2004). Moreover, the different IHC technical steps are highly dependent on fixation conditions that significantly modify membrane staining (Press *et al*., 2002; Penault-Llorca *et al*., 1994; Rhodes *et al*., 2002a, b). Consequently, significant variability of IHC results has been demonstrated in inter-laboratory quality control studies. For scoring of IHC staining, the 0 to 3+ visual system developed for the HercepTest (Dako, Glostrup. Denmark) is widely in use (Figure 22.1). While there is little difficulty in assigning the 0 and 3+ scores, interpretation is more problematic for the two intermediate levels. For cases scoring 2+ (10–15% of all breast cancers), the concordance with gene amplification by fluorescence *in situ* hybridization (FISH) is barely 25%, and yet a proportion of these 2+ cases are true HER-2/*neu* amplified tumors. These cases, therefore, require a second line amplification test.

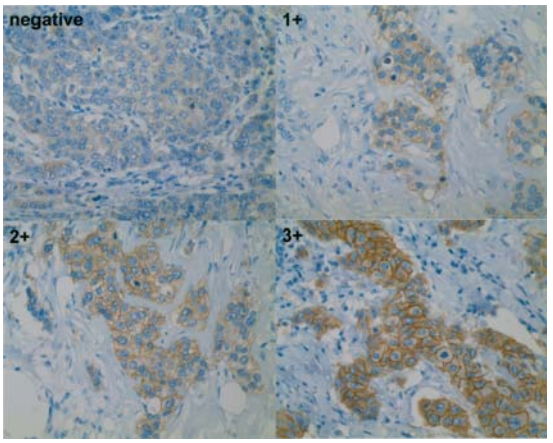

FIGURE 22.1. Examples of scoring of HER-2/*neu* immunohistochemical staining. (Top left) Negative staining: no staining, only cytoplasmic staining or less than 10% cells with membrane staining. (Top right) 1+ staining: more than 10% cells with membrane staining which is however incomplete. (Bottom left) 2+ staining: more than 10% cells with complete membrane staining which is however not strong in intensity. (Bottom right) 3+ staining: more than 10% cells with complete intense membrane staining. (Reprinted with permission from Purnomosari *et al*., 2006)

Gene amplification can be assessed by different methods. Southern blotting is the gold standard, but is time consuming, complicated and requires a lot of DNA which makes it an unattractive method for daily pathology practice. Therefore, traditionally FISH has for long been the most popular method for HER-2/*neu* gene amplification testing. However, FISH is expensive, technically challenging and sensitive to differences in digestion methods, and the commercially available kits have a limited half life. This method is, therefore, not a practical primary screening tool (Jacobs *et al*. 1999), although it has been recognized as such by the United States Food and Drug Administration (Birner *et al*., 2001). Its use, therefore, is usually limited to equivocal cases. Chromogenic ISH (CISH) overcomes many of the disadvantages of FISH but is less sensitive and quantitative (Tanner *et al*., 2000; Hanna and Kwok, 2006). A relatively new PCR based technique to assess HER-2/*neu* amplification is Multiplex Ligation-dependent Probe Amplification (MLPA). In this chapter we review the value of MLPA for detection of HER-2/*neu* amplification in breast cancer in comparison with other available methods.

Multiplex ligation-dependent probe amplification

The MLPA technique was first described in 2002 by Schouten *et al*. (2002) and is summarized in Figure 22.2. This technique uses a mixture of hemi-probe sets

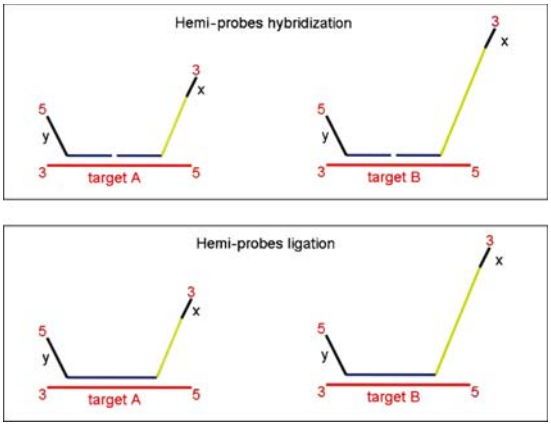

FIGURE 22.2. Principle of Multiplex Ligation–dependent Probe Amplification (MLPA). MLPA uses a mixture of hemi-probe sets that consist of two oligonucleotides, both having PCR primer sequences (x/y) on the outer ends, while both on the inner ends having a sequence complementary to a part of the target sequence (A or B). One of the primers has a spacer (in green) of variable length in between the PCR primer sequence and the complementary target sequence. When the complementary target sequences of both hemi-probes hybridize adjacent to each other on the target sequence (top figure), they can be ligated to each other (bottom figure), and subsequently amplified using the PCR primer sequences. Because the PCR primers are the same for all hemi-probe sets, they can be amplified in a single PCR, which will provide amplicons of unique and defined lengths due to the specific spacer length within each probe set

that consists of two oligonucleotides, both having PCR primer sequences on the outer ends and a sequence complementary to a part of the target sequence on the inner ends. One of the primers has a spacer (in green) of variable length in between the PCR primer sequence and the complementary target sequence. When the complementary target sequences of both hemi-probes hybridize adjacent to each other on the target sequence, they can be ligated to each other, and subsequently amplified using the PCR primer sequences. Because the PCR primers are the same for all hemi-probe sets, they can be amplified in a single PCR, which will provide amplicons of unique and defined lengths due to the specific spacer length within each probe set. Up to 40 probe sets can be run in one reaction. MLPA has found applications to assess gene copy number changes (Raju *et al*., 2006; Arkblad *et al*., 2006; Vorstman *et al*., 2006), gene expression (Eldering *et al*., 2003; Hess *et al*., 2004), and methylation (Nygren *et al*., 2005; Procter *et al*., 2006; Dikow *et al*., 2007).

Due to the short lengths of the target sequences of the hemiprobes, MLPA can not only be applied to fresh frozen material, but is also suitable for paraffin embedded material. Depending on the quality of the DNA, 20–200 ng of DNA suffice, although reproducibility may be less with very small amounts of DNA. The ability to carry out a multiplex copy number assessment on small amounts of paraffin embedded material makes MLPA a very attractive method in pathology. Obviously, it remains a non-morphological method that requires proper morphological control of the input material. In cases with a low percentage of relevant material, manual or microdissection may be necessary.

Multiplex Ligation-dependent Probe Amplification for detection of HER-2/*neu* amplification

Several studies have employed MLPA for detection of HER-2/*neu* amplification. The first study was by Purnomosari *et al*. (2006), using a commercially available kit from MRC-Holland (www.mrc-holland.nl). The P012 probe mix contains three sets of hemi-probes that recognise different

sequences of the human *erb*B2/HER-2 gene. In addition to these HER-2/*neu* specific probes, nine control hemi-probe sets are present: one probe for Topoisomerase IIα (TOP2A), a gene located on chromosome 17 at a short distance from the HER-2 gene, as well as eight probes for single copy sequences on regions on other chromosomes that harbor infrequent copy number changes in breast cancers according to Comparative Genomic Hybridization experiments: MOX2, NP220, EMS1, IL1A, NFKB1, APP, IL2, and ING1.

A typical protocol comprises the following (Purnomosari *et al.*, 2006): 50–500 ng target DNA/5 µl of 10 mM Tris (pH 8)-0.1 mM EDTA is denatured for 5 min at 98°C after which 3 µl of the probe mix is added. The mixture is heated at 95°C for 1 min and incubated at 60°C overnight (16 h). Ligation is performed with the temperature-stable Ligase-65 enzyme (MRC-Holland) for 15 min at 54°C. Next, the ligase is inactivated by incubation for 5 min at 98°C. Ten microliters of this ligation mix is premixed with 30 µl of PCR buffer and placed in a PCR machine at 60°C. Subsequently, a 10-µl mix is added containing deoxynucleoside triphosphate, Taq polymerase, and one unlabeled and one carboxyfluorescein-labeled PCR primer, which are complementary to the universal primer sequences. PCR is carried out for 33 cycles (30 s at 95°C, 30 s at 60°C, and 1 min at 72°C). The fragments can be analyzed on an ABI model 310 capillary sequencer (Applied Biosystems) using Genescan-TAMRA 500 size standards (Applied Biosystems). Fragment analysis can be performed with Genescan software. DNA from Centre d'Etude Polymorphisme du Humain (CEPH) can be used as control sample and is analyzed simultaneously with breast cancer samples in every run.

To objectify the interpretation of the fragment analysis, the relative quantity of the amplified probes in each sample needs to be determined using an Excel template. For this purpose, the relative peak areas for each probe are calculated as fractions of the total sum of peak areas in a certain sample. Subsequently, the fraction of each peak is divided by the average peak fractions of the corresponding probe in control samples. Finally, the values have to be normalized using the values obtained for the autosomal control probes, which serve as a reference for the copy number of 2.0. Cases that show a copy number above 2 for at least two of the probes on the HER-2/*neu* locus are considered to be amplified. Two further studies by Moerland *et al.* (2006) and Moelans *et al.* (2007) used similar protocols.

Correlations between HER-2/*neu* Multiplex Ligation-dependent Probe Amplification and immunohistochemistry

In the study of Purnomosari *et al.* (2006), 60 frozen and accompanying formaldehyde fixed and paraffin embedded breast cancer samples were obtained from women treated at Sardjito Hospital, Yogyakarta, Indonesia. Table 22.1 shows the correlation between MLPA results in comparison with HER-2/*neu* immunohistochemistry (IHC) using the CB11 antibody from Novocastra, which was interpreted according to the DAKO scoring system. Of the 60 cases, 36 were HER-2/*neu* negative by IHC and 7, 8, and 9 of the cases showed 1+, 2+ and 3+ HER-2/*neu* overexpression, respectively. A total of 13/60 cases (22%)

TABLE 22.1. Comparison between HER-2/*neu* immunohistochemistry (IHC) to assess protein overexpression and multiplex ligation-dependent probe amplification (MLPA) to detect HER-2/*neu* gene amplification. (Purnomosari *et al*., 2006.)

		MLPA	
IHC score	N	Normal	Amplified
0	36	36	0
1+	7	7	0
2+	8	3	5
3+	9	1	8
Total	60	47	13

showed gene amplification by MLPA. Of these amplified cases, 8 (62%) showed 3+ IHC, 5 (38%) 2+ IHC and no cases were 1+ or IHC negative. None of the 36 IHC negative and 0/7 1+ cases were amplified. Five of the eight (63%) 2+ cases were amplified, and as many as 8/9 (89%) IHC 3+ tumors showed gene amplification by MLPA assay. These data made it clear that MLPA is a good method to identify the HER-2/*neu* amplified cases within the IHC 2+ group. On the other hand, not all IHC 3+ cases are HER-2/*neu* amplified. Figure 22.3 shows an example of a HER-2/*neu* MLPA test on an HER-2 amplified invasive breast cancer sample compared to a HER-2 normal sample.

In the study of Moerland *et al*. (2006), 47 breast cancers were studied by MLPA, of which 19 showed a clear amplification (40%). At least a twofold amplification of the HER-2 gene was shown in 1/8 (13%) of the IHC 0/1+ tumors and in 10/30 (33%) of the IHC 2+ tumors. Of the IHC 3+ tumors 8/9 (89%) showed amplification (Table 22.2). Both in the Hercep 2+ and 3+ groups, strong amplification could be detected, whereas all samples were standardized for at least 50% tumor cells.

In the study of Moelans *et al*. (2007), using the same MLPA kit and the DAKO Hercep test, 582 breast cancers were studied. IHC showed 2+ tumors in 8.8% and 10.1% of cases were IHC 3+. MLPA showed clear amplification in 11.5% and an intermediate amplification (significantly higher than controls but < 2) in 3.8% of cases. At least a twofold amplification of the HER-2 gene was shown in 11/472 (2.3%) of the IHC 0/1+ tumors and in 4/51 (7.8%) of the IHC 2+ tumors. Of the IHC 3+ tumors 52/59 (88.1%) showed amplification (Table 22.3).

Correlation between Multiplex Ligation-dependent Probe Amplification and other amplification detection methods

The study of Moerland *et al*. (2006) also compared HER-2 gene amplification by MLPA and FISH (PathVysion kit, Vysis). In a series of 46 formaldehyde-fixed paraffin-embedded breast carcinomas, HER-2 gene amplification by FISH was found in 7/9, 10/30, and 1/7 in IHC 3+, 2+ and 0/1+ cases, respectively (Table 22.4). They also applied digitalized automated spot counting that was 100% concordant with manual FISH scoring. All but one FISH positive cases (17/18) were confirmed by MLPA for the presence of the gene amplification. The overall concordance of FISH and MLPA was 96% (44/46) (Table 22.5).

Furthermore, both the level of amplification and equivocal results correlated well between both methods (Table 22.6). This underlines that MLPA is a reliable and reproducible technique that can be used either as an alternative or as an additional test to determine HER-2 status in invasive breast cancers.

The study of Moelans *et al*. (2007) also compared HER-2 gene amplification by MLPA and FISH (PathVysion kit). In a

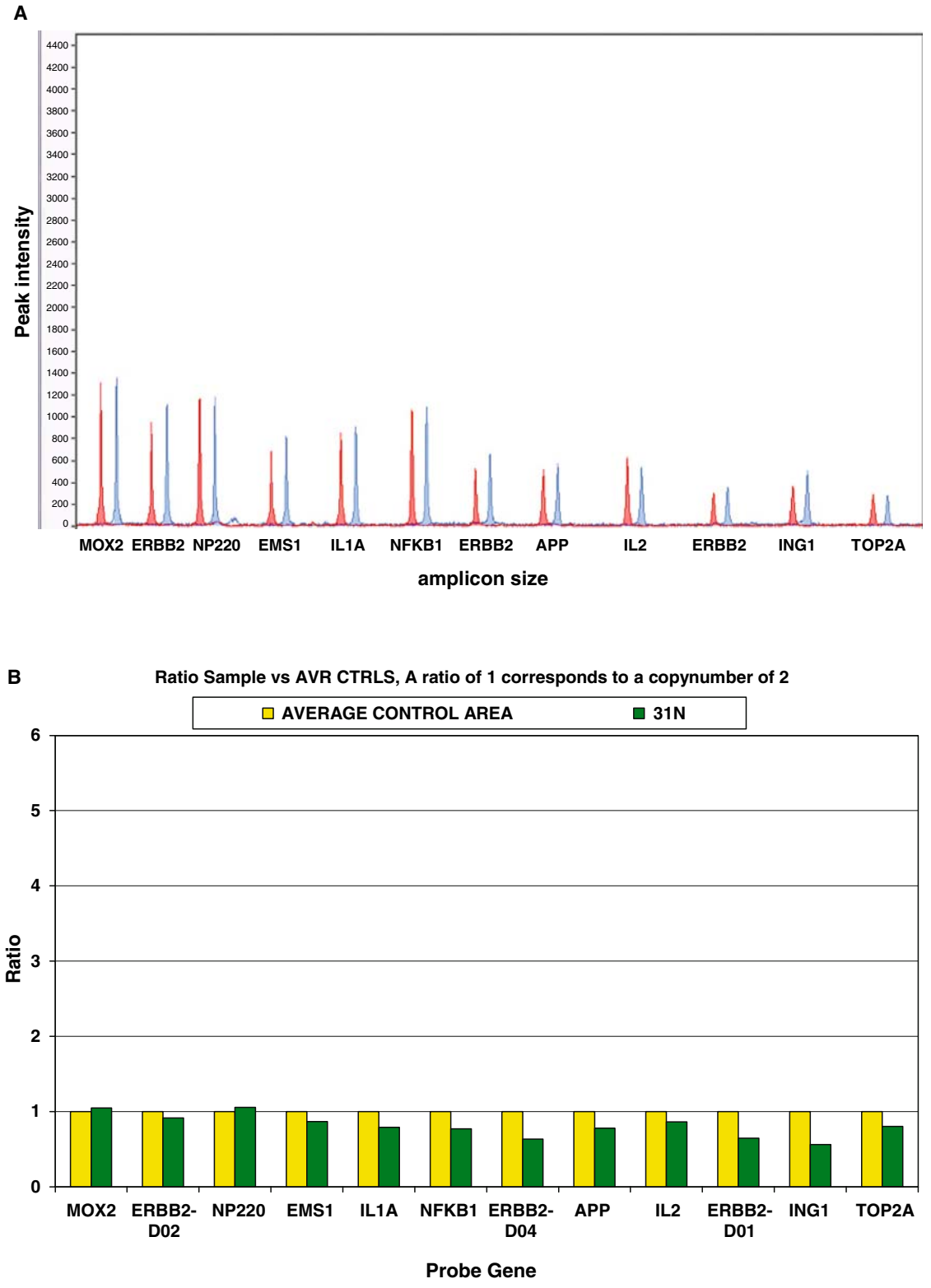

FIGURE 22.3. Examples of a HER-2/*neu* MLPA test in two invasive breast cancers and copy number calculations. (A) Breast cancer without HER-2 amplification showing on the left tumor peaks (red) comparable to each other and to the control (blue) peaks, further demonstrated by (B) copy number calculations yielding a ratio of around 1 for all probes.

(continued)

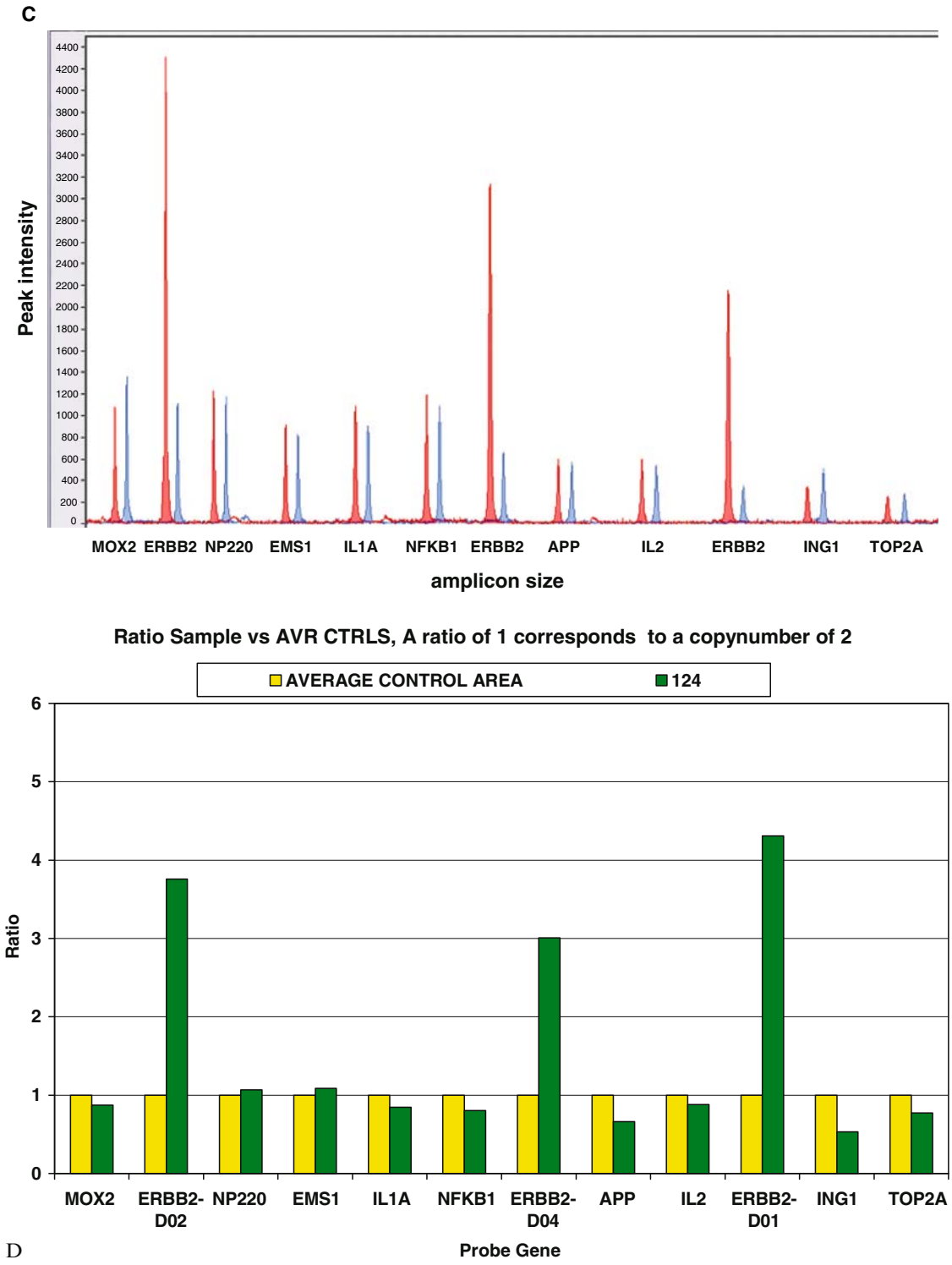

FIGURE 22.3. (continued) (C) Invasive breast cancer with HER-2/neu amplification as demonstrated by three red HER-2 peaks which are clearly higher than the red autosomal control peaks for this sample and the blue normal control peaks pointing to increased HER-2 gene copy number, further demonstrated by (D) copy number calculations yielding a ratio between 3 and 5 for the HER-2 probes compared to ratios around 1 for the control probes. Please note that the chromosome 17 located TOP2A gene (far right) shows normal copy number, denying chromosome 17 polysomy as an explanation for the HER-2 gene amplification

TABLE 22.2. Comparison between HER-2/*neu* immunohistochemistry (IHC) to assess protein overexpression and multiplex ligation-dependent probe amplification (MLPA) to detect HER-2/*neu* gene amplification. (Moerland *et al*., 2006.)

		MLPA	
IHC score	N	Normal	Amplified
0/1+	8	7	1
2+	30	20	10
3+	9	1	8
Total	47	28	19

TABLE 22.3. Comparison between HER-2/*neu* immunohistochemistry (IHC) to assess protein overexpression and multiplex ligation-dependent probe amplification (MLPA) to detect HER-2/*neu* gene amplification. (Moelans *et al*., submitted 2007.)

		MLPA	
IHC score	N	Normal	Intermediate/ amplified
0/1+	472	446	15/11
2+	51	44	3/4
3+	59	3	4/52
Total	582	493	22/67

TABLE 22.4. Comparison between HER-2/*neu* immunohistochemistry (IHC) to assess protein overexpression and FISH to detect HER-2/*neu* gene amplification. (Moerland *et al*., 2006.)

		FISH	
IHC score	N	Normal	Amplified
0/1+	7	6	1
2+	30	20	10
3+	9	2	7
Total	46	28	18

TABLE 22.5. Comparison between HER-2/*neu* amplification by MLPA and FISH in 46 cases of invasive breast cancer. (Moerland *et al*., 2006.)

	FISH	
MLPA	Normal	Amplified
Normal	27	1
Amplified	1	17
Total	28	18

TABLE 22.6. Comparison between HER-2/*neu* amplification ratios by MLPA and FISH in 13 cases of HER-2 amplified invasive breast cancer. (Moerland *et al*., 2006.)

		HER2 ratio	
Sample	IHC score	FISH	MLPA
1	2+	2.2	2.9
2	3+	4.6	3.9
3	3+	2.7	5.6
4	3+	2.9	4.6
5	3+	2.7	5.6
6	3+	2.0	5.2
7	3+	3.3	3.3
8	3+	2.5	3.6
9	2+	2.4	6.9
10	2+	2.2	4.0
11	2+	3.8	8.5
12	2+	2.5	7.7
13	2+	2.4	5.9

TABLE 22.7. Comparison between HER-2/*neu* immunohistochemistry (IHC) to assess protein overexpression and FISH to detect HER-2/*neu* gene amplification. (Moelans *et al*., submitted 2007.)

		FISH	
IHC score	N	Normal	Amplified
0/1+	28	22	6
2+	21	17	4
3+	22	3	19
Total	71	42	29

series of 71 formaldehyde-fixed paraffin-embedded breast carcinomas, HER-2 gene amplification by FISH was found in 19/22, 4/21 and 6/28 in IHC 3+, 2+ and 0/1+ cases, respectively (Table 22.7).

In the same series of 71 formaldehyde-fixed paraffin-embedded breast carcinomas, HER-2 gene amplification by FISH was found in 212/2, 6/16 and 2/30 MLPA amplified, intermediate and normal, respectively

(Table 22.8). Similar data were obtained with CISH.

Discussion

Several methods are in use for the detection of HER-2/*neu* gene amplification or protein overexpression, including immunostaining of the protein, FISH, quantitative Southern blotting, and real time PCR. The most widely applied test for HER-2/*neu* is IHC. Depending on the antibody and scoring system used, HER-2/*neu* overexpression rates in the literature vary between 14% (Van de Vijver *et al*., 1988) and 60% (Roche and Ingle, 1999). The subjectivity of IHC generally tends to decrease with increasing positivity, such that inter observer correlation is higher for strongly positive cases. Chromosome 17 polysomy has been postulated to play a role in other studies showing discrepancies between protein expression and gene amplification. Pauletti *et al*. (2000) attributed such 3+ positive, FISH-negative cases to chromosome 17 polysomy, and also found this subset of patients to have similar clinical outcomes to patients without the HER-2/*neu* gene alteration. In the literature, concordance rates between IHC and FISH range from 79% to 100% for 3+ cases (Tubbs *et al*., 2001; Lebeau, 2001) and between 12% and 36% for 2+ cases (Perez *et al*., 2002; Ridolfi, 2000), demonstrating the importance of a gene amplification test.

TABLE 22.8. Comparison between HER-2/*neu* amplification by MLPA and FISH in 71 cases of invasive breast cancer. (Moelans *et al*., submitted 2007.)

	FISH	
MLPA	Normal	Amplified
Normal	28	2
Intermediate	10	6
Amplified	4	21
Total	55	16

Recently, two new methods have been described for the measurement of gene copy number; multiplex amplifiable probe hybridization (MAPH) (Armour *et al*., 2000) and MLPA (Schouten *et al*., 2002). Both techniques rely on comparative quantitation of specifically bound probes that are amplified by PCR with universal primers. The introduction of universal primers has advantages in that multiplexing numerous targets simultaneously becomes much easier, and when fluorescence detection of products is being used, only one fluorescent primer is required, thus reducing the cost compared to buying fluorescent probes for each target. Technically, FISH has disadvantages comparing to MLPA in determining part-gene deletions and remains a relatively low throughput when compared to other molecular genetic techniques available. The latter limitation also holds for Southern blotting, where only a few samples can be run per gel, a limited number of loci can be queried per blot, and the tests may take several days (Sellner and Taylor, 2004). Real-time PCR can also be used as a semi-quantitative technique when an internal amplification control is incorporated and has the advantage of not requiring post-PCR analysis. However, the number of targets that can be interrogated in a reaction is limited by the number of fluorophores available and the detection capabilities of the instrument. In general, PCR-based techniques for gene dosage determination can offer a less labor intensive alternative with higher throughput.

Recent studies have evaluated MLPA for HER-2/*neu* gene amplification testing. In general, there is a good concordance between IHC and MLPA, and MLPA was clearly

able to identify the amplified cases among the equivocal IHC 2+ cases. However, some IHC 0/1+ cases appear to be amplified, and some IHC 3+ cases lack amplification. In view of the very good concordance between MLPA and FISH, it therefore seems that MLPA may at least have additional value to IHC, but may even be attractive for upfront amplification testing.

Compared to FISH, MLPA has many practical advantages. FISH probes loose their fluorescence in time, so the kits have a limited half life and stained slides cannot be kept indefinitely. Furthermore, interpretation has to be done under a fluorescence microscope in a darkened room, which is unpractical for the pathologist. The consequence of the fact that a 100x oil objective is needed to view the small spots is that overview is lost and heterogeneity may be easily missed. The probes for MLPA can be kept, the method works on small amounts of DNA extracted from paraffin-embedded tissue, and can be done in a high-throughput way. An additional advantage of the MRC-Holland kit is that a TOP2A probe is included. TOP2A is located close to the HER-2 locus, is often coamplified with HER-2, and this is related to response to HER-2 targeting therapies (Durbecq *et al*., 2004; Knoop *et al*., 2005; Tanner *et al*., 2006). Besides being a chromosome 17 polysomy control, it may also help to predict response to therapy. A disadvantage is that MLPA is not a morphological method. Therefore, proper tissue selection guided by control H&E sections, and manual-/microdissection in cases with a low percentage of tumor epithelium may be necessary. The lower limit of tumor percentage still allowing meaningful MLPA analysis needs to be established. Obviously, the higher the amplification, the easier it will be to detect amplification in a background of non-amplified cells.

As DCIS often shows HER-2 amplification while the invasive surrounding parts are negative, one has to be careful with blocks showing extensive DCIS which may yield false-positive results. Also in these cases, manual-/microdissection may be necessary.

No studies have yet been published evaluating MLPA as a predictor or response to HER-2 targeting therapy. In view of the high correlation with FISH, we expect MLPA to have equal predictive power as FISH (Mass *et al*., 2005; Chorn, 2006; Hofmann *et al*., 2007).

Cicromogenh in situ hybridization, which has recently been introduced as an alternative to FISH, circumvents many of the disadvantages of FISH. The kits last, stained slides can be kept well, interpretation can be done with a light microscope using dry objective, making it easier to screen a full slide, skip DCIS and detect heterogeneity. The disadvantage of not having a chromosome 17 probe can be overcome using serial sections when deemed necessary. FISH and CISH have been proven to correlate well (Tanner *et al*., 2000; Gong *et al*., 2005; Hanna and Kwok, 2006), but CISH is less quantitative than FISH and MLPA, and is not a high throughput method. Time and future comparative studies will tell whether CISH or MLPA will prevail as HER-2 amplification tests.

In view of the increasing appreciation of the high value of new methods such MLPA and CISH, there will in the future probably be a discussion whether such methods need to be applied next to IHC in

all cases. Although this will increase the costs of HER-2 testing, this will easily be compensated for when a small fraction of patients can be spared a very expensive therapy with significant side effects. Even better, it may be that high throughput amplification tests completely replace IHC. Future studies need to address these cost-effectiveness issues.

In conclusion, MLPA is a quick, cheap and easy method to detect HER-2/*neu* amplification in frozen and paraffin material in daily laboratory practice. MLPA is an attractive alternative to FISH for amplification testing in IHC equivocal cases, but may also be well suited for upfront HER-2 amplification testing in invasive breast cancer.

Acknowledgements. Supported by grant IN-2001-008 of the Dutch Cancer Society and an educational grant from the American Women's club of The Hague.

REFERENCES

Arkblad, E.L., Darin, N., Berg, K., Kimber, E., Brandberg, G., Lindberg, C., Holmberg, E., Tulinius, M., and Nordling, M. 2006. Multiplex ligation-dependent probe amplification improves diagnostics in spinal muscular atrophy. *Neuromuscul. Disord. 16*: 830–838.

Armour, J.A., Sismani, C., Patsalis, P.C., and Cross, G. 2000. Measurement of locus copy number by hybridisation with amplifiable probes. *Nucleic Acids Res. 28*: 605–609.

Baak, P.A., Chin, D., Van Diest, P.J., Matze-Cok, P., and Bacus, S.S. 1991. Comparative long term prognostic value of quantitative Her2/Neu protein expression, DNA ploidy, morphometric and clinical features in paraffin-embedded invasive breast cancer. *Lab. Invest. 64*: 215–222.

Bargmann, C.I., Hung, M.C., and Weinberg, R.A. 1986. The neu oncogene encodes an epidermal growth factor receptor-related protein. *Nature 319*: 226–230.

Birner, P., Oberhuber, G., Stani, J., Reithofer, C., Samonigg, H., Hausmaninger, H., Kubista, E., Kwasny, W., Kandioler-Eckersberger, D., Gnant, M., and Jakesz, R.; Austrian Breast & Colorectal Cancer Study Group. 2001. Evaluation of the United States Food and Drug Administration-approved scoring and test system of HER-2 protein expression in breast cancer. *Clin. Cancer Res. 7*: 1669–1675.

Chorn, N. 2006. Accurate identification of HER2-positive patients is essential for superior outcomes with trastuzumab therapy. *Oncol. Nurs. Forum, 33*: 265–272.

Dikow, N., Nygren, A.O., Schouten, J.P., Hartmann, C., Kramer, N., Janssen, B., and Zschocke, J. 2007. Quantification of the methylation status of the PWS/AS imprinted region: comparison of two approaches based on bisulfite sequencing and methylation-sensitive MLPA. *Mol. Cell Probes 21*: 208–215.

Di Leo, A., Dowsett, M., Horten, B., and Penault-Llorca, F. 2002. Current status of HER2 testing. *Oncology 63*(Suppl 1): 25–32.

Durbecq, V., Paesmans, M., Cardoso, F., Desmedt, C., Di Leo, A., Chan, S., Friedrichs, K., Pinter, T., Van Belle, S., Murray, E., Bodrogi, I., Walpole, E., Lesperance, B., Korec, S., Crown, J., Simmonds, P., Perren, T.J., Leroy, J.Y., Rouas, G., Sotiriou, C., Piccart, M., and Larsimont D. 2004. Topoisomerase-II alpha expression as a predictive marker in a population of advanced breast cancer patients randomly treated either with single-agent doxorubicin or single-agent docetaxel. *Mol. Cancer Ther. 3*: 1207–1214.

Eldering, E., Spek, C.A., Aberson, H.L., Grummels, A., Derks, I.A., De Vos, A.F., McElgunn, C.J., and Schouten, J.P. 2003. Expression profiling via novel multiplex assay allows rapid assessment of gene regulation in defined signalling pathways. *Nucleic Acids Res. 31*: e153.

Ginestier, C., Charafe-Jauffret, E., Penault-Llorca, F., Geneix, J., Adelaide, J., Chaffanet, M., Mozziconacci, M.J., Hassoun, J., Viens, P., Birnbaum, D., and Jacquemier, J. 2004. Comparative multi-methodological measurement of ERBB2 status in breast cancer. *J. Pathol. 202*: 286–298.

Gong, Y., Gilcrease, M., and Sneige, N. 2005. Reliability of chromogenic in situ hybridization for detecting HER-2 gene status in breast cancer: comparison with fluorescence in situ hybridization

and assessment of interobserver reproducibility. *Mod. Pathol. 18* : 1015–1021.

Hanna, W. 2001. Testing for HER2 status. *Oncology 61*(Suppl 2): 22–30.

Hanna, W.M., and Kwok, K. 2006. Chromogenic in-situ hybridization: a viable alternative to fluorescence in-situ hybridization in the HER2 testing algorithm. *Mod. Pathol. 19* : 481–487.

Hess, C.J., Denkers, F., Ossenkoppele, G.J., Waisfisz, Q., McElgunn, C.J., Eldering, E., Schouten, J.P., and Schuurhuis, G.J. 2004. Gene expression profiling of minimal residual disease in acute myeloid leukaemia by novel multiplex-PCR-based method. *Leukemia 18*: 1981–1988.

Hofmann, M., Stoss, O., Gaiser, T., Kneitz, H., Heinmoller, P., Gutjahr, T., Kaufmann, M., Henkel, T., and Ruschoff, J. 2008. Central HER2 IHC and FISH analysis in a trastuzumab (Herceptin®) phase II monotherapy study: assessment of test sensitivity and impact of chromosome 17 polysomy. *J. Clin. Pathol. 61*: 89–94.

Jacobs, T.W., Gown, A.M., Yaziji, H., Barnes, M.J., and Schnitt, S.J. 1999. Comparison of fluorescence in situ hybridization and immunohistochemistry for the evaluation of HER-2/neu in breast cancer. *J. Clin. Oncol. 17*: 1974–1982.

Knoop, A.S., Knudsen, H., Balslev, E., Rasmussen, B.B., Overgaard, J., Nielsen, K.V., Schonau, A., Gunnarsdottir, K., Olsen, K.E., Mouridsen, H., and Ejlertsen B.; Danish Breast Cancer Cooperative Group. 2005. Retrospective analysis of topoisomerase IIa amplifications and deletions as predictive markers in primary breast cancer patients randomly assigned to cyclophosphamide, methotrexate, and fluorouracil or cyclophosphamide, epirubicin, and fluorouracil: Danish Breast Cancer Cooperative Group. *J. Clin. Oncol. 23*: 7483–7490.

Konecny, G.E., Thomssen, C., Luck, H.J., Untch, M., Wang, H.J., Kuhn, W., Eidtmann, H., du Bois, A., Olbricht, S., Steinfeld, D., Mobus, V., von Minckwitz, G., Dandekar, S., Ramos, L., Pauletti, G., Pegram, M.D., Janicke, F., and Slamon, D.J. 2004. Her-2/neu gene amplification and response to paclitaxel in patients with metastatic breast cancer. *J. Natl. Cancer Inst. 96*: 1141–1151.

Lebeau, A., Deimling, D., Kaltz, C., Sendelhofert, A., Iff, A., Luthardt, B., Untch, M., and Lohrs, U. 2001. Her-2/neu analysis in archival tissue samples of human breast cancer: comparison of immunohistochemistry and fluorescence in situ hybridization. *J. Clin. Oncol. 19*: 354–363.

Mass, R.D., Press, M.F., Anderson, S., Murphy, M., and Slamon, D. 2001. Improved survival benefit from Herceptin (trastuzumab) in patients selected by fluorescence in situ hybridization (FISH). *Proc. Am. Soc. Clin. Oncol. 20*: 85.

Mass, R.D., Press, M.F., Anderson, S., Cobleigh, M.A., Vogel, C.L., Dybdal, N., Leiberman, G., and Slamon, D.J. 2005. Evaluation of clinical outcomes according to HER2 detection by fluorescence in situ hybridization in women with metastatic breast cancer treated with trastuzumab. *Clin. Breast Cancer 6*: 240–246.

Moelans, C., De Weger, R.A., Van Blokland, M., Ezendam, C., Griesdoorn, V., Elshof, S.M., Rozemuller, E., Tilanus, M., and Van Diest, P.J. 2007. HER-2/neu amplification testing in breast cancer by multiplex ligation-dependent probe amplification in comparison with immunohistochemistry and in situ hybridization. *Cell. Oncol.* in press.

Moerland, E., Van Hezik, R.L.H.P.M., Van der Aa, T.C.J.M., Van Beek, M.W.P.M., and Van den Brule, A.J.C. 2006. Detection of HER2 amplification in breast carcinomas: comparison of Multiplex Ligation-dependent Probe Amplification (MLPA) and Fluorescence In Situ Hybridization (FISH) combined with automated spot counting. *Cell. Oncol. 28*: 151–159.

Nygren, A.O., Ameziane, N., Duarte, H.M., Vijzelaar, R.N., Waisfisz, Q., Hess, C.J., Schouten, J.P., and Errami, A. 2005. Methylation-specific MLPA (MS-MLPA): simultaneous detection of CpG methylation and copy number changes of up to 40 sequences. *Nucleic Acids Res. 33*: e128.

Pauletti, G., Dandekar, S., Rong, H., Ramos, L., Peng, H., Seshadri, R., and Slamon, D.J. 2000. Assessment of methods for tissue-based detection of the HER-2/neu alteration in human breast cancer: a direct comparison of fluorescence in situ hybridization and immunohistochemistry. *J. Clin. Oncol. 18*: 3651–3664.

Penault-Llorca, F., Adelaide, J., Houvenaeghel, G., Hassoun, J., Birnbaum, D., and Jacquemier, J. 1994. Optimization of immunohistochemical detection of ERBB2 in human breast cancer: impact of fixation. *J. Pathol. 173*: 65–75.

Perez, E.A., Roche, P.C., Jenkins, R.B., Reynolds, C.A., Halling, K.C., Ingle, J.N., and Wold, L.E.

2002. HER2 testing in patients with breast cancer: poor correlation between weak positivity by immunohistochemistry and gene amplification by fluorescence in situ hybridization. *Mayo Clin. Proc. 77*: 148–154.

Popescu, N.C., King, C.R., and Kraus, M.H. 1989. Localization of the human erbB-2 gene on normal and rearranged chromosomes 17 to bands q12-21.32. *Genomics 4*: 362–366.

Press, M.F., Slamon, D.J., Flom, K.J., Park, J., Zhou, J.Y., and Bernstein, L. 2002. Evaluation of HER-2/neu gene amplification and overexpression: comparison of frequently used assay methods in a molecularly characterized cohort of breast cancer specimens. *J. Clin. Oncol. 20*: 3095–3105.

Procter, M., Chou, L.S., Tang, W., Jama, M., and Mao, R. 2006. Molecular diagnosis of Prader-Willi and Angelman syndromes by methylation-specific melting analysis and methylation-specific multiplex ligation-dependent probe amplification. *Clin. Chem. 52*: 1276–1283.

Purnomosari, D., Aryandono, T., Setiaji, K., Nugraha, S.B., Pals, G., and Van Diest, P.J. 2006. Comparison of multiplex ligation probe amplification to immunohistochemistry for assessing HER-2/neu amplification in invasive breast cancer. *Biotechnic & Histochemistry. 81*(2–3) : 79–85

Raju, U., Lu, M., Sethi, S., Qureshi, H., Wolman, S.R., and Worsham, M.J. 2006. Molecular classification of breast carcinoma in situ. *Curr. Genomics 7*: 523–532.

Rhodes, A., Jasani, B., Anderson, E., Dodson, A.R., and Balaton, A.J. 2002a. Evaluation of HER-2/neu immunohistochemical assay sensitivity and scoring on formalin-fixed and paraffin-processed cell lines and breast tumors: a comparative study involving results from laboratories in 21 countries. *Am. J. Clin. Pathol. 118*: 408–417.

Rhodes, A., Jasani, B., Couturier, J., McKinley, M.J., Morgan, J.M., Dodson, A.R., Navabi, H., Miller, K.D., and Balaton, A.J. 2002b. A formalin-fixed, paraffin-processed cell line standard for quality control of immunohistochemical assay of HER-2/neu expression in breast cancer. *Am. J. Clin. Pathol. 117*: 81–89.

Ridolfi, R.L., Jamehdor, M.R., and Arber, J.M. 2000. HER-2/neu testing in breast carcinoma: a combined immunohistochemical and fluorescence in situ hybridization approach. *Mod. Pathol. 13*: 866–873.

Roche, P.C., and Ingle, J.N. 1999. Increased HER2 with U.S. Food and Drug Administration-approved antibody. *J. Clin. Oncol. 17*: 434.

Schouten, J.P., McElgunn, C.J., Waaijer, R., Zwijnenburg, D., Diepvens, F., and Pals, G. 2002. Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res. 30*: e57.

Sellner, L.N., and Taylor, G.R. 2004. MLPA and MAPH: new techniques for detection of gene deletions. *Hum. Mutat. 23*: 413–419.

Slamon, D.J., Clark, G.M., Wong, S.G., Levin, W.J., Ullrich, A., and McGuire, W.L. 1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science 235*: 177–182.

Slamon, D.J., Godolphin, W., Jones, L.A., Holt, J.A., Wong, S.G., Keith, D.E., Levin, W.J., Stuart, S.G., Udove, J., and Ullrich, A. 1989. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science 244*: 707–712.

Sparano, J.A. 2001. Cardiac toxicity of trastuzumab (Herceptin): implications for the design of adjuvant trials. *Semin Oncol 28*(1 Suppl 3): 20–27.

Tanner, M., Gancberg, D., Di, L.A., Larsimont, D., Rouas, G., Piccart, M.J., and Isola, J. 2000. Chromogenic in situ hybridization: a practical alternative for fluorescence in situ hybridization to detect HER-2/neu oncogene amplification in archival breast cancer samples. *Am. J. Pathol. 157*: 1467–1472.

Tanner, M., Isola, J., Wiklund, T., Erikstein, B., Kellokumpu-Lehtinen, P., Malmstrom, P., Wilking, N., Nilsson, J., and Bergh, J. 2006. Topoisomerase IIalpha gene amplification predicts favorable treatment response to tailored and dose-escalated anthracycline-based adjuvant chemotherapy in HER-2/neu-amplified breast cancer: Scandinavian Breast Group Trial 9401. *J. Clin. Oncol. 24*: 2428–2436.

Tubbs, R.R., Pettay, J.D., Roche, P.C., Stoler, M.H., Jenkins, R.B., and Grogan, T.M. 2001. Discrepancies in clinical laboratory testing of eligibility for trastuzumab therapy: apparent immunohistochemical false-positives do not get the message. *J. Clin. Oncol. 19*: 2714–2721.

Van de Vijver, M.J., Peterse, J.L., Mooi, W.J., Wisman, P., Lomans, J., Dalesio, O., and Nusse, R. 1988. Neu-protein overexpression in breast cancer. Association with comedo-type ductal carcinoma in

situ and limited prognostic value in stage II breast cancer. *N. Engl. J. Med. 319*: 1239–1245.

Vorstman, J.A., Jalali, G.R., Rappaport, E.F., Hacker, A.M., Scott, C., and Emanuel, B.S. 2006. MLPA: a rapid, reliable, and sensitive method for detection and analysis of abnormalities of 22q. *Hum. Mutat. 27*: 814–821.

Wright, C., Nicholson, S., Angus, B., Sainsbury, J.R., Farndon, J., Cairns, J., Harris, A.L., and Horne, C.H. 1992. Relationship between c-erbB-2 protein product expression and response to endocrine therapy in advanced breast cancer. *Br. J. Cancer 65*: 118–121.

23
Operable Breast Cancer: Neoadjuvant Treatment (Methodology)

Manfred Kaufmann, Sibylle Loibl, and Gunter von Minckwitz

INTRODUCTION

Primary operable breast cancer has been treated by neoadjuvant systemic therapy (NST) to make breast conservation possible in some patients with operable disease who otherwise would require a mastectomy. Nowadays, the surgical defect is expected to be as limited as possible. Neoadjuvant systemic therapy has become widely accepted as the treatment of choice for patients with locally advanced disease, large inoperable tumors or inflammatory disease to convert inoperable to operable primary tumors. Although the terms *primary systemic therapy* and *preoperative therapy* are more accurate descriptions of this treatment, NST is the term that has come into broad use, and that is the term we will use here (Kaufmann *et al*., 2003, 2006).

DIAGNOSIS

The diagnosis of breast cancer has to be histologically confirmed by core cut biopsy. It is recommended to take 3–5 core biopsies for diagnosis and to evaluate predictive and prognostic markers that are necessary for treatment decision. Imaging is an important adjunct to neoadjuvant therapy. Before starting therapy, it helps to identify the extent of the disease; during therapy it can be used to evaluate response, and after NST, it may assist with evaluation of the extent of residual tumor and to guide surgery. The imaging techniques used in the baseline examination that best delineate the malignancy should be repeated during treatment to document tumor response. Clinical examination based on palpable change in tumor size is the most common and easiest method for monitoring treatment effect and has been acknowledged as a factor of prognostic importance. It has been demonstrated that the treatment effect is frequently overestimated with clinical examination, whereas the effect is usually underestimated with ultrasound measurement (von Minckwitz *et al*., 2005).

It seems advisable to perform ultrasound and clinical examination in combination with mammography for response evaluation to rule out over- and under-estimation

of response. In the case of ambiguous results, multicentricity, and lobular invasive cancers, a quantitative contrast-enhanced MRI is helpful (Manton *et al*., 2006). Some studies have shown MRI to be superior to mammography, ultrasound, and clinical examination in evaluating the extent of the tumor (Esserman *et al*., 1999). The agreement of pathologic residual tumor size with mammography or sonography residual tumor is moderate, especially in lobular invasive cancers (Chagpar *et al*., 2006; Peintinger *et al*., 2006). At the time of surgery, MRI may, therefore, help to identify residual disease more accurately and assist guiding the surgeon. However, changes in imaging generally manifest themselves later than changes in underlying tumor function, e.g., vascular density and permeability (Wasser *et al*., 2003). Newer techniques, such as proton magnetic resonance spectroscopy, diffusion weighted imaging, interstitial fluid pressure, and Doppler ultrasound are under investigation (Mardor *et al*., 2003; Taghian *et al*., 2005).

TREATMENT DECISION

Treatment decision is based on various known predictive and prognostic markers. If the aim is to target the most dangerous tumor cells, it is reasonable to believe that NST is appropriate for each patient who has an indication to receive adjuvant therapy. Groups of patients need to be identified to whom it is appropriate to offer NST after histological diagnosis of breast cancer. Offering NST to a patient means providing comprehensive information on the risks and benefits of systemic treatment in general as well as its schedule, and an explanation for what purpose this treatment should be given before surgery.

For operable breast cancer, NST may be offered to all patients who are expected to be candidates for adjuvant systemic chemotherapy. As a prerequisite, all necessary information for this decision should be available at the time of the recommendation. According to current recommendations adjuvant systemic treatment decisions are based on endocrine responsiveness of the tumor, lymph node involvement, age and menopausal status at the time of diagnosis, tumor size, grade, and HER-2/neu receptor status. Whereas steroid hormone receptors and grade can be determined accurately on the tissue from the diagnostic core biopsy, information on lymph node involvement and tumor size usually will be derived from imaging tests and will have some degree of uncertainties Sometimes, in borderline cases (e.g., a postmenopausal patient with a T 1–2, hormone sensitive tumor) reliable information on lymph node status is warranted for decision. This recommendation for the primary use of chemotherapy is based on a set of randomized trials showing equal efficacy for the post- and preoperative use of non-anthracycline or anthracycline containing regimen (Fisher *et al*., 1998; Scholl *et al*., 1994). Equal long term efficacy has also been shown for the post- and preoperative use of docetaxel in one trial.

There is a group of selected patients who are expected to be candidates for adjuvant endocrine therapy alone. This addresses mainly postmenopausal, frail patients where surgery alone or in combination with chemotherapy is associated with an increased risk due to age and/or relevant life expectancy limiting comorbidities. However, there is no information

on the comparative efficacy of pre and postoperative use of hormonal agents. As a consequence, NST should be avoided in patients for whom the need and the type of adjuvant therapy cannot be defined. In this subset of patients there is a possibility of over-treatment if the individual risk is overestimated.

Chemotherapy

Neoadjuvant systemic therapy is considered as safe and effective as the same systemic treatment given postoperatively (Davidson and Morrow, 2005; Mauri *et al*., 2005). Chemotherapeutic regimens are equally efficacious regardless of whether they were administered postoperatively or preoperatively (Fisher *et al*., 1998; Scholl *et al*., 1994). Equal long-term efficacy was also shown in a trial of docetaxel given either postoperatively or preoperatively.

Anthracycline/taxane-based chemotherapy regimens have been extensively studied in prospective randomized trials and are the most frequently prescribed treatments in patients with operable breast cancer (Evans *et al*., 2005; Green *et al*., 2005). Regimens that have been tested in large multicenter phase III trials and yielded pCR rates of at least 15% and up to 20% are AC followed by docetaxel (AC-Doc), docetaxel/doxorubicin/cyclophosphamide (TAC), epirubicin/paclitaxel-cyclophosphamide/methotrexate/5-fluorouracil (ET-CMF) and a dose-dense sequence of epirubicin and paclitaxel (E-T). Administration strategies have included the sequential, concurrent, and both sequential and concurrent delivery of agents as well as dose-dense approaches. So far, however, no strategy has been found to be clearly superior to the others in patients with operable breast cancer. For this reason, the type of chemotherapy has not been standardized. Treatment with paclitaxel in the dose-dense and weekly schedules has appeared to be more promising than the 3weekly schedule, but the efficacy of docetaxel depends less on the schedule used (Green *et al*., 2005). Most of the trials of docetaxel in sequence or combination with an anthracycline-based regimen have yielded pCR rates in excess of 20%.

Until now, an increase in the pCR rate as the result of a superior treatment has not been consistently proven to translate into an improved long-term outcome. This is illustrated by two trials. In a small trial conducted by the Aberdeen Group, in which a significant increase in the pCR rate was observed in patients showing an early response to an anthracycline-containing regimen who had taxane added to their treatment, a significant survival benefit was seen (Smith *et al*., 2002). In the larger NSABP B-27 trial, however, although the pCR rate was double in the group who had a taxane added to an anthracycline-containing regimen, there was no significant improvement in the long-term outcome in these patients. In the NSABP B-27 trial, there was a significant increase in relapse-free survival rate in the preoperative docetaxel arm compared with the AC alone arm, with a P value of 0.03. In addition, the disease-free survival rate was 5% better at 5 years in the preoperative docetaxel arm than in the AC alone arm, with a P value of 0.10. No overall survival benefit has been observed as yet. However, because the difference in the pCR rate was only 13%, not all patients with pCRs remain relapse-free, and not all patients

with non-pCRs suffer relapse, it would be unrealistic to expect the improvements shown by the Aberdeen trial. Nonetheless, because the patients who achieved a pCR from a superior NST also showed improved survival, this trial did confirm that pCR can serve as a surrogate marker for favorable long term prognosis. However, as of the most recent report of this trial, this gain was insufficient to improve survival in the total population.

In a retrospective analysis of data from the NSABP B-27 study, one group of patients was identified who did substantially benefit from the longer treatment and/or the addition of docetaxel. These were the patients who showed a partial clinical remission after the first four cycles of Adriamycin/cyclophosphamide, and the improvement consisted of a significantly better disease-free survival rate in patients who received four further cycles of docetaxel than in those who did not. Such improvement was not seen in patients who showed either a complete or no clinical remission after AC. The value of an early response assessment immediately after the second cycle of chemotherapy in order to predict pCR has prospectively been shown recently in a pilot trial of the Gepartrio study of the German Breast Group.

A problem of most of these trials is that they have also compared a shorter with a longer treatment schedule, which makes it difficult to judge whether these results are an effect of the regimen or the prolonged duration of treatment. Some trials have compared different combination treatments with short durations of 3–6 cycles but did not show a significant improvement [e.g., EDoc (epirubicin/docetaxel) versus EC (epirubicin/cyclophosphamide], ADoc (adriamycin/docetaxel) (q2w, q3w).

Tailoring Therapy

In some trials, therapy is tailored according to the patient's response to neoadjuvant therapy. In the Aberdeen trial, patients who responded to the first part of the treatment were randomized to continue preoperatively with the same therapy or to change to a non-cross-resistant regimen including a taxane. In the Gepartrio (German Breast Group) trial, therapy is tailored in non-responders, whereas the M. D. Anderson group randomized patients with residual tumor exceeding $1\,cm^3$ to the postoperative therapy depending on their response to the neoadjuvant therapy (Thomas *et al.*, 2004). The Aberdeen and M. D. Anderson trials were able to demonstrate a survival benefit by changing the therapy. So far it is not yet clear which group, the responders or nonresponders, benefits the most from changing to a non-cross-resistant regimen. The Gepartrio trial could not demonstrate an increased pCR rate for the patients who changed to a non-cross resistant therapy if they did not show an early response.

Endocrine Therapy

Endocrine therapy alone is also efficacious preoperatively and has primarily been tested in postmenopausal women with hormone receptor-positive tumors who needed mastectomy but were not fit for chemotherapy. Studies have shown that neoadjuvant endocrine treatment alone might be as good as chemotherapy in postmenopausal patients with endocrine responsive disease (Smith *et al.*, 2005). Its use as NST alone would be appropriate mainly for frail postmenopausal patients in whom surgery with or without chemotherapy would be associated with an increased risk because of advanced age

or relevant and life-limiting comorbidities. Data have consistently shown that the percentage of mastectomies is significantly reduced in patients who receive aromatase inhibitors (AIs), and that the risk of tumor progression during the first 4 months of such treatment is low (≤ 10%). However, there is a lack of information on the comparative efficacy of hormonal agents given preoperatively and postoperatively.

Although the clinical response rate in women who received the aromatase inhibitor letrozole or anastrozole was significantly higher than that in women treated with tamoxifen, the pCR rate for all primary endocrine treatments is low, ranging from 1% to 8% (Eiermann *et al*., 2001). In a trial conducted by Cameron *et al*. (1997), first-line hormone therapy was only offered to women with estrogen receptor (ER)-moderate/-rich (> 20 fmol/protein tumor), and CHOP (cyclophosphamide, hydroxydaunomycin, oncovin [vincristine], and prednisone) was reserved for those women whose tumors did not respond to hormone therapy or who had ER-negative/-poor tumors. There was no difference in survival between those women given hormone therapy initially and those given chemotherapy. The two key factors that predicted poor survival were the number of involved axillary nodes after preoperative systemic therapy ($P < 0.00001$) and a lack of response to preoperative therapy ($P < 0.05$). These data suggest that the prognosis is good for many women with ER-responsive tumors who receive preoperative hormone therapy alone. In elderly patients, tamoxifen treatment alone compared with tamoxifen as adjuvant therapy after surgery was associated with no difference in overall and disease-free survival (Mustacchi *et al*., 2003).

Aromatase inhibitors were associated with a larger proportion of local responses and breast conservation procedures than was tamoxifen. There are no large trials demonstrating that breast conservation after neoadjuvant endocrine therapy is as safe as mastectomy in terms of local regional control.

However, the concurrent use of chemotherapy and tamoxifen may be detrimental in terms of both survival and toxicity (von Minckwitz *et al*., 2001). Endocrine treatment should be started after surgery and any chemotherapy. The use of other endocrine treatments, such as those that suppress ovarian function, in combination with aromatase inhibitors in premenopausal women (alone, in sequence with, or in combination with chemotherapy) is investigational.

There are limited data on the use of preoperative endocrine treatment in premenopausal patients. A combination of gonadotropin releasing hormone analogue (GnRH) and letrozole in premenopausal patients with ER and PR ≥ 10% with a median duration of 5 and 4 months, respectively, demonstrated a pCR of 3% and a rate of breast conservation of 42%. A tailored approach of a chemoendocrine therapy with or without an AI demonstrated a pCR rate of 7% and 11%, respectively and a BCS rate of 62% and 58%, respectively (Della Pasqua *et al*., 2006; Torrisi *et al*., 2007). However, none of the approaches can be used as a routine therapy. Proper timing of ovarian function suppression (OFS) in relation to chemotherapy is not clearly defined. As has been stated before, chemotherapy and tamoxifen should be delivered sequentially but no such data exist for OFS. Hence, the concomitant use of any indicated chemotherapy with GnRH might be considered as acceptable in women

with a desire of pregnancy (Del Mastro *et al.*, 2006). A combined chemoendocrine approach including an AI is investigational. Whereas the AIs are well established in the neoadjuvant endocrine treatment of postmenopausal women with hormone-responsive tumors. The IMPACT trial could not demonstrate a significant benefit in terms of the clinical response rate for the AIs over tamoxifen.

Therapy for HER-2/neu–Overexpressing Tumors

Trastuzumab should be incorporated into the regimen in patients with HER-2/neu positive disease, but concomitant use with an anthracycline of less cardiac toxicity (epirubicin/pegylated doxorubicin) should only occur in clinical trials. The concomitant application of trastuzumab and epirubicin seems safe as could be demonstrated in the Geparquattro trial of the GBG and the MD-Anderson trial (Buzdar *et al.*, 2007). The Geparquattro trial by the German Breast Group, is investigating in the neoadjuvant protocol the additional use of trastuzumab for HER2/neu positive patients, concurrent to the cytotoxic treatment until 1 year thereafter (GBG 40 protocol). This will be the largest prospectively treated neoadjuvant HER-2/neu population. Other antibodies such as Bevacizumab an anti-VEGF antibody, lapatinib-a dual anti HER tyrosinkinase inhibitor are investigational and current running trials will investigate these new treatments.

Predictive Marker of Response and Resistance

Predicting the chance of response to treatment before starting NST is an important research goal. Patients with a low chance of a pCR, and especially a low chance of a clinically useful response, might then be spared unnecessary toxicity. As stated above, patients whose *tumors* express markers predictive of chemo-response are the best candidates for neoadjuvant chemotherapy. Negative hormone receptor status is one of the strongest predictive markers for chemo-response in general. Tumors completely lacking such receptors were found to be particularly sensitive to preoperative cytotoxic agents, but despite a pathological complete remission rate exceeding 40%, survival of patients with this phenotype was reported in several studies to be shorter than those with receptor-positive tumors, supporting the use of postoperative adjuvant therapies in the former subgroup of patients, especially in cases with no response to preoperative therapy (Colleoni *et al.*, 2004; Hennessy *et al.*, 2005; Ring *et al.*, 2004).

Recently, investigations have concentrated on predicting a response to specific therapies based on various biological markers. HER-2/neu was one of the most intensively studied marker. However, so far there is no clear evidence that HER-2/neu-positive breast cancers respond better than negative ones to anthracycline and taxane based chemotherapy (Bozzetti *et al.*, 2006; Loibl *et al.*, 2005). HER-2/neu negative breast cancer patients might respond better to a taxane than to an anthracycline treatment. Topoisomerase IIα has been demonstrated to be an independent predictor of clinical tumor response, especially to anthracycline, but in a gene array analysis it failed to predict pCR (MacGrogan *et al.*, 2003; Rody *et al.*, 2007). It is unlikely that a single biologic marker will ever be

able to differentiate definitively between responders and non-responders (Burcombe *et al*., 2005).

An alternative to measuring one specific marker is to use a combination of different markers and/or the establishment of a specific gene expression profile that can predict response to neoadjuvant therapy. Gene expression profiling has become quite popular, but has so far failed to identify a definitive population which should be spared of chemotherapy, nor has this approach identified a population which has more than 80% chance of a pCR.

It is well known that patients with a pCR have a better overall survival than those who did not achieve a pCR. Data are limited regarding the prognostic significance of residual in situ ductal cancer (DCIS) (Abrial *et al*., 2005; Jones *et al*., 2006). Apart from the pCR in the breast, one of the most important prognostic factors after neoadjuvant therapy is the post-treatment lymph node status (Klauber-DeMore *et al*., 2006). Chemosensitivity testing is investigational and should not be used outside clinical trials.

LOCAL TREATMENT

Surgical Management of the Breast

Breast surgery after primary chemotherapy should be performed according to the guidelines for breast surgery without prior systemic therapy.

Surgical margins should be free of invasive or noninvasive breast cancer. Otherwise, re-excision (breast-conserving re-excision or mastectomy) should be performed.

Adequate cosmetic results should be anticipated.

If cosmetically acceptable, the whole previously involved area of the breast can be excised. Otherwise (e.g., in case of a clinically complete response and unfavorable relation of tumor to breast size), a biopsy of adequate size should be taken from a representative area. In this case a wire-guided biopsy is recommended.

Breast conservation techniques include lumpectomy, wide excision (segmental resection), and quadrantectomy (Senofsky *et al*., 1998). For medium and large breasts, dermoglandular flaps can be used to fill the excision site. If intramammary glandular flaps are used or if reduction mammoplasty techniques are used, the initial tumor location should be marked by clips on the pectoralis muscle to allow planning of subsequent breast irradiation (Loibl *et al*., 2006).

When mastectomy is indicated, postoperative radiotherapy is also indicated in most of these patients (see below), and therefore breast reconstruction should usually be delayed until 6–12 months after completion of breast irradiation.

Data on breast conservation after neoadjuvant chemotherapy are limited, but several studies have demonstrated that NST can increase the feasibility of breast conservation without adversely affecting survival. There are some factors such as tumor size, lymphatic invasion, nodal status, or diffuse microcalcifications (Chen *et al*., 2005; Sadetzki *et al*., 2005), which might influence the success of BCS. Also, some data suggest that converting young patients who were not candidates from breast conservation to lumpectomy candidates with NST may be associated with a higher risk of local recurrence. However, this does not appear to compromise patient overall outcomes. Surgical planning and execution should take into account the size of the original tumor and the response to NST. Before starting treatment, the tumor region should be marked either by inserting a titanium clip or by marking the skin

with tattoos or with temporary marking plus taking a photograph of that region (Kaufmann *et al*., 2003).

Surgical Management of the Axilla

The standard surgical procedure for staging the axilla has been axillary dissection, aiming at removal of at least 10 axillary lymph nodes. Sentinel lymph node biopsy (SLN) before the start of NST is an option that has been advocated by some investigators, but is not mandatory. The accuracy of SLN biopsy for determining lymph node status before primary surgery has been confirmed in a metaanalysis by Kim *et al*. (2006), who pooled data from 69 trials with more than 8,000 pts. Thirty percent of patients with T1 tumors had involved lymph nodes.

SLN biopsy *after* NST is an acceptable approach. In patients with clinically tumor-free axillary lymph nodes after NST, sentinel lymph node biopsy may be performed, with accuracy that is comparable to that in primary surgery patients (Mamounas *et al*., 2005). The identification rate (pooled estimate 90%) and the false-negative rate (pooled estimate 12%) for SLN biopsy reported in a recent meta-analysis were similar for patients with or without preoperative chemotherapy (Xing *et al*., 2006). The SLN biopsy procedure and histologic examination should be performed according to the consensus recommendations (Kuehn *et al*., 2005). However, this procedure is still controversial after neoadjuvant chemotherapy, and should therefore be used with caution in patients presenting with grossly positive nodes, and only in patients with a clinically negative axilla after chemotherapy patients should be informed by the surgeon that this approach is unproven. Intraoperative frozen sections of the SLN should be performed. In the subsequent paraffin histology, step sections as well as immunohistochemical stainings for epithelial markers such as cytokeratins should be performed. If the frozen section and the definitive histology show no tumor cells in the SLN, axillary dissection is not needed and the axilla can be considered tumor-free. If SLN mapping fails or if the SLN is positive for metastases (> 0.2 mm), then standard axillary dissection should be carried out. We cannot yet identify any patients with certainty who might forego excision of the primary tumor site. Whether we could spare the patient the operation on the axilla has not been systematically investigated in those patients who respond well to NST and have a clinically negative axilla.

Radiotherapy

It has been proven that systemic treatment and radiotherapy are independent factors influencing the outcome of patients with operable breast cancer, and that both improve locoregional control, disease-free, and overall survival (Clarke *et al*., 2005). However, the addition of preoperative radiotherapy to NST in patients with operable tumors has not yet been adequately proven to be effective. There are limited data demonstrating additional benefits in terms of higher rates of pCR and breast conservation especially in T3 and T4 tumors. Regardless, because up to one third of patients with clinical complete remission after NST still have pathologic evidence of residual tumor in the breast, radiotherapy alone cannot replace adequate surgery (Ring *et al*., 2003).

It appears that postmastectomy radiation therapy is beneficial in patients with

an initial T3 or T4 tumor who subsequently achieved a pCR, because the rate of locoregional recurrence remains high in these patients (Huang *et al.*, 2004). Postmastecomy radiotherapy is not justified in patients with T1 or T2 disease and one to three involved lymph nodes because of the low 5-year risk of local regional recurrence in these patients (Garg *et al.*, 2004). However, because the number of involved lymph nodes can be altered by NST, post-mastectomy radiotherapy should also be considered in patients who present initially with clinically positive lymph nodes.

PATHOLOGY

To perform neoadjuvant treatment in primary breast cancer an interdisciplinary team including a breast pathologist is necessary. There are several prerequisites which need to be fulfilled for histopathological assessment in NST. Mandatory before the start of any NST is to perform a core biopsy to obtain the diagnosis. To offer a tailored therapy to each patient, predictive parameters and biomarkers should be investigated in the pretherapeutic biopsy. These markers include the histological subtype, the tumor grading, the ER and PR percentages and intensity and the HER-2/neu status by immunohistochemistry or by FISH.

As the management of patients with NST is a interdisciplinary task, the pathologist needs sufficient information about the clinical situation to be able to perform an optimal histopathological assessment in a minimal amount of processing time. For each tumor resection specimen, the information given to the pathologist is: (1) a NST has been performed, (2) the clinical pretherapeutic tumor size, localization and extent of the disease (one lesion or multicentricity), (3) and the clinical and/or sonographic response, and (4) the localization of residual tumor foci determined by preoperative imaging studies.

In the pathology report of the surgical specimen after NST the total size of the tumor bed as well as, in cases of therapy-induced regression, the size of largest foci of invasive as well as noninvasive tumor should be reported. Furthermore therapy-induced regressive cellular changes such as sclerosis and inflammation have to be documented. The pathologist should determine the tumor-free margins in all six directions of the specimen, the regression score (using one of the national or international scoring systems), to repeat the ER, PR, HER-2/neu status at least in initially negative disease.

Axillary lymph nodes should be examined and reported. The pathology report should give the total number of nodes, the number of positive nodes with information on the size of the metastatic foci, regressive changes, and the number of nodes with therapy-induced regressive changes, but without residual tumor cells. All tumor parameters should be reported according to the TNM system with the addition of "y" to indicate the status post-therapy. Quality control can be assured by spot check and monitoring of the pathology report.

Several neoadjuvant trials demonstrate a difference between lobular invasive and ductal invasive carcinomas in terms of response to NST and BCS (Loibl *et al.*, 2006; Wenzel *et al.*, 2007). However, there is still a debate if the histological subtype is an independent prognostic factor and if

the worse response to NST in this subgroup translates into a worse overall survival.

In conclusion, neoadjuvant therapy is an important tool to transform primary inoperable into operable breast cancer. However, in the last decade it has become an equally important method as adjuvant cytotoxic therapy for primary operable breast cancer. Neoadjuvant treatment is a perfect situation to establish predictive markers and to test new treatment options, because the pCR can serve as a surrogate marker for long term outcome. However, the best prognostic marker for disease free and overall survival is still the nodal status determined at the time of definite surgery. It has become clear in the last years that the chemotherapy should be completed before surgery unless progression of the tumor is observed, and therefore no postoperative chemotherapy is usually necessary.

REFERENCES

Abrial, S.C., Penault-Llorca, F., Delva, R., Bougnoux, P., Leduc, B., Mouret-Reynier, M.A., Mery-Mignard, D., Bleuse, J.P., Dauplat, J., Cure, H., and Chollet, P. 2005. High prognostic significance of residual disease after neoadjuvant chemotherapy: a retrospective study in 710 patients with operable breast cancer. *Breast Cancer Res. Treat. 94*: 255–263.

Bozzetti, C., Musolino, A., Camisa, R., Bisagni, G., Flora, M., Bassano, C., Martella, E., Lagrasta, C., Nizzoli, R., Personeni, N., Leonardi, F., Cocconi, G., and Ardizzoni, A. 2006. Evaluation of HER-2/neu amplification and other biological markers as predictors of response to neoadjuvant anthracycline-based chemotherapy in primary breast cancer: the role of anthracycline dose intensity. *Am. J. Clin. Oncol. 29*: 171–177.

Burcombe, R.J., Makris, A., Richman, P.I., Daley, F.M., Noble, S., Pittam, M., Wright, D., Allen, A., Dove, J., and Wilson, G.D. 2005. Evaluation of ER, PgR, HER-2 and Ki-67 as predictors of response to neoadjuvant anthracycline chemotherapy for operable breast cancer. *Br. J. Cancer 92*: 147–155.

Buzdar, A.U., Valero, V., Ibrahim, N. K., Francis, D., Broglio, K. R., Theriault, R. L., Pusztai, L., Green, M. C., Singletary, S. E., Hunt, K. K., Sahin, A. A., Esteva, F., Symmans, W. F , Ewer, M. S., Buchholz, T. A., and Hortobagyi, G.N. 2007. Neoadjuvant therapy with paclitaxel followed by 5-fluorouracil, epirubicin, and cyclophosphamide chemotherapy and concurrent trastuzumab in human epidermal growth factor receptor 2-positive operable breast cancer: an update of the initial randomized study population and data of additional patients treated with the same regimen. *Clin. Cancer Res. 13*: 228–233.

Cameron, D.A., Anderson, E.D., Levack, P., Hawkins, R.A., Anderson, T.J., Leonard, R.C., Forrest, A.P., and Chetty, U. 1997. Primary systemic therapy for operable breast cancer – 10-year survival data after chemotherapy and hormone therapy. *Br. J. Cancer 76*: 1099–1105.

Chagpar, A.B., Middleton, L.P., Sahin, A.A., Dempsey, P., Buzdar, A.U., Mirza, A.N., Ames, F.C., Babiera, G.V., Feig, B.W., Hunt, K.K., Kuerer, H.M., Meric-Bernstam, F., Ross, M.I., and Singletary, S.E. 2006. Accuracy of physical examination, ultrasonography, and mammography in predicting residual pathologic tumor size in patients treated with neoadjuvant chemotherapy. *Ann. Surg. 243*: 257–264.

Chen, A.M., Meric-Bernstam, F., Hunt, K.K., Thames, H.D., Outlaw, E.D., Strom, E.A., McNeese, M.D., Kuerer, H.M., Ross, M.I., Singletary, S.E., Ames, F.C., Feig, B.W., Sahin, A.A., Perkins, G.H., Babiera, G., Hortobagyi, G.N., and Buchholz, T.A. 2005. Breast conservation after neoadjuvant chemotherapy.*Cancer 103*: 689–695.

Clarke, M., Collins, R., Darby, S., Davies, C., Elphinstone, P., Evans, E., Godwin, J., Gray, R., Hicks, C., James, S., MacKinnon, E., McGale, P., McHugh, T., Peto, R, Taylor, C., and Wang, Y. 2005. Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15-year survival: an overview of the randomised trials. *Lancet 366*: 2087–2106.

Colleoni, M., Viale, G., Zahrieh, D., Pruneri, G., Gentilini, O., Veronesi, P., Gelber, R.D., Curigliano, G., Torrisi, R., Luini, A., Intra, M., Galimberti, V., Renne, G., Nole, F., Peruzzotti, G., and Goldhirsch, A. 2004. Chemotherapy is

more effective in patients with breast cancer not expressing steroid hormone receptors: a study of preoperative treatment. *Clin. Cancer Res. 10*: 6622–6628.

Davidson, N.E., and Morrow, M. 2005. Sometimes a great notion–an assessment of neoadjuvant systemic therapy for breast cancer. *J. Natl. Cancer Inst. 97*: 159–161.

Del Mastro, L., Catzeddu, T., Boni, L., Bell, C., Sertoli, M.R., Bighin, C., Clavarezza, M., Testa, D., and Venturini, M. 2006. Prevention of chemotherapy-induced menopause by temporary ovarian suppression with goserelin in young, early breast cancer patients. *Ann. Oncol. 17*: 74–78.

Della Pasqua, S., Balduzzi, A., and Torrisi, R. 2006. Preoperative concurrent chemo- and endocrine therapies for women with large operable breast cancer expressing steroid hormone receptors. *J. Clin. Oncol. 24*: suppl abstr.

Eiermann, W., Paepke, S., Appfelstaedt, J., Llombart-Cussac, A., Eremin, J., Vinholes, J., Mauriac, L., Ellis, M., Lassus, M., Chaudri-Ross, H.A., Dugan, M., and Borgs, M. 2001. Preoperative treatment of postmenopausal breast cancer patients with letrozole: A randomized double-blind multicenter study. *Ann. Oncol. 12*: 1527–1532.

Esserman, L., Hylton, N., Yassa, L., Barclay, J., Frankel, S., and Sickles, E. 1999. Utility of magnetic resonance imaging in the management of breast cancer: evidence for improved preoperative staging. *J. Clin. Oncol. 17*: 110–119.

Evans, T.R., Yellowlees, A., Foster, E., Earl, H., Cameron, D.A., Hutcheon, A.W., Coleman, R.E., Perren, T., Gallagher, C.J., Quigley, M., Crown, J., Jones, A.L., Highley, M., Leonard, R. C., and Mansi, J.L. 2005. Phase III randomized trial of doxorubicin and docetaxel versus doxorubicin and cyclophosphamide as primary medical therapy in women with breast cancer: an anglo-celtic cooperative oncology group study. *J. Clin. Oncol. 23*: 2988–2995.

Fisher, B., Bryant, J., Wolmark, N., Mamounas, E., Brown, A., Fisher, E.R., Wickerham, D.L., Begovic, M., DeCillis, A., Robidoux, A., Margolese, R.G., Cruz, A.B., Jr., Hoehn, J.L, Lees, A.W., Dimitrov, N.V., and Bear, H.D. 1998. Effect of preoperative chemotherapy on the outcome of women with operable breast cancer. *J. Clin. Oncol. 16*: 2672–2685.

Garg, A.K., Strom, E.A., McNeese, M.D., Buzdar, A.U., Hortobagyi, G.N., Kuerer, H.M., Perkins, G.H., Singletary, S.E., Hunt, K.K., Sahin, A., Schechter, N., Valero, V., Tucker, S.L., and Buchholz, T.A. 2004. T3 disease at presentation or pathologic involvement of four or more lymph nodes predict for locoregional recurrence in stage II breast cancer treated with neoadjuvant chemotherapy and mastectomy without radiotherapy. *Int. J. Radiat. Oncol. Biol. Phys. 59*: 138–145.

Green, M.C., Buzdar, A.U., Smith, T., Ibrahim, N.K., Valero, V., Rosales, M.F., Cristofanilli, M., Booser, D.J., Pusztai, L., Rivera, E., Theriault, R.L., Carter, C., Frye, D., Hunt, K.K., Symmans, W.F., Strom, E.A., Sahin, A.A., Sikov, W., and Hortobagyi, G.N. 2005. Weekly paclitaxel improves pathologic complete remission in operable breast cancer when compared with paclitaxel once every 3 weeks. *J. Clin. Oncol. 23*: 5983–5992.

Hennessy, B.T., Hortobagyi, G.N., Rouzier, R., Kuerer, H., Sneige, N., Buzdar, A.U., Kau, S.W., Fornage, B., Sahin, A, Broglio, K., Singletary, S.E., and Valero, V. 2005. Outcome after pathologic complete eradication of cytologically proven breast cancer axillary node metastases following primary chemotherapy. *J. Clin. Oncol. 23*: 9304–9311.

Huang, E.H., Tucker, S.L., Strom, E.A., McNeese, M. D., Kuerer, H.M., Buzdar, A.U., Valero, V., Perkins, G.H., Schechter, N.R., Hunt, K.K., Sahin, A.A., Hortobagyi, G.N., and Buchholz, T.A. 2004. Postmastectomy radiation improves local-regional control and survival for selected patients with locally advanced breast cancer treated with neoadjuvant chemotherapy and mastectomy. *J. Clin. Oncol. 22*: 4691–4699.

Jones, R.L., Lakhani, S.R., Ring, A.E., Ashley, S., Walsh, G., and Smith, I.E. 2006. Pathological complete response and residual DCIS following neoadjuvant chemotherapy for breast carcinoma. *Br. J. Cancer 94*: 358–362.

Kaufmann, M., von Minckwitz G., Smith, R., Valero, V., Gianni, L., Eiermann, W., Howell, A., Costa, S. D., Beuzeboc, P., Untch, M., Blohmer, J.U., Sinn, H.P., Sittek, R., Souchon, R., Tulusan, A.H., Volm, T., and Senn, H.J. 2003. International expert panel on the use of primary (preoperative) systemic treatment of operable breast cancer:

review and recommendations. *J. Clin. Oncol. 21*: 2600–2608.

Kaufmann, M., Hortobagyi, G.N., Goldhirsch, A., Scholl, S., Makris, A., Valagussa, P., Blohmer, J.U., Eiermann, W., Jackesz, R., Jonat, W., Lebeau, A., Loibl, S., Miller, W., Seeber, S., Semiglazov, V., Smith, R., Souchon, R., Stearns, V., Untch, M., and von Minckwitz G. 2006. Recommendations from an international expert panel on the use of neoadjuvant (primary) systemic treatment of operable breast cancer: an update. *J. Clin. Oncol. 24*: 1940–1949.

Kim, T., Giuliano, A.E., and Lyman, G.H. 2006. Lymphatic mapping and sentinel lymph node biopsy in early-stage breast carcinoma: a metaanalysis. *Cancer 106*: 4–16.

Klauber-DeMore, N., Ollila, D.W., Moore, D.T., Livasy, C., Calvo, B.F., Kim, H.J., Dees, E.C., Sartor, C.I., Sawyer, L.R., Graham, M., and Carey, L.A. 2006. Size of residual lymph node metastasis after neoadjuvant chemotherapy in locally advanced breast cancer patients is prognostic. *Ann. Surg. Oncol. 13*: 685–691.

Kuehn, T., Bembenek, A., Decker, T., Munz, D.L., Sautter-Bihl, M.L., Untch, M., and Wallwiener, D. 2005. A concept for the clinical implementation of sentinel lymph node biopsy in patients with breast carcinoma with special regard to quality assurance. *Cancer 103*: 451–461.

Loibl, S., Blohmer, J.-U., and Raab, G. 2005. Prospective central assessment of HER-2 status by FISH is not a predictive factor for pathologic complete response (pCR) in 648 breast cancer patients treated preoperatively with an anthracycline/taxane based regimen in the multicenter GeparTrio trial. *Breast Cancer Res. Treat. 94*: 46 abstr.

Loibl, S., von Minckwitz G., Raab, G., Blohmer, J.-U., Dan Costa, S., Gerber, B., Eidtmann, H., Petrich, S., Hilfrich, J., Jackisch, C., du Bois, A., and Kaufmann, M. 2006. Surgical procedures after neoadjuvant chemotherapy in operable breast cancer: results of the GEPARDUO trial. *Ann. Surg. Oncol. 13*: 1434–1442.

MacGrogan, G., Rudolph, P., Mascarel, Id.I, Mauriac, L., Durand, M., Avril, A., Dilhuydy, J.M., Robert, J., Mathoulin-Pelissier, S., Picot, V., Floquet, A., Sierankowski, G., and Coindre, J.M. 2003. DNA topoisomerase IIalpha expression and the response toprimary chemotherapy in breast cancer. *Br. J. Cancer 89*: 666–671.

Mamounas, E.P., Brown, A., Anderson, S., Smith, R., Julian, T., Miller, B., Bear, H.D., Caldwell, C.B., Walker, A.P., Mikkelson, W.M., Stauffer, J.S., Robidoux, A., Theoret, H., Soran, A., Fisher, B., Wickerham, D.L., and Wolmark, N. 2005. Sentinel node biopsy after neoadjuvant chemotherapy in breast cancer: results from National Surgical Adjuvant Breast and Bowel Project Protocol B-27. *J. Clin. Oncol. 23*: 2694–2702.

Manton, D. J., Chaturvedi, A., Hubbard, A., Lind, M. J., Lowry, M., Maraveyas, A., Pickles, M.D., Tozer, D.J., and Turnbull, L.W. 2006. Neoadjuvant chemotherapy in breast cancer: early response prediction with quantitative MR imaging and spectroscopy. *Br. J. Cancer 94*: 427–435.

Mardor, Y., Pfeffer, R., Spiegelmann, R., Roth, Y., Maier, S.E., Nissim, O., Berger, R., Glicksman, A., Baram, J., Orenstein, A., Cohen, J.S., and Tichler, T. 2003. Early detection of response to radiation therapy in patients with brain malignancies using conventional and high b-value diffusion-weighted magnetic resonance imaging. *J. Clin. Oncol. 21*: 1094–1100.

Mauri, D., Pavlidis, N., and Ioannidis, J.P. 2005. Neoadjuvant versus adjuvant systemic treatment in breast cancer: a meta-analysis. *J. Natl. Cancer Inst. 97*: 188–194.

Mustacchi, G., Ceccherini, R., Milani, S., Pluchinotta, A., De Matteis, A, Maiorino, L., Farris, A., Scanni, A., and Sasso, F.. 2003. Tamoxifen alone versus adjuvant tamoxifen for operable breast cancer of the elderly: long-term results of the phase III randomized controlled multicenter GRETA trial. *Ann. Oncol. 14*: 414–420.

Peintinger, F., Kuerer, H.M., Anderson, K., Boughey, J.C., Meric-Bernstam, F., Singletary, S.E., Hunt, K.K., Whitman, G.J., Stephens, T., Buzdar, A.U., Green, M.C., and Symmans, W.F. 2006. Accuracy of the combination of mammography and sonography in predicting tumor response in breast cancer patients after neoadjuvant chemotherapy. *Ann. Surg. Oncol. 13*: 1443–1449.

Ring, A., Webb, A., Ashley, S., Allum, W.H., Ebbs, S., Gui, G., Sacks, N.P., Walsh, G., and Smith, I.E. 2003. Is surgery necessary after complete clinical remission following neoadjuvant chemotherapy for early breast cancer? *J. Clin. Oncol. 21*: 4540–4545.

Ring, A.E., Smith, I.E., Ashley, S., Fulford, L.G., and Lakhani, S.R. 2004. Oestrogen receptor status, pathological complete response and prognosis in patients receiving neoadjuvant chemotherapy for early breast cancer. *Br. J. Cancer 91*: 2012–2017.

Rody, A., Karn, T., Gatje, R., Ahr, A., Solbach, C., Kourtis, K., Munnes, M., Loibl, S, Kissler, S, Ruckhaberle, E., Holtrich, U., von Minckwitz G., and Kaufmann, M. 2007. Gene expression profiling of breast cancer patients treated with docetaxel, doxorubicin, and cyclophosphamide within the GEPARTRIO trial: HER-2, but not topoisomerase II alpha and microtubule-associated protein tau, is highly predictive of tumor response. *Breast 16*: 86–93.

Sadetzki, S., Oberman, B., Zipple, D., Kaufman, B., Rizel, S., Novikov, I., and Papa, M.Z. 2005. Breast conservation after neoadjuvant chemotherapy. *Ann. Surg. Oncol. 12*: 480–487.

Scholl, S. M., Fourquet, A., Asselain, B., Pierga, J. Y., Vilcoq, J.R., Durand, J.C., Dorval, T., Palangie, T., Jouve, M., Beuzeboc, P. 1994. Neoadjuvant versus adjuvant chemotherapy in premenopausal patients with tumours considered too large for breast conserving surgery: preliminary results of a randomised trial: S6. *Eur. J. Cancer 30A*: 645–652.

Senofsky, G.M., Gierson, E.D., Craig, P.H., Gamagami, P.P., and Silverstein, M.J. 1998. 1998. Local excision, lumpectomy, and quadrantectomy. Surgical considerations. Surgery of the Breast: Principles and Art. Lippincott-Raven, Philadelphia, pp. 129–135.

Smith, I.C., Heys, S.D., Hutcheon, A.W., I. D. Miller, I.D., Payne, S., Gilbert, F.J., Ah-See, A.K., Eremin, O., Walker, L.G., Sarkar, T.K., Eggleton, S.P., and Ogston, K.N. 2002. Neoadjuvant chemotherapy in breast cancer: significantly enhanced response with docetaxel. *J. Clin. Oncol. 20*: 1456–1466.

Smith, I.E., Dowsett, M., Ebbs, S.R., Dixon, J. M., Skene, A., Blohmer, J.U., Ashley, S.E. Francis, S., Boeddinghaus, I., and Walsh, G. 2005. Neoadjuvant treatment of postmenopausal breast cancer with anastrozole, tamoxifen, or both in combination: the Immediate Preoperative Anastrozole, Tamoxifen, or Combined with Tamoxifen (IMPACT) multicenter double-blind randomized trial. *J. Clin. Oncol. 23*: 5108–5116.

Taghian, A.G., bi-Raad, R., Assaad, S.I., Casty, A., Ancukiewicz, M., Yeh, E., Molokhia, P., Attia, K., Sullivan, T., Kuter, I., Boucher, Y., and Powell, S.N. 2005. Paclitaxel decreases the interstitial fluid pressure and improves oxygenation in breast cancers in patients treated with neoadjuvant chemotherapy: clinical implications. *J. Clin. Oncol. 23*: 1951–1961.

Thomas, E., Holmes, F.A., Smith, T.L., Buzdar, A.U., Frye, D.K., Fraschini, G., Singletary, S.E., Theriault, R.L., McNeese, M.D., Ames, F., Walters, R., and Hortobagyi, G.N. 2004. The use of alternate, non-cross-resistant adjuvant chemotherapy on the basis of pathologic response to a neoadjuvant doxorubicin-based regimen in women with operable breast cancer: long-term results from a prospective randomized trial. *J. Clin. Oncol. 22*: 2294–2302.

Torrisi, R., Colleoni, M., Veronesi, P., Rocca, A., Peruzzotti, G., Severi, G., Medici, M., Renne, G., Intra, M., Luini, A., Nole, F., Viale, G., and Goldhirsch, A. 2007. Primary therapy with ECF in combination with a GnRH analog in premenopausal women with hormone receptor-positive T2-T4 breast cancer. *Breast 16*: 73–80.

von Minckwitz, G., Costa, S.D., Raab, G., Blohmer, J.U., Eidtmann, H., Hilfrich, J., Merkle, E., Jackisch, C., Gademann, G., Tulusan, A.H., Eiermann, W., Graf, E., and Kaufmann, M. 2001. Dose-dense doxorubicin, docetaxel, and granulocyte colony-stimulating factor support with or without tamoxifen as preoperative therapy in patients with operable carcinoma of the breast: a randomized, controlled, open phase IIb study. *J. Clin. Oncol. 19*: 3506–3515.

von Minckwitz, G., Raab, G., Caputo, A., Schutte, M., Hilfrich, J., Blohmer, J.U., Gerber, B., Costa, S.D., Merkle, E., Eidtmann, H., Lampe, D., Jackisch, C., du Bois, A., and Kaufmann, M. 2005. Doxorubicin with cyclophosphamide followed by docetaxel every 21 days compared with doxorubicin and docetaxel every 14 days as preoperative treatment in operable breast cancer: the GEPARDUO study of the German Breast Group. *J. Clin. Oncol. 23*: 2676–2685.

Wasser, K., Klein, S.K., Fink, C., Junkermann, H., Sinn, H.P., Zuna, I., Knopp, M.V., and Delorme, S. 2003. Evaluation of neoadjuvant chemotherapeutic response of breast cancer using dynamic MRI with high temporal resolution. *Eur. Radiol. 13*: 80–87.

Wenzel, C., Bartsch, R., Hussian, D., Pluschnig, U., Altorjai, G., Zielinski, C.C., Lang, A., Haid, A., Jakesz, R., Gnant, M., and Steger, G.G. 2007. Invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC) of breast differ in response following neoadjuvant therapy with epidoxorubicin and docetaxel + G-CSF. *Breast Cancer Res. Treat*. published online: 24 October 2006.

Xing, Y., Foy, M., Cox, D.D., Kuerer, H.M., Hunt, K.K., and Cormier, J.N. 2006. Meta-analysis of sentinel lymph node biopsy after preoperative chemotherapy in patients with breast cancer. *Br. J. Surg. 93*: 539–546.

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Chemotherapy for Breast Cancer

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INTRODUCTION

Breast cancer ranks as the second most common cause of cancer death among women in the United States. Anticancer agents are an important component of breast cancer therapy. Drugs frequently used to treat breast cancer include methotrexate, 5-fluorouracil (5-FU), cyclophosphamide, anthracyclines, taxanes, trastuzumab, tamoxifen, and aromatase inhibitors. These agents inhibit breast cancer progression by a variety of different mechanisms.

More than 210,000 new cases of breast cancer are diagnosed in the United States each year reported by Centers for Disease Control and Prevention 2006. Approximately one in seven women in the United States will be diagnosed with breast cancer during her lifetime reported by Jemal *et al*. (2004). Breast cancer is the cause of death of over 40,000 women in the United States each year, which ranks it as the second most common cause of cancer death among women. Many drugs have been demonstrated to extend survival of breast cancer patients. Anticancer agents frequently used to treat breast cancer include methotrexate, 5-fluorouracil (5-FU), cyclophosphamide, anthracyclines, taxanes, trastuzumab, tamoxifen, and aromatase inhibitors. Mechanisms by which these agents inhibit breast cancer progression vary from drug to drug.

METHOTREXATE AND 5-FLUOROURACIL

DNA synthesis requires thymidine 5′-triphosphate (TTP). TTP is synthesized from thymidine 5′-monophosphate (TMP). Thymidylate synthetase generates TMP by catalyzing transfer of a methyl group from N^5,N^{10}-methylenetetrahydrofolate to 2′-deoxyuridine 5′-monophosphate (dUMP). Methotrexate and 5-FU treatment each prevent TMP synthesis. Thymidylate synthetase is irreversibly inhibited by 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP), which is produced from 5-FU. In contrast, methotrexate treatment blocks TMP synthesis by preventing N^5,N^{10}-methylenetetrahydrofolate synthesis.

Methotrexate contains a single glutamic acid residue. Folylpoly-γ-glutamate synthetase (FPGS) catalyzes addition of one or

more glutamic acid moieties to methotrexate. Methotrexate and its polyglutamylated derivatives inhibit dihydrofolate (DHF) reductase (DHFR). DHFR reduces folate to DHF and DHF to tetrahydrofolate (THF). Serine hydroxymethyltransferase converts THF to N^5,N^{10}-methylenetetrahydrofolate. Methotrexate treatment reduces TMP production by eliminating a source for N^5,N^{10}-methylenetetrahydrofolate synthesis. Reduction of TMP levels by treatment with methotrexate or 5-FU inhibits TTP production. Inhibition of TTP production blocks cell proliferation by preventing DNA synthesis.

CYCLOPHOSPHAMIDE

Hepatic metabolism converts cyclophosphamide to 4-hydroxycyclophosphamide. Tautomerization of 4-hydroxycyclophosphamide yields aldophosphamide. Acrolein and *N,N*-bis-2-(2-chloroethyl) phosphorodiamidate are produced from spontaneous cleavage of aldophosphamide. DNA is alkylated by *N,N*-bis-2-(2-chloroethyl) phosphorodiamidate at multiple sites. The N^7 position of guanine is a site that is particularly susceptible to alkylation by *N,N*-bis-2-(2-chloroethyl) phosphorodiamidate. Alkylation of the N^7 position of guanine caused by cyclophosphamide treatment stabilizes the enol tautomer of guanine, which causes guanine to pair with thymine instead of cytosine. DNA damage caused by cyclophosphamide treatment induces apoptotic cell death reported by Meyn *et al.* (1994), and Schwartz and Waxman (2001).

ANTHRACYCLINES

Anthracyclines frequently used to treat breast cancer include doxorubicin (Adriamycin) and epirubicin. Anthracyclines disrupt DNA structure by intercalating between adjacent DNA base pairs. Disruption of DNA structure by anthracyclines inhibits synthesis of both DNA and RNA reported by Goodman *et al.* (1974), and Goodman *et al.* (1977). Intercalation of anthracyclines within DNA also induces DNA cleavage that is mediated by topoisomerase II isoforms reported by Tewey *et al.* (1984), and Capranico *et al.* (1990). Topoisomerase II isoforms include topoisomerase IIα, topoisomerase IIβ-1, and topoisomerase IIβ-2 reported by Tsai-Pflugfelder *et al.* (1988), Chung *et al.* (1989), Jenkins *et al.* (1992), and Davies *et al.* (1993). DNA cleavage mediated by topoisomerase II isoforms likely makes an important contribution to the cytotoxicity of anthracyclines because decreased expression of topoisomerase IIα is correlated with decreased anthracycline sensitivity reported by Järvinen *et al.* (2000). These anthracyclines induce apoptotic cell death.

TAXANES

The mitotic spindle of a dividing cell functions to distribute chromatids to each daughter cell. Mitotic spindles are composed of microtubules. Cellular control of microtuble polymerization and depolymerization is essential for proper spindle function. Taxanes disrupt mitotic spindle function by stabilizing microtubules reported by Schiff and Horwitz (1980), Kumar (1981), and Rowinsky *et al.* (1988). Microtubules are assembled from tubulin heterodimers. Tubulin heterdimers are composed of α-tubulin and β-tubulin. Taxanes stabilize microtubules by binding to β-tubulin. Disruption of mitotic spindle function by taxanes prevents cell division. Taxanes frequently used to treat breast cancer include paclitaxel and docetaxel.

TRASTUZUMAB

Trastuzumab (Herceptin) is a humanized mouse monoclonal immunoglobulin G_1 (IgG_1) κ antibody that binds to the extracellular domain of HER-2 reported by Hudziak *et al*. (1989), and Carter *et al*. (1992). HER-2 is amplified and overexpressed in 20–30% of breast cancers and associated with a poor prognosis. Endocytic degradation of HER-2 is accelerated by binding to trastuzumab. Induction of HER-2 degradation by trastuzumab decreases activity of signal transduction cascades downstream of HER-2 that promote cell cycle progression and inhibit apoptosis. These signal transduction cascades include Raf/MEK/ERK and PI3K/PDK/Akt pathways. Deactivation of these signal transduction cascades by trastuzumab treatment prevents cell proliferation. Trastuzumab treatment is most effective for breast cancers with either high HER-2 protein levels or amplification of the gene encoding HER-2 reported by Vogel *et al*. (2002).

TAMOXIFEN

Tamoxifen and its metabolites inhibit proliferation of breast cancer cells by binding to estrogen receptors (ERs) reported by Borgna and Rochefort (1981). ER isoforms include ERα and ERβ reported by Green *et al*. (1986), Mosselman *et al*. (1996), Moore *et al*. (1998), Ogawa *et al*. (1998a), and Shoda *et al*. (2002). ERα and ERβ bind together in both homodimeric and heterodimeric combinations reported by Cowley *et al*. (1997), Pace *et al*. (1997), Petterson *et al*. (1997), and Ogawa *et al*. (1998b). ERs are transcription factors that induce expression of proteins that promote cell cycle progression and inhibit apoptosis. These proteins include cyclin D1, which promotes cell cycle progression, and Bcl-2, which prevents apoptosis reported by Dong *et al*. (1999), and Perillo *et al*. (2000). Binding of ERs to estrogens stimulates transcription of these genes. Tamoxifen and its metabolites compete with estrogens for the same ERα and ERβ ligand binding sites. Binding of tamoxifen or its metabolites to ERs prevents estrogens from promoting cell proliferation because the transactivation potential of ERs bound to tamoxifen is less than that of ERs bound to estrogens. Treatment of MCF-7 cells with tamoxifen decreased levels of Bcl-2 mRNA as well as Bcl-2 protein reported by Zhang *et al*. (1999).

The transactivation potential of ERs bound to tamoxifen or its metabolites is dependent upon whether ERα or ERβ isoforms are present. Tamoxifen is a partial agonist for ERα homodimers, but is a pure antagonist for ERβ homodimers reported by Barkhem *et al*. (1998). Hepatic metabolism converts tamoxifen to 4-hydroxytamoxifen (4-HT), which is similar to tamoxifen in that it is a partial agonist for ERα homodimers yet is a pure antagonist for ERβ homodimers. Estrogenic effects of tamoxifen treatment are likely responsible for its stabilization of bone mineral density (BMD) as well as its association with increased frequencies of endometrial cancer and thromboembolic disease reported by Bernstein *et al*. (1999), Bergman *et al*. (2000), and Yoneda *et al*. (2002).

Tumor biopsy specimens from breast cancer patients are analyzed for ERα expression by immunohistochemisty (IHC) to determine whether tamoxifen treatment is appropriate reported by Harvey *et al*. (1999). Tamoxifen is only administered to breast cancer patients with ERα positive

(ER+) tumors. The therapeutic benefit of tamoxifen treatment is substantially higher in breast cancer patients with ER+ tumors than those with ER negative (ER−) tumors reported by Chang *et al*. (1999), and Chang *et al*. (2000). Coadministration of tamoxifen together with chemotherapeutic drugs is more effective than administration of the same chemotherapeutic drugs without tamoxifen for treatment of breast cancer patients with ER+ tumors, but not for treatment of those with ER- tumors reported by Fisher *et al*. (2001).

AROMATASE INHIBITORS

Estrogen biosynthesis is dependent upon aromatase. Aromatase inhibitors prevent proliferation of breast cancer cells by blocking estrogen production. There are two classes of aromatase inhibitors that differ in chemical structure and mechanism of action. Nonsteroidal aromatase inhibitors, such as anastrozole and letrozole, bind reversibly to aromatase. In contrast, steroidal aromatase inhibitors, such as exemestane, bind irreversibly to aromatase. Anastrozole and letrozole have each been reported to be superior to tamoxifen in first-line therapy of postmenopausal patients with hormone receptor positive advanced breast cancer reported by Nabholtz *et al*. (2000), Mouridsen *et al*. (2001), Mouridsen *et al*. (2003), and Mouridsen *et al*. (2004).

In conclusion, although much progress has been achieved in breast cancer treatment, metastatic breast cancer remains a generally incurable and fatal disease as 50% of the patients die from the disease. Cytotoxic drug treatment is an important weapon against cancer. However, cancerous cells frequently develop drug resistance to these agents. Drug resistance can occur by diverse mechanisms such as: increased expression of membrane transport proteins which transport the drug out of the cells, increased expression of signaling pathways or even lack of expression of a critical receptor necessary for the particular drug, *e.g*., loss of the estrogen receptor negates the effects of tamoxifen. Thus there are desperate needs to develop novel approaches to combat breast cancer drug resistance.

A variety of anticancer agents have been observed to extend survival of breast cancer patients. Novel drugs for treatment of breast cancer patients will undoubtedly become available in the near future. Additional therapeutic options include radiation and surgery. The large number of choices available underscores the need to identify the optimal treatment for each individual breast cancer patient. It is likely that selection of breast cancer therapy will increasingly depend upon molecular features. Expression of ERα and HER-2 are but two of the many characteristics that may impact breast cancer treatment decisions in the future.

Acknowledgements. JAM, RAF and LSS were supported in part by a grant from the NIH (R01098195). AMM was supported in part from a grant from Associazione Italiana Ricerca sul Cancro (AIRC Regional grants) and in part by a grant from the CARISBO Foundation.

REFERENCES

Barkhem, T., Carlsson, B., Nilsson, Y., Enmark, E., Gustafsson, J-Å. and Nilsson, S. 1998. Differential response of estrogen receptor α and

estrogen receptor β to partial estrogen agonists/ antagonists. *Mol. Pharmacol. 54*: 105–112.

Bergman, L., Beelen, M.L.R., Gallee, M.P.W., Hollema, H., Benraadt J., and van Leeuwen, F.E. 2000. Risk and prognosis of endometrial cancer after tamoxifen for breast cancer. *Lancet 356*: 881–887.

Bernstein, L., Deapen, D., Cerhan, J.R., Schwartz, S.M., Liff, J., McGann-Maloney, E., Perlman, J.A., and Ford, L. 1999. Tamoxifen therapy for breast cancer and endometrial cancer risk. *J. Natl. Cancer Inst. 91*: 1654–1662.

Borgna, J-L., and Rochefort, H. 1981. Hydroxylated metabolites of tamoxifen are formed in vivo and bound to estrogen receptor in target tissues. *J. Biol. Chem. 256*: 859–868.

Capranico, G., Kohn, K.W., and Pommier, Y. 1990. Local sequence requirements for DNA cleavage by mammalian topoisomerase II in the presence of doxorubicin. *Nucleic Acids Res. 18*: 6611–6619.

Carter, P., Presta, L., Gorman, C.M., Ridgway, J.B.B., Henner, D., Wong, W.L.T., Rowland, A.M., Kotts, C., Carver, M.E., and Shepard, H.M. 1992. Humanization of an anti-p185^{HER-2} antibody for human cancer therapy. *Proc. Natl. Acad. Sci. USA. 89*: 4285–4289.

Centers for Disease Control and Prevention. 2006. Cancer: symptoms of breast cancer. Atlanta, GA, Centers for Disease Control and Prevention. http://www.cdc.gov/ cancer/ breast/ basic_info/ symptoms.htm

Chang, J., Powles, T.J., Allred, D.C., Ashley, S.E., Clark, G.M., Makris, A., Assersohn, L., Gregory, R.K., Osborne, C.K., and Dowsett, M. 1999. Biologic markers as predictors of clinical outcome from systemic therapy for primary operable breast cancer. *J. Clin. Oncol. 17*: 3058–3063.

Chang, J., Powles, T.J., Allred, D.C., Ashley, S.E., Makris, A., Gregory, R.K., Osborne, C.K., and Dowsett, M. 2000. Prediction of clinical outcome from primary tamoxifen by expression of biologic markers in breast cancer patients. *Clin. Cancer Res. 6*: 616–621.

Chung, T.D.Y., Drake, F.H., Tan, K.B., Per, S.R., Crooke, S.T., and Mirabelli, C.K. 1989. Characterization and immunological identification of cDNA clones encoding two human DNA topoisomerase II isozymes. *Proc. Natl. Acad. Sci. USA. 86*: 9431–9435.

Cowley, S.M., Hoare, S., Mosselman, S., and Parker, M.G. 1997. Estrogen receptors α and β form heterodimers on DNA. *J. Biol. Chem. 272*: 19858–19862.

Davies, S.L., Jenkins, J.R., and Hickson, I.D. 1993. Human cells express two differentially spliced forms of topoisomerase IIβ mRNA. *Nucleic Acids Res. 21*: 3719–3723.

Dong, L., Wang, W., Wang, F., Stoner, M., Reed, J.C., Harigai, M., Samudio, I., Kladde, M.P., Vyhlidal, C., and Safe, S. 1999. Mechanisms of transcriptional activation of bcl-2 gene expression by 17β-estradiol in breast cancer cells. *J. Biol. Chem. 274*: 32099–32107.

Fisher, B., Anderson, S., Tan-Chiu, E., Wolmark, N., Wickerham, D.L., Fisher, E.R., Dimitrov, N.V., Atkins, J.N., Abramson, N., Merajver, S., Romond, E.H., Kardinal, C.G., Shibata, H.R., Margolese, R.G., and Farrar, W.B. 2001. Tamoxifen and chemotherapy for axillary node-negative, estrogen receptor-negative breast cancer: findings from National Surgical Adjuvant Breast and Bowel Project B-23. *J. Clin. Oncol. 19*: 931–942.

Goodman, M.F., Bessman, M.J., and Bachur, N.R. 1974. Adriamycin and daunorubicin inhibition of mutant T4 DNA polymerase. *Proc. Natl. Acad. Sci. USA. 71*: 1193–1196.

Goodman, M.F., Lee, G.M., and Bachur, N.R. 1977. Adriamycin interactions with T4 DNA polymerase. Two modes of template-mediated inhibition. *J. Biol. Chem. 252*: 2670–2674.

Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J-M., Argos, P., and Chambon, P. 1986. Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. *Nature. 320*: 134–139.

Harvey, J.M, Clark, G.M., Osborne, C.K., and Allred, D.C. 1999. Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. *J. Clin. Oncol. 17*: 1474–1481.

Hudziak, R.M., Lewis, G.D., Winget, M., Fendly, B.M., Shepard, H.M., and Ullrich, A. 1989. p185^{HER-2} monoclonal antibody has antiproliferative effects in vitro and sensitizes human breast tumor cells to tumor necrosis factor. *Mol. Cell Biol. 9*: 1165–1172.

Järvinen, T.A.H., Tanner, M., Rantanen, V., Bärlund, M., Borg, Å., Grénman, S., and Isola, J.

2000. Amplification and deletion of topoisomerase IIα associate with ErbB-2 amplification and affect sensitivity to topoisomerase II inhibitor doxorubicin in breast cancer. *Am. J. Pathol. 156*: 839–847.

Jemal, A., Tiwar, R.C., Murray, T., Ghafoor, A., Samuels, A., Ward, E., Feuer, E.J., and Thun, M.J. 2004. Cancer statistics. *CA. Cancer J. Clin. 54*: 829.

Jenkins, J.R., Ayton, P., Jones, T., Davies, S.L., Simmons, D.L., Harris, A.L., Sheer, D., and Hickson, I.D. 1992. Isolation of cDNA clones encoding the β isozyme of human DNA topoisomerase II and localisation of the gene to chromosome 3p24. *Nucleic Acids Res. 20*: 5587–5592.

Kumar, N. 1981. Taxol-induced polymerization of purified tubulin. Mechanism of action. *J. Biol. Chem. 256*: 10435–10441.

Meyn, R.E., Stephens, L.C., Hunter, N.R., and Milas, L. 1994. Induction of apoptosis in murine tumors by cyclophosphamide. *Cancer Chemother. Pharmacol. 33*: 410–414.

Moore, J.T., McKee, D.D., Slentz-Kesler, K., Moore, L.B., Jones, S.A., Horne, E.L., Su J-L., Kliewer, S.A., Lehmann, J.M., and Willson, T.M. 1998. Cloning and characterization of human estrogen receptor β isoforms. *Biochim. Biophys. Res. Commun. 247*: 75–78.

Mosselman, S., Polman, J., and Dijkema, R. 1996. ERβ: identification and characterization of a novel human estrogen receptor. *FEBS. Lett. 392*: 49–53.

Mouridsen, H., Gershanovich, M., Sun, Y., Pérez-Carrión, R., Boni, C., Monnier, A., Apffelstaedt, J., Smith, R., Sleeboom, H.P., Jäenicke, F., Pluzanska, A., Dank, M., Becquart, D., Bapsy, P.P., Salminen, E., Snyder, R., Lassus, M., Verbeek, J.A., Staffler, B., Chaudri-Ross, H.A., and Dugan, M. 2001. Superior efficacy of letrozole versus tamoxifen as first-line therapy for postmenopausal women with advanced breast cancer: results of a phase III study of the International Letrozole Breast Cancer Group. *J. Clin. Oncol. 19*: 2596–2606.

Mouridsen, H., Gershanovich, M., Sun, Y., Pérez-Carrión, R., Boni, C., Monnier, A., Apffelstaedt, J., Smith, R., Sleeboom, H.P., Jaenicke, F., Pluzanska, A., Dank, M., Becquart, D., Bapsy, P.P., Salminen, E., Snyder, R., Chaudri-Ross, H., Lang, R., Wyld, P., and Bhatnagar, A. 2003. Phase III study of letrozole versus tamoxifen as first-line therapy of advanced breast cancer in postmenopausal women: analysis of survival and update of efficacy from the International Letrozole Breast Cancer Group. *J. Clin. Oncol. 21*: 2101–2109.

Mouridsen, H., Sun, Y., Gershanovich, M., Perez-Carrion, R., Becquart, D., Chaudri-Ross, H.A., and Lang, R. 2004. Superiority of letrozole to tamoxifen in the first-line treatment of advanced breast cancer: evidence from metastatic subgroups and a test of functional ability. *Oncologist. 9*: 489–496.

Nabholtz, J.M., Buzdar, A., Pollak, M., Harwin, W., Burton, G., Mangalik, A., Steinberg, M., Webster, A., and von Euler, M. 2000. Anastrozole is superior to tamoxifen as first-line therapy for advanced breast cancer in postmenopausal women: results of a North American multicenter randomized trial. *J. Clin. Oncol. 18*: 3758–3767.

Ogawa, S., Inoue, S., Watanabe, T., Orimo, A., Hosoi, T., Ouchi, Y., and Muramatsu, M. 1998a. Molecular cloning and characterization of human estrogen receptor βcx: a potential inhibitor of estrogen action in human. *Nucleic Acids Res. 26*: 3505–3512.

Ogawa, S., Inoue, S., Watanabe, T., Hiroi, H., Orimo, A., Hosoi, T., Ouchi, Y., and Muramatsu, M. 1998b. The complete primary structure of human estrogen receptor β (hERβ) and its heterodimerization with ER α in vivo and in vitro. *Biochim. Biophys. Res. Commun. 243*: 122–126.

Pace, P., Taylor, J., Suntharalingam, S., Coombes, R.C., and Ali, S. 1997. Human estrogen receptor β binds DNA in a manner similar to and dimerizes with estrogen receptor α. *J. Biol. Chem. 272*: 25832–25838.

Perillo, B., Sasso, A., Abbondanza, C., and Palumbo, G. 2000. 17β-Estradiol inhibits apoptosis in MCF-7 cells, inducing bcl-2 expression via two estrogen-responsive elements present in the coding sequence. *Mol. Cell Biol. 20*: 2890–2901.

Pettersson, K., Grandien, K., Kuiper, G.G.J.M., and Gustafsson, J-Å. 1997. Mouse estrogen receptor β forms estrogen response element-binding heterodimers with estrogen receptor α. *Mol. Endocrinol. 11*: 1486–1496.

Rowinsky, E.K., Donehower, R.C., Jones, R.J., and Tucker, R.W. 1988. Microtubule changes

and cytotoxicity in leukemic cell lines treated with Taxol. *Cancer Res. 48*: 4093–4100.

Schiff, P.B., and Horwitz, S.B. 1980. Taxol stablizes microtubules in mouse fibroblast cells. *Proc. Natl. Acad. Sci. USA. 77*: 1561–1565.

Schwartz, P.S., and Waxman, D.J. 2001. Cyclophosphamide induces caspase 9-dependent apoptosis in 9L tumor cells. *Mol. Pharmacol. 60*: 1268–1279.

Shoda, T., Hirata, S., Kato, J., and Hoshi, K. 2002. Cloning of the novel isoform of the estrogen receptor beta cDNA (ERβ isoform M cDNA) from the human testicular cDNA library. *J. Steroid Biochem. Mol. Biol. 82*: 201–208.

Tewey, K.M., Chen, G.L., Nelson, E.M., and Liu, L.F. 1984. Intercalative antitumor drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II. *J. Biol. Chem. 259*: 9182–9187.

Tsai-Pflugfelder, M., Liu, L.F., Liu, A.A., Tewey, K.M., Whang-Peng, J., Knutsen, T., Huebner, K., Croce, C.M., and Wang, J.C. 1988. Cloning and sequencing of cDNA encoding human DNA topoisomerase II and localization of the gene to chromosome region 17q21-22. *Proc. Natl. Acad. Sci. USA. 85*: 7177–7181.

Vogel, C.L., Cobleigh, M.A., Tripathy, D., Gutheil, J.C., Harris, L.N., Fehrenbacher, L., Slamon, D.J., Murphy, M., Novotny, W.F., Burchmore, M., Shak, S., Stewart, S.J., and Press, M. 2002. Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER-2-overexpressing metastatic breast cancer. *J. Clin. Oncol. 20*: 719–726.

Yoneda, K., Tanji, Y., Ikeda, N., Miyoshi, Y., Taguchi, T., Tamaki, Y., and Noguchi, S. 2002. Influence of adjuvant tamoxifen treatment on bone mineral density and bone turnover markers in postmenopausal breast cancer patients in Japan. *Cancer Lett. 186*: 223–230.

Zhang, G-J., Kimijima, I., Onda, M., Kanno, M., Sato, H., Watanabe, T., Tsuchiya, A., Abe, R., and Takenoshita, S. 1999. Tamoxifen-induced apoptosis in breast cancer cells relates to down-regulation of bcl-2, but not bax and bcl-X_L, without alteration of p53 protein levels. *Clin. Cancer Res. 5*: 2971–2977.

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Locally Advanced Breast Cancer: Role of Chemotherapy in Improving Prognosis

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INTRODUCTION

Locally advanced breast cancer (LABC) was originally defined as inoperable breast cancer. Inoperable breast cancer has the following features: tumor with direct extension to the chest wall or skin (T4), tumor of any size with supraclavicular, or infraclavicular nodes (N3), or findings used to describe inflammatory breast cancer (IBC) such as progressive breast erythema, warmth, edema, and induration.

Some decades ago a relevant proportion of women presented with locally advanced disease at diagnosis. Today, as a consequence of wide-spread screening campaigns, the number of women with inoperable or very large operable tumors at diagnosis has dramatically fallen in Western countries. The majority of patients show T1 or T2 disease at diagnosis, and in most of them a breast conserving surgery can be safely performed. In view of this changed scenario, the denomination of LABC is also adopted today for operable larger tumors (> 5 cm). The attitude of surgeons towards large though technically resectable tumors has changed in the last decades, shifting from an early to a delayed surgery approach.

Few studies have been published about the long-term prognosis of LABC (either inflammatory or not), at least in part because of the poor prognosis of these patients, and also because of the heterogeneity of this classification. The largest database of IBC is the Surveillance, Epidemiology, and End Results registry of the NCI (Chang *et al*., 1998), which reported the survival rates for 913 white and 121 African American women with IBC involving dermal invasion of lymphatic ducts. Among whites, 3-year survival for IBC patients treated between 1975–1979 was 32%, and increased to 42% for patients treated between 1988–1992.

At the MD Anderson Cancer Center, Ueno *et al*. (1997) reviewed their 20 years of experience with a combined-modality approach against inflammatory breast carcinoma. An estimated 28% of patients were currently free of disease beyond 15 years. Panades *et al*. (2005)

performed a retrospective analysis of 308 IBC patients who were curatively treated in British Columbia between 1980 and 2000, and analyzed locoregional relapse-free survival (LRFS) and breast cancer-specific survival (BCSS) by treatment intent and treatment received. Patients receiving more intensive chemotherapy had improved 10-year BCSS compared with standard CT (43.7% vs. 26.3%; P = 0.04).

It is quite difficult to evaluate the role of neoadjuvant chemotherapy in patients with LABC separately from those with operable tumor, because the large majority of clinical trials conducted in the last decades placed these two subsets of patients together. It is reasonable to analyze the published data evaluating whether they can provide answers to the following questions:

1. Does primary chemotherapy result into a substantial prognostic advantage when compared to a standard approach consisting of locoregional (Radiotherapy ± Surgery) treatment followed by chemotherapy?
2. Is it possible to identify an end point for neoadjuvant chemotherapy significantly correlated to long-term outcome?
3. Are we able to define the best regimen, dose, timing, and sequence today?
4. What is the current role of targeted therapy, and what will it be in the near future?
5. Can we properly tailor the treatment by utilizing molecular and biological markers and/or advanced imaging techniques?
6. Can an individualized multistep approach result in a prognostic gain?

PREOPERATIVE VERSUS POSTOPERATIVE CHEMOTHERAPY

The NSABP B18 trial (Fisher *et al.*, 1998) seemed to properly address this issue, at least when operable tumors are considered. There was no significant difference in disease-free and overall survival between patients receiving preoperative and postoperative chemotherapy. However, a subset analysis at 9 years (Wolmark *et al.*, 2001) revealed that women who were 49 years and younger had a significant advantage in terms of disease-free and overall survival when given preoperative chemotherapy (55% vs. 46% and 71% vs. 65%, respectively). Conversely, in women who were 50 years and older, the disease-free and overall survival was less with preoperative chemotherapy (56% vs. 60% and 67% vs. 75%, respectively). It is possible that the benefit of preoperative chemotherapy, when compared with adjuvant treatment, might be age-dependent.

It is uncommon to find randomized trials addressing this issue in very large or even inoperable tumors, because it appears more reasonable to administer chemotherapy before locoregional treatment in such patients. Semiglazov *et al.* (1994) randomized 271 patients, with stage IIb–IIIa breast cancer to receive as preoperative treatment chemotherapy plus radiotherapy, or radiation therapy only. All patients underwent mastectomy and complete axillary clearance, and then received 4–6 courses of the same regimen. Pathologic complete regression of the tumor was observed in 29.1% of the patients in group I and in 19.4% of the patients in group II. The estimated 5-year

overall survival percentages were 86.1% for group I, and 78.3% for group II ($P > 0.05$). Five-year disease-free survival percentages were 81.0% and 71.6%, respectively ($p < 0.05$).

The role of neoadjuvant as compared to adjuvant chemotherapy was assessed by Scholl *et al.* (1994) in 414 premenopausal patients with T2-T3 N0-N1 M0 breast cancer. Women were randomized to receive either four cycles of neoadjuvant chemotherapy (cyclophosphamide, doxorubicin, 5-fluorouracil), followed by local-regional treatment (group I) or four cycles of adjuvant chemotherapy after primary irradiation ± surgery (group II). Surgery was limited to those patients with a persisting mass after irradiation. With a median follow-up of 54 months, a statistically significant difference ($P = 0.039$) in survival in favor of the neoadjuvant chemotherapy group was observed.

The European Cooperative Trial in Operable Breast Cancer Study Group (Gianni *et al.*, 2005a) randomized 1,355 T2-3 breast cancer patients to three groups: Surgery→doxorubicin→CMF, Surgery→doxorubicin/paclitaxel→CMF and doxorubicin/paclitaxel→CMF→Surgery. The rate of patients undergoing breast conserving surgery was significantly higher in the neoadjuvant arm (65% vs. 34%; $p < 0.001$). However, no differences in terms of DFS and OS were observed between preoperative and postoperative treatments. A recent meta-analysis (Mauri *et al.*, 2005) addressed directly the question of neoadjuvant versus adjuvant chemotherapy. Nine randomized clinical trials involving 3,946 patients were included. No difference was observed between the two arms for death, disease progression or distant recurrence.

It is almost obvious that in women with operable disease a 1- or 2-month anticipation of chemotherapy administration, *per se*, cannot substantially change prognosis. The only reasonable way for optimizing the efficacy of the preoperative approach is to design a multistep therapeutic approach, utilizing most of available chemotherapy (or targeted) agents, when necessary. An early assessment of response should be performed, and treatment should be modified in those women who are unlikely to achieve a satisfactory pathological response.

In truly LABC patients, who are not candidate for surgery at diagnosis, the intensity of chemotherapy is very important in determining the long-term outcome. A multistep approach appears even more necessary in these patients, together with innovative maintenance treatments, because the risk of relapse is very high in this group, even in the presence of a satisfactory pathological response to front-line treatment. These concepts will be widely discussed in the successive paragraphs.

CLINICOPATHOLOGICAL END POINTS AND LONG-TERM OUTCOME

The complete disappearance of all viable tumor cells, as shown by histopathologic examination of the removed breast and axillary tissue, indicates the complete eradication of locoregional disease by systemic medical treatment and is widely proposed as a surrogate indication of the almost complete eradication of distant micrometastatic residual disease. This is supported by different trials. In the NSABP-B18

trial (Fisher *et al*., 1998) the outcome was better in women whose tumors showed a pCR than in the others (RFS rates, 85.7% and 76.9%, respectively; $P < 0.0001$), even when baseline prognostic variables were controlled.

Kuerer *et al*. (1999) reported the long-term outcome of 372 LABC patients who received preoperative chemotherapy in two prospective neoadjuvant trials conducted at the M.D. Anderson Cancer Center. Sixteen percent of the patients in this study (n = 60) had a pathologic complete primary tumor response. Twelve percent of patients (n = 43) had no microscopic evidence of invasive cancer in their breast and axillary specimens. The 5-year overall and disease-free survival rates were significantly higher in the group who had a pCR (89% and 87%, respectively) than in the group who had less than a pCR (64% and 58%, respectively; $P < 0.01$).

More recently, Gonzalez-Angulo *et al*. (2005) analyzed the long-term outcome of 226 patients treated at the same institution, and identified as having pCR after neoadjuvant chemotherapy. One hundred and forty-two out of 226 patients had stage III disease or inflammatory breast cancer at diagnosis. Multivariate Cox regression analysis using combined stage, revealed that stages IIIB, IIIC and IBC (HR 4.24; $p < 0.0001$), identification of ≤ 10 lymph-nodes (HR 2.94; $p = 0.004$), and premenopausal status (HR 3.08; $p = 0.015$) were predictive for distant metastasis. Freedom from distant metastasis at 10 years was 97% for no factors, 88% for one factor, 77% for two factors, and 31% for three factors ($p < 0.0001$).

The complete eradication of the tumor in both breast and axilla is predictive of a high probability, but not certainty of cure. A better definition of pathological complete response would include those patients showing absence of invasive tumor also in the axilla. It is quite clear that some pretreatment features, such as very large tumor, massive axillary node involvement, high biological aggressiveness (IBC, HER2 overexpression, younger age, etc.) can render the achievement of the pCR less predictive of cure. It is reasonable to hypothesize that an old and/or very aggressive tumor is more likely associated with the presence of chemo-resistant distant tumor clones, resulting in a relevant risk of distant relapse in spite of the achievement of loco-regional pCR.

SEARCHING FOR THE BEST NEOADJUVANT TREATMENT

Drugs

Anthracycline/taxane-based chemotherapy regimens have been studied extensively in prospective randomized trials, and are the most frequently prescribed primary treatments in patients with either early or locally-advanced breast cancer (Table 25.1). However, a clear superiority of taxane-including neoadjuvant chemotherapy in terms of disease free survival and overall survival has not yet been demonstrated. To evaluate the prognostic impact of the addition of paclitaxel to doxorubicin-based neoadjuvant chemotherapy, Mazouni *et al*. (2007) performed a pooled analysis of results from seven consecutive neoadjuvant chemotherapy trials conducted at M.D. Anderson Cancer Center from 1974 to 2001, including 1,079 patients. Four hundred and twenty-six (39.5%) patients received taxane-based neoadjuvant therapy.

TABLE 25.1. Anthracycline-taxane neoadjuvant therapy. Randomized trials.

Author	Treatment	N. of pts.	pCR (%)
Bear HD (2006)	AC vs. AC→D	2,411	13.7 vs. 26.1°
Smith IC (2002)	CVAPr vs. CVAPr→D	104	15 vs. 30.8°
Evans TR (2005)	AC vs. AD	363	24 vs. 21°
Green MC (2005)	P→FAC wk vs. 3 wk	258	28.2 vs. 15.7#
Von Minckwitz (2005)	AD vs. AC→D	913	7.0 vs. 14.3#
Frasci G. (2006)	PET vs. ET	200*	16.0 vs. 6.0#

Abbreviations: A = adriamycin, C = cyclophosphamide, D = docetaxel, E = epirubicin, F = fluorouracil, P = platinum, T = paclitaxel, V = vincristine, °only T4 tumor, °only breast is considered, #both breast and axilla are considered.

pCR rates and survival times were analyzed as a function of chemotherapy regimen and estrogen receptor status. Multivariate logistic and Cox regression analysis were carried out to identify variables associated with pCR and survival. Patients with ER-negative cancer had higher overall pCR rate than those with ER-positive tumors (20.1% versus 4.9%, $P < 0.001$). In ER-negative patients, the pCR rates were 29% and 15% with or without a taxane ($P < 0.001$), respectively. In ER-positive patients, the pCR rates were 8.8% and 2.0% with or without a taxane ($P < 0.001$). In multivariate analysis, clinical tumor size ($P < 0.001$), ER-negative status ($P < 0.001$), and inclusion of a taxane ($P = 0.01$) were independently associated with pCR.

Similar data had been reported by Cristofanilli *et al.* (2004), in a retrospective analysis of 240 IBC patients treated at the same Institution in six consecutive trials between 1973 and 2000. Patients were treated with FAC in the first four trials (1973–1993), and with FAC followed by paclitaxel in the last two trials (1994–2000). The median overall survival (OS) and progression-free survival (PFS) were better in the patients treated with paclitaxel, and these differences reached statistical significance in the patients with ER-negative disease (median OS: 32 months vs. 54 months; $P = 0.03$).

Several randomised phase III trials have been carried out aimed at determining whether the addition of docetaxel (either sequentially or in combination), to standard anthracycline-based regimens translated into a substantial therapeutic advantage.

In the National Surgical Adjuvant Breast and Bowel Project Protocol B-27 (Bear *et al.*, 2003) preoperative AC followed by docetaxel yielded a higher pathologic complete response rate than AC alone (13.7% v 26.1%; $P < 0.001$). The superiority of docetaxel-including regimen was evident in both ER-negative and ER-positive patients. The NSABP B27 trial results have been recently updated (Bear *et al.*, 2006). In spite of doubling the pCR rate in the group of patients receiving docetaxel, a statistical improvement in OS and DFS was not observed in this arm. A number of factors might explain this. First, the study was powered to detect a 25% reduction in the hazard ratio for mortality. None of the trials testing the addition of a taxane in the adjuvant setting has demonstrated benefits of this magnitude. Extrapolating from the NSABP B18 survival curves, doubling

of the pCR rate from 13% to 26% could be predicted to increase the number of surviving patients by ~ 2%. The NSABP B27 trial was not powered to detect such small differences in DFS and OS. If we take relapse-free survival into consideration, there were 231 events in the AC→ docetaxel arm as compared to 258 events in the AC arm. This 10% event rate reduction would have required > 10,000 patients enrolled to be detected statistically. The interpretation of the results of NSABP B27 trial could be potentially confounded by the longer duration of preoperative chemotherapy in the docetaxel arm.

Another neoadjuvant study from Scotland (Smith *et al*., 2002) suggests that sequential addition of docetaxel improved response rate even when duration of treatment was not different. In that study patients with large or locally advanced breast cancer received four cycles of cyclophosphamide 1,000 mg/m^2, doxorubicin 50 mg/m^2, vincristine 1.5 mg/m^2, and prednisolone 40 mg for 5 days. Those who responded were randomized to receive further four CVAP cycles or four docetaxel (100 mg/m^2) cycles. All nonresponders received 4 × docetaxel. The pCR rate was significantly higher (31% vs. 15%) in patients who received docetaxel.

A well designed randomized trial was carried out in UK (Evans *et al*., 2005), which compared six cycles of doxorubicin-cyclophosphamide (AC) to six cycles of doxorubicin-docetaxel (AD), as primary treatment in patients with either large operable or locally advanced breast cancer. Twenty-three percent of patients had inoperable or inflammatory disease. There was no significant difference in either the pathologic complete response rates in the breast (AC 24%, AD 21%) or in the number of patients with positive axillary nodes at surgery (AC 61%, AD 66%). At a median follow-up of 32 months, there was no significant difference between the two groups for the number of relapses. The authors concluded that their data did not suggest a benefit for simultaneous AD over AC. Interestingly, the pCR rate in AC arm observed in this study was quite similar to that reported by sequential AC→ docetaxel in NSABP B27 study, and substantially superior to that achieved by four AC cycles in the same trial, although a relevant proportion of patients with T4 disease was included in this trial.

Chemotherapy agents other than anthracyclines and taxane have also been tested in the neoadjuvant setting (Table 25.2). Phase II studies have explored the effect of a combination of cisplatin with either taxanes or anthracylines. Ezzat *et al*. (2004) treated 126 consecutive patients with noninflammatory LABC (T2 > 4 cm, T3 or T4, N0-N3, M0) with three to four cycles of the neoadjuvant PC (paclitaxel 135 mg/m^2 and cisplatin 75 mg/m^2) every 21 days. Pathologic complete response (pCR) was achieved in 29 patients (24%). The projected 5-year overall survival (OS) and disease-free survival (DFS) was 85% and 63%, respectively. The docetaxel/cisplatin combination was tested by Lee *et al*. (2004) as primary chemotherapy in 57 locally advanced breast carcinoma (LABC). Pathologic complete response in the breast was achieved in 15 patients (26%) and pCR in the breast and the axilla was achieved in 11 patients (20%).

The epirubicin+cisplatin+fluorouracil (ECF) as continuous infusion regimen in association with a gonadotropin-releasing hormone (GnRH) analogue was evaluated by Torrisi *et al*. (2007) in 36 premenopausal

TABLE 25.2. Phase II trials with other drugs.

Author	Treatment	N. of pts.	pCR (%)
Ezzat AA (2004)	PT	126	24
Lee YJ (2004)	PD	57	26
Torrisi R (2007)	PEF	36	11
Natoli C (2007)	EC→DX	44	17.1
Lebowitz PF (2004)	DX	30	10
Braud AC (2002)	ECV	30	32
Limentani SA (2006)	DV	59	20

Abbreviations: C = cyclophosphamide, D = docetaxel, E = epirubicin, F = fluorouracil, P = platinum, T = paclitaxel, V = vinorelbine, X = xeloda

women with T2-T4a-d N0-2 M0 ER and/or progesterone receptor positive breast cancer. A pCR was observed in four patients (11%). Capecitabine has also been tested in the neoadjuvant setting, in the last few years. Natoli *et al*. (2007) evaluated the efficacy of dose-dense epirubicin plus cyclophosphamide followed by docetaxel plus capecitabine in 44 patients with stage II or IIIA breast cancer. Seven patients (17.1%) exhibited a pathologic complete response. The intent-to-treat pCR rate was 19% (95% CI: 9–33%). Lebowitz *et al*. (2004) treated 30 stage II/III patients with the docetaxel/capecitabine regimen. A pathological complete response in the breast was achieved in 10% of patients after four cycles of treatment.

Numerous clinical trials have been conducted which evaluated the role of vinorelbine in locally-advanced disease. Braud *et al*. (2002) reported a very high pCR rate (32%) with the administration of a dose-dense epirubicin-cyclophosphamide-vinorelbine combination. Limentani *et al*. (2006) yielded a 98% ORR and a 20% pCR rate among 59 IIA–IIIB patients after treatment with dose-dense docetaxel-vinorelbine.

The addition of a taxane to an anthracycline-based chemotherapy results in a higher pCR rate. However, it is still uncertain whether this small advantage can translate into a meaningful survival advantage. It is mandatory to address this issue separately in patients with different hormone receptor and/or HER-2 status, since the benefit of chemotherapy may vary dramatically according to these features. The addition of other drugs like platin-compounds, capecitabine, vinorelbine, to anthracycline and taxanes, as well as the adoption of combinations non including anthracyclines, taxanes, or both also deserves to be evaluated in well designed randomized trials. Finally, some other factors like dose, schedule, sequence etc., might have a major role in determining response and long-term outcome.

Dose and Schedule?

Dose intensification of particular chemotherapy agents can produce high, complete and overall response rates in women with locally advanced or metastatic breast cancer. However, high dose chemotherapy (HDCT) with stem cell rescue is currently a controversial strategy, not only as a component of the standard of care, but even as investigative field. The results of several pilot trials evaluating the role of HDCT in the neoadjuvant setting have been reported in the last decade. Stewart *et al*. (2005) evaluated one cycle of mitoxantrone 63 mg/m^2 – vinblastine 12.5 mg/m^2 – cyclophosphamide 6 g/m^2 (MVC) with autologous blood stem cell transplantation (ASCT) after four cycles of fluorouracil-adriamycin-cyclophosphamide (FAC). Twenty-five patients were treated preoperatively for LABC and 67 were treated postoperatively. The 7-year event-free and overall survival rates were 53% and 62%, respectively, with no significant difference between pre- and postoperative groups. The outcomes of 56 IBC patients, receiving high-dose chemotherapy (HDC) with cyclophosphamide, thiotepa, and carboplatin (CTCb) were reported by Schwartzberg *et al*. (1999). There were two (4%) infectious deaths after HDC. The probabilities of overall (OS) and event-free survival (EFS) at 3 years for all 56 patients were 72%

and 53%, respectively. These trials do not answer the question whether HDCT is better than standard dose chemotherapy in improving long-term outcome of patients with LABC, since the median follow-up too short in all cases. However, a DFS rate of 40–50% at 3–4 years is not so different from that reported with standard anthracycline-based chemotherapy. The shortening of the administration interval is another way to deliver a higher cumulative dose without administering very high drug doses at each cycle. It has been hypothesized that the administration of standard doses at shorter intervals is more effective in avoiding tumor regrowth than the delivery of a single very high dose treatment.

The validity of the dose-dense approach seems to be confirmed, at least in the adjuvant setting, by the results of the first report of Intergroup Trial C9741/ Cancer and Leukemia Group B Trial 9741 (Citron *et al*., 2003). Using a 2 × 2 factorial design, the investigators compared sequential doxorubicin → paclitaxel → cyclophosphamide with concurrent doxorubicin/cyclophosphamide→ paclitaxel, and the q3wk with the q2wk interval. A protocol-specified analysis was performed at a median follow-up of 36 months: 315 patients had experienced relapse or died. Dose-dense treatment improved the primary end point, DFS (Hazard Ratio = 0.74; P = 0.010), and OS (Hazard Ratio = 0.69; P = 0.013). Four-year DFS was 82% for the dose-dense regimens and 75% for the others. There was no difference in either DFS or OS between the concurrent and sequential schedules.

The results of the SWOG/Intergroup 9623 (Moore *et al*., 2007) which compared HDCT to a sequential dose-dense chemotherapy in the adjuvant setting have been recently reported. Estimated 5-year DFS was 80% for dose-dense therapy and 75% for HDCT. The authors concluded that in the adjuvant treatment of breast cancer, the era of the dose escalation to the limits of tolerability seems to be over. The improvement in survival will more likely be achieved with the inclusion of more effective chemotherapy agents and targeted therapies, with appropriate use of predictive markers, as well as through optimizing drug scheduling.

There is numerous evidence in favour of a better therapeutic index for the weekly paclitaxel administration. Green *et al*. (2005), reported a 28.8% pCR rate with weekly paclitaxel followed by 4 FAC (5-fluorouracil, doxorubicin and cyclophosphamide) cycles before surgery, as compared to a 13.6% pCR rate with the tri-weekly paclitaxel followed by FAC.

The SICOG 9908 trial (Frasci *et al*., 2006) aimed at evaluating whether a weekly cisplatin, epirubicin, and paclitaxel (PET) regimen could increase the pathological complete response (pCR) rate in comparison with a tri-weekly epirubicin and paclitaxel administration in locally-advanced breast cancer (LABC) patients. Patients with stage IIIB disease were randomized to receive either 12 weekly cycles of cisplatin 30 mg/m^2, epirubicin 50 mg/m^2, and paclitaxel 120 mg/m^2 (PET) plus granulocyte-colony stimulating factor support, or four cycles of epirubicin 90 mg/m^2 + paclitaxel 175 mg/m^2 (ET) every 3 weeks. The treatment duration was similar in the two arms, but a much higher cumulative dose of epirubicin and paclitaxel, besides the cisplatin addition, characterized the weekly arm.

Overall, 200 patients (PET/ET = 100/100) were included in this study. A pCR in both breast and axilla occurred in 16 (16%)

PET patients and in 6 (6%) ET patients (P = 0.02). The higher activity of PET was evident only in ER-negative (27.5% vs. 5.4%; P = 0.026), and in HER2/neu positive (31% vs. 5%; P = 0.037) tumors. The two arms yielded similar pCR rate in ER-positive (PET/ET = 7.5%/7.1%) and HER2/neu negative (PET/ET = 10%/6%) patients. The same authors (Frasci *et al*., 2005) evaluated the antitumor activity of 8 weekly PET cycles in patients with large operable breast cancer (T2-T3 N0-1; T > 3 cm). Sixty-three patients (T2/T3 = 30/33; N0/N+ = 8/55) were enrolled.. At pathological assessment, 28 patients (45%) showed absence of invasive residual disease in breast and 34 (55%) had negative axilla. In 20 women (32%) both breast and axilla were found disease-free. Taking into account the main biologic features at diagnosis, the absence of ERs was associated with a higher probability of pCR achievement (ER- vs. ER+ = 66% vs. 14%; p = 0.00005). A significantly higher pCR probability was also observed in HER-2 3+ patients (60% vs. 23%; p = 0.01).

There has been an extensive debate regarding the best way to administer anthracyclines and taxane. It has been hypothesized that the sequential administration of optimal dose of doxorubicin (75 mg/m^2 alone rather than 60 mg/m^2 in combination) followed by optimal dose of docetaxel (100 mg/m^2 rather than 75 mg/m^2 given in combination), might result in a higher antitumor activity either in neoadjuvant or in the adjuvant setting. In the Geparduo study (von Minckwitz *et al*., 2005) 913 women with untreated operable breast cancer (T2-3, N0-2, M0) were randomly assigned to receive either doxorubicin 50 mg/m^2 plus docetaxel 75 mg/m^2 every 14 days for four cycles with filgrastim support (ADOC), or doxorubicin 60 mg/m^2 plus cyclophosphamide 600 mg/m^2 every 21 days followed by docetaxel 100 mg/m^2 every 21 days for four cycles each (AC-DOC). The pCR rate was significantly greater with AC-DOC (14.3%; n = 63) than with ADOC (7.0%; n = 31). If only primary tumor in the breast was considered the pCR rate was 22.3% with AC-DOC and 11.0% with ADOC. Therefore, the pCR rate in the breast obtained with the sequential treatment looks similar to that reported with the same regimen in the NSABP B27 trial. Conversely, in spite of the dose-dense approach, four cycles of doxorubicin/docetaxel in this study gave the same pCR rate in the breast than that achieved by four cycles of doxorubicin/cyclophosphamide in NSABP B27, and lower than that reported with six cycles of doxorubicin/docetaxel every 3 weeks in the UK trial (Evans *et al*., 2005). The possible explanation for this is easy. Patients in the dose-dense concurrent arm received a total dose of doxorubicin and docetaxel of 200 mg/m^2 and 300 mg/m^2, respectively; as compared to cumulative doses of doxorubicin, docetaxel and cyclophosphamide of 240 mg/m^2, 400 mg/m^2 and 2,400 mg/m^2, respectively in the sequential arm. The cumulative doses of doxorubicin and cyclophosphamide and docetaxel in the two arms of the UK trial were 360 mg, 3600 mg and 450 mg, respectively. It means that both arms of UK study received higher cumulative doses than the comparable arms in the NSABP-B27 (AC × 4) and Geparduo (AD × 4).

The AGO study (Untch *et al*., 2002) compared a dose-dense (q2wk) sequence of three cycles of epirubicin (150 mg/m^2) followed by three cycles of paclitaxel (250 mg/m^2) with four every 3 weeks

cycles of a combination of both drugs (Epi 90 mg/m^2 and Paclitaxel 175 mg/m^2). Treatment had the same duration of 12 weeks, but the total dose for both drugs was higher in the sequential dose-dense treatment (EPI 450 mg/m^2 vs. 270 mg/m^2; Paclitaxel 750 mg/m^2 vs. 525 mg/m^2). The pCR rate (absence of invasive tumor in the breast and axilla) was significantly higher in the sequential dose-dense arm (18% vs. 10%). Both these studies suggest that the sequential use of an anthracycline with a taxane is associated with better results than their concurrent use, perhaps as a result of the higher total dose of chemotherapy delivered in the sequential arm.

Although the mortality rate associated with the administration of high-dose chemotherapy has dramatically declined in the last decades, this approach still represents a risky therapeutic strategy, and should be considered with caution, especially in patients with a reasonably high chance of cure with standard approaches. The data from the SWOG/Intergroup 9623 seems to demonstrate that a more intensive standard chemotherapy with shorter administration interval, may be at least as effective and less toxic than HDCT in the adjuvant setting. The CALGB 9741 findings seem to demonstrate that when the total dose delivered is the same, the shortening of the interval may result in higher antitumor activity. The shortening of administration interval is not sufficient to improve prognosis, if both the single and cumulative doses for each drug are not adequate. The weekly schedule may represent the best way to administer paclitaxel, in view of the particular pharmacokinetics of this drug. The administration of 80–150 mg/m^2 of paclitaxel each week produces tumoricidal, but not myelotoxic serum concentration. In view of that, we are able to deliver a cumulative dose of paclitaxel exceeding 400 mg/m^2 over 3 weeks, with less myelotoxicity than that associated to 200–250 mg/m^2 given as a 1- to 24-h infusion every 3 weeks. There is a substantial amount of clinical evidence in favour of the weekly schedule in both the locoregional and metastatic breast cancer setting. There is no evidence in favour of the weekly schedule for drugs like docetaxel, anthracyclines, and alkylating agents.

ROLE OF TARGETED THERAPY

Trastuzumab

Human epidermal growth factor (HER-2) receptor is overexpressed in 15–25% of breast cancers. Trastuzumab, a humanized receptor antibody directed against HER-2, in combination with chemotherapy has been proven to improve DFS and OS in metastatic breast cancer, and, more recently, in the adjuvant setting in patients with HER-2 positive breast cancer. A number of small phase II trials have studied different combinations of preoperative trastuzumab and chemotherapy. In this studies, the pCR rate ranged from 12–45% (Table 25.3).

Burstein *et al*. (2003) treated 40 HER-2/neu 2+ or 3+ women with trastuzumab plus paclitaxel for 12 weeks, before surgery. The pCR rate in both breast and axilla was 18%. Primary therapy with trastuzumab and another taxane, docetaxel, is also highly active. Coudert *et al*. (2006) treated 33 HER-2 3+ patients with weekly trastuzumab + docetaxel 100 mg/m^2 every 3 weeks for six cycles. Pathological complete response in breast and lymph-nodes was registered in 14 patients (47%). Forty-eight

TABLE 25.3. Neoadjuvant studies with trastuzumab.

Author	Treatment	N. of pts.	pCR (%)
Burstein HJ (2003)	T+P × 12 wk	40	18
Coudert BP (2006)	T+D q3wk × 6	33	47
Hurley J (2006)	T+D+Pl × 12 wk	48	17
Harris L (2007)	T+V × 12 wk	28	20
Torrisi R (2006)	T+oral V + hormone × 12 wk	40	14 (ER+)
Limentani SA (2007)	T+DV	31	39
Buzdar A (2007)	P→FEC vs. P→FEC + T × 24 wk	64	26 vs. 60

Abbreviations: C = cyclophosphamide, D = docetaxel, E = epirubicin, F = fluorouracil, P = paclitaxel, Pl = platinum T = trastuzumab, V = vinorelbine

HER-2 2+ or 3+ patients with LABC were treated with 12 weeks of trastuzumab, docetaxel and cisplatin by Hurley *et al.* (2006). The pCR rate in breast and axilla was 17%, being 13% and 22% in Fish-positive and Fish-negative, respectively.

Trastuzumab in combination with weekly vinorelbine has also been tested in the neoadjuvant setting. Harris *et al.* (2007) treated 28 stage II/III HER-2 3+ patients with 12 weekly cycles of trastuzumab plus vinorelbine 25 mg/m^2. The observed pCR rate was 20%, with no episodes of symptomatic cardiac toxicity. Torrisi *et al.* (2006) have recently evaluated the combination of oral vinorelbine 55 mg/m^2 d 1&3 q3wk and trastuzumab 6 mg/kg q3wk + hormone treatment (in hormone receptor positive) × 24 weeks, in 40 patients with large operable or LABC. The pCR rate was promising in hormone receptor positive patients (14%), whereas it was disappointing in hormone receptor negative patients (1/16). The results of a phase II trial evaluating the activity of dose-dense docetaxel-vinorelbine (q2wk with G-CSF support) plus weekly trastuzumab have been recently published (Limentani *et al.*, 2007) . Twelve out of 31 enrolled patients (39%) had pCR in breast and axilla, 14 patients (45%) showed pCR in the breast.

So far only one randomized phase III trial has assessed the use of preoperative trastuzumab in conjunction with chemotherapy. Buzdar *et al.* (2007) compared a 6-month course of preoperative chemotherapy consisting of four cycles of paclitaxel followed by four cycles of FEC, with the same chemotherapy with simultaneous weekly trastuzumab for 24 weeks in non-inflammatory stage II and IIIA, HER-2 positive breast cancer patients. The pCR rate was 60% in all patients enrolled in the trastuzumab-chemotherapy arm as compared to 26% in the chemotherapy-alone arm. Three recurrences and one death have been observed in the chemotherapy-alone arm, with no recurrence observed in the trastuzumab-chemotherapy arm, with an estimated DFS at 3 years of 85.3% and 100%, respectively.

Lapatinib

Lapatinib (Tykerb, GlaxoSmith Kline) is an orally active small molecule that inhibits the tyrosine kinases of HER-2 and epidermal growth factor receptor type 1 (EGFR). In preclinical studies, lapatinib was not cross-resistant with trastuzumab. Spector *et al.* (2006) treated 34 women with refractory inflammatory breast cancer

with Lapatinib 1,500 mg orally once daily. Eleven patients had biopsy-documented HER-2/neu positive tumor. Eight of 11 (72%) patients in this cohort achieved a major clinical response. There were no responses in HER-2/neu-negative patients.

Lapatinib has also been recently administered in combination with paclitaxel in relapsed/refractory IBC patients (Cristofanilli *et al*., 2006). Twenty out of 21 (95%) patients with biopsy-proven HER-2/neu positive tumor achieved a clinical major response. Two out of two patients who were erb-B1 positive/HER2/neu non-overexpressing also achieved a major response.

Bevacizumab

Vascular endothelial growth factor (VEGF) is a potent molecule that mediates tumor angiogenesis. Bevacizumab is a monoclonal antibody specifically targeting VEGF. Wedam *et al*. (2006) treated 21 patients with inflammatory and locally advanced breast cancer with bevacizumab for cycle 1 (15 mg/kg on day 1) followed by six cycles of bevacizumab with doxorubicin (50 mg/m^2) and docetaxel (75 mg/m^2) every 3 weeks. A significant decrease in phosphorylated VEGFR2 in tumor cells and increase in tumor apoptosis were seen after bevacizumab alone. Moreover, a reduced angiogenesis was documented with magnetic resonance imaging (MRI).

Other molecules

Ras proteins belong to the G-protein superfamily that plays a critical role in cell growth and regulation. Although the frequency of Ras mutations in breast cancer is low (< 2%), hyperactivation of Ras protein and its downstream effectors is common as a result of either overexpression of upstream components, such as HER-2/neu, or estrogen-dependent aberrant pathways. Ras protein overexpression in breast cancer has been associated with poor prognosis. Farnesyltransferase mediates Ras modification which is essential for its downstream effects. Tipifarnib is an orally available Farnesyltransferase inhibitor, which has demonstrated antitumor activity in patients with metastatic breast cancer. Sparano *et al*. (2006) evaluated the combination of Tipifarnib (200 mg bid d 2–7) with doxorubicin-cyclophosphamide q2wk in 21 LABC patients. Seven (33%) patients achieved a pCR in the breast. The serial biopsies performed, showed an at least 50% Farnesyltransferase inhibition after tipifarnib. The rate of pCR achieved in the breast after such a short-term treatment seems very encouraging. Moreover, it is intriguing that a breast pCR occurred in 5 (42%) of 12 estrogen receptor-positive or borderline-positive tumors, suggesting particular benefit in estrogen receptor-positive disease.

The administration of anthracycline-taxane chemotherapy in combination with trastuzumab produces a very high pCR rate in HER-2-overexpressing breast cancer patients. The preliminary DFS data from the MDACC trial also look very promising, the estimated 3-year DFS rate being 100% in the trastuzumab arm. The cardiotoxicity associated with trastuzumab justifies some concerns on its use in combination with doxorubicin. A less troublesome cardiotoxicity can be expected when epirubicin, rather than doxorubicin, is combined with trastuzumab. In the MDACC trial 10 patients showed a > 10% decrease of the LVEF, but none developed congestive heart failure.

Too few data are available to define the best chemotherapy regimen to be combined with trastuzumab. However, some findings are indisputable: HER-2-overexpressing tumors require an adequately dosed and prolonged chemotherapy, thus a short-term treatment combining just one agent with trastuzumab would be not enough to maximize the chance of getting a pCR.

The inclusion of anthracyclines may not be necessary since very promising pCR rates have been obtained with other combinations as recently reported by Limentani *et al*. (2006) and Coudert *et al*. (2006). Trials evaluating lapatinib alone or in combination with trastuzumab are ongoing or ready to start in the adjuvant and neoadjuvant setting. There is a great expectation on these results, and let us hope that this new molecule will further improve the fate of HER-2-positive early breast cancer patients.

CORRECT INDIVIDUALIZATION OF THE TREATMENT

Hormone receptor status

Most groups have identified negative hormone receptor status as the strongest predictive factor of response to neoadjuvant chemotherapy. A subset analysis taking hormone receptor status into account was performed in some of the above mentioned trials. There is a general agreement that the benefit of aggressive anthracycline/taxane-based chemotherapy is much more evident in ER-negative patients, whereas a very modest therapeutic gain can be obtained with this approach in ER-positive patients.

In the ECTO trial (Gianni *et al*., 2005a) the odds ratio for ER-negative patients was 5.77 (95% confidence interval, 3.49–9.52; $P < 0.0001$). In the MDACC retrospective analysis conducted by Mazouni *et al*. (2007) the pCR rate in the taxane-group was 29% in ER-negative and 8.8% in ER-positive patients. Colleoni *et al*. (2004) reviewed the pretreatment biopsies and histologic specimens at final surgery of 399 patients with large or locally advanced breast cancer (cT2-T4, N0-2, M0) who were treated with preoperative chemotherapy. The chance of obtaining pathological complete remission was 33.3% in ER-negative versus 7.5% in ER-positive patients. In the SICOG 9908 phase III trial (Frasci *et al*. 2006) the pCR rate achieved with the dose-dense PET regimen was much higher in ER-negative (27.5% vs. 5.4%), whereas it was similar to the standard approach in ER-positive (7.4% vs. 7.1%) patients.

Gene expression profile

Recently, gene expression profiles have been suggested to have a good predictive value. Gene expression markers that predict the likelihood of response to neoadjuvant paclitaxel and doxorubicin in locally-advanced breast cancer were identified by Gianni *et al*. (2005b). Of 89 assessable patients, 11 (12%) had a pCR. In univariate analysis, 24 genes correlated with pCR with $P < 0.05$.

Hess *et al*. (2006) developed a multigene predictor of pathologic complete response (pCR) to preoperative weekly paclitaxel and fluorouracil-doxorubicin-cyclophosphamide (T/FAC) chemotherapy and assessed its predictive accuracy on independent cases. The authors developed predictors of pCR from 82 cases and assessed

accuracy on 51 independent cases. A nominally best 30-probe set was selected for independent validation. This 30-probe set pharmacogenomic predictor correctly identified all but one of the patients who achieved pCR (12 of 13 patients) and all but one of those who were predicted to have residual disease had residual cancer (27 of 28 patients).

Early assessment of tumor response

Assessment of response to neoadjuvant chemotherapy early during the treatment is helpful in choosing between maintenance of successful therapy and discontinuation of unsuccessful treatment. Conventional methods like physical examination, mammography and ultrasound have shown to be suboptimal in accurate assessment. Several studies have shown the usefulness of [18 F]fluorodeoxyglucose positron emission tomography (FDG-PET) in the assessment of response. Early in the course of chemotherapy, a significant decline in the standardized uptake value (SUV) occurs in responders, in contrast to no change or a slight increase in those who do not respond to treatment.

Rousseau *et al*. (2006) assessed prospectively the efficacy of sequential FDG-PET to evaluate early response to neoadjuvant chemotherapy in stage II and III breast cancer patients. Images were acquired in 64 patients after administration of FDG (5 MBq/kg) at baseline and after the first, second, third, and sixth courses of chemotherapy. Decrease in the standardized uptake value (SUV) with FDG-PET was compared with pathologic response. Standardized uptake value (SUV) data decreased markedly to background levels in 94% (34 of 36) of responders. When using 60% of SUV at baseline as the cutoff value, the sensitivity, specificity, and negative predictive value of FDG-PET were 61%, 96%, and 68% after one course of chemotherapy, 89%, 95%, and 85% after two courses, and 88%, 73%, and 83% after three courses, respectively.

MRI may also be better than traditional methods in monitoring response to chemotherapy and may help determine early in the treatment if a particularly chemotherapy regimen is working for a patient, or if alternative drugs should be used.

Padhani *et al*. (2006) prospectively documented changes in contrast agent kinetics in patients with primary breast cancer treated with systemic chemotherapy after one or two cycles. Dynamic magnetic resonance (MR) examinations were performed before treatment and after the first and second cycle of neoadjuvant chemotherapy. Size (an increase, no change or $< 15\%$ reduction) and transfer constant range (an increase, no change or $< 11\%$ reduction) were equally accurate for predicting the absence of pathologic response after two cycles of treatment (sensitivity, specificity, and area under ROC curve were 100%, 90%, and 0.93, respectively, for size and 100%, 75%, and 0.94, respectively, for transfer constant range).

How can the baseline tumor characteristics (hormone receptor and HER-2/neu status, gene profile etc.) and the early assessment of response by imaging, work together in order to optimize the therapeutic strategy in the single patient ? Let us imagine a specific scenario in which the two tools can usefully cooperate. The 30-probe set pharmacogenomic predictor developed by Hess *et al*. (2006) has a high (92%) sensitivity but a low positive predictive value (52%). It means that almost all pCR can be predicted by the baseline gene profile, but a relevant number

of patients, predicted to achieve a pCR, will achieve a lesser response. Findings reported by Rousseau *et al*. (2006) with FDG-PET and Padhani *et al*. (2006) with contrast enhanced-MRI show that it is possible with functional imaging of the primary tumor to accurately predict (sensitivity and specificity ~ 90%) the pathologic response after 1–2 chemotherapy cycles. A tumor which does not show after two cycles of chemotherapy a 60% or more SUV reduction at PET, and/or > 15% decrease in size and > 11% decrease in the transfer constant rate, will almost never show a pathological complete response at the end of the treatment, even if it is genetically identified as likely pCR. This kind of patient should be a candidate to shift to a more aggressive chemotherapy. What shall we do for patients who are genetically identified as unlikely to achieve a pCR? We should take into account that paradoxically these patients have a better prognosis independently of chemotherapy administration. Indeed, a lot of patients can achieve a long-term survival and even cure in spite of failing to achieve pCR. Also in this case an early imaging evaluation can be useful. Chemotherapy should be continued only in the presence of a relevant SUV reduction at PET and size and transfer constant rate decrease at MRI. Conversely, alternative treatments like endocrine therapy (if justified), targeted drugs, etc., should be considered.

TAILORED NEOADJUVANT CHEMOTHERAPY MAY IMPROVE THE PROGNOSIS

The results of the meta-analysis conducted on nine trials comparing neoadjuvant vs. adjuvant chemotherapy, leads to less enthusiasm towards the neoadjuvant approach. However, given the design of these trials, it is unlikely to expect significant differences in terms of survival, when the unique difference consists in the delivery of the same chemotherapy a couple of months before. It is not reasonable to hypothesize that the burden of chemoresistant tumor cells could substantially change within a few months.

The only reasonable way to evaluate the role of preoperative chemotherapy is to design a multistep therapeutic approach, which utilizes most of available chemotherapy agents, when necessary. To date there have been very few attempts to verify whether a treatment change according to the type of response observed, can result into a substantial prognostic gain.

Thomas *et al*. (2004) evaluated the use of an alternate, non-cross-resistant adjuvant chemotherapy regimen in women with a good clinical but poor pathologic response to a preoperative doxorubicin-based regimen. Patients with locally advanced breast cancer received three cycles of vincristine, doxorubicin, cyclophosphamide, and prednisone (VACP) every 21 days followed by surgery. Patients with less than 1 cm^3 residual tumor at mastectomy received an additional five cycles of VACP. Those with more than 1 cm^3 residual tumor were randomly assigned to receive an additional five cycles of VACP or five cycles of vinblastine, methotrexate with calcium leucovorin rescue, and fluorouracil (VbMF). Patients receiving VbMF achieved higher relapse-free survival (RFS) and overall survival (OS) than those who received additional VACP, although the differences did not reach statistical significance. The Aberdeen trial (Smith *et al*., 2002) followed a quite different philosophy. The shift

was performed before surgery in good responders while in the previous trial it was performed after surgery in poorly responders. Again, the change of treatment (docetaxel instead of continuation of anthracycline-based chemotherapy) resulted in higher pCR rate and better 5-year DFS rate (90% vs. 72%; p = 0.04).

Both these trials had a design not optimizing the opportunities the neoadjuvant approach can offer. Surgery should represent only the last step on a route, having several intermediate stops, in order to decide whether that single patient is likely to achieve the best response with that regimen, or otherwise requires a chemotherapy shift. Figures 25.1–25.3 depict some models of study design evaluating preoperative chemotherapy in different subsets of patients, identified by means of clinical, immunohistochemical and genetic parameters. The model outlined in Figure 25.1 includes patients with a very aggressive tumor non-overexpressing HER-2/neu, but expected to be highly responsive to an aggressive chemotherapy approach (i.e., ER-negative, HER-2 negative, poor prognosis but highly responsive genetic signature). The rationale is to compare a standard preplanned preoperative approach to a multistep individualized treatment. Disease-free Survival is the main end point of such a study. In the tailored arm a first assessment of response is performed after two chemotherapy cycles. Those patients showing at FDG-PET + MRI a rapid decrease in both size and functional parameters (likely to achieve pCR), will receive the same regimen (AC) for two more cycles, resulting in a less toxic treatment than the preplanned AC × 4 →DOC × 4. In suboptimal responders a shift to a noncross-resistant regimen (i.e., Docetaxel/capecitabine or docetaxel/vinorelbine) should be performed since it theoretically offers more chance than docetaxel alone to achieve the best possible pathological response. In nonresponding patients a non-cross-resistant regimen should be combined with a targeted agent (bevacizumab), since other mechanisms besides a change in chemotherapy may be necessary to circumvent tumor resistance in these patients. A second assessment after 6–12 weeks is performed. If absence of residual

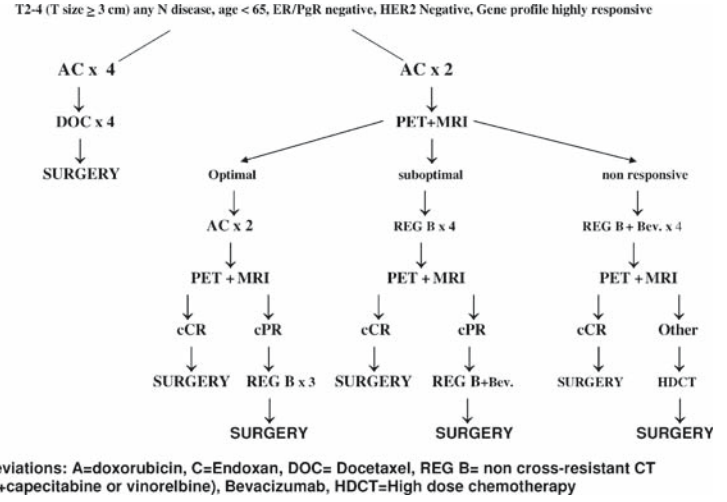

FIGURE 25.1. Model of study design for triple negative patients

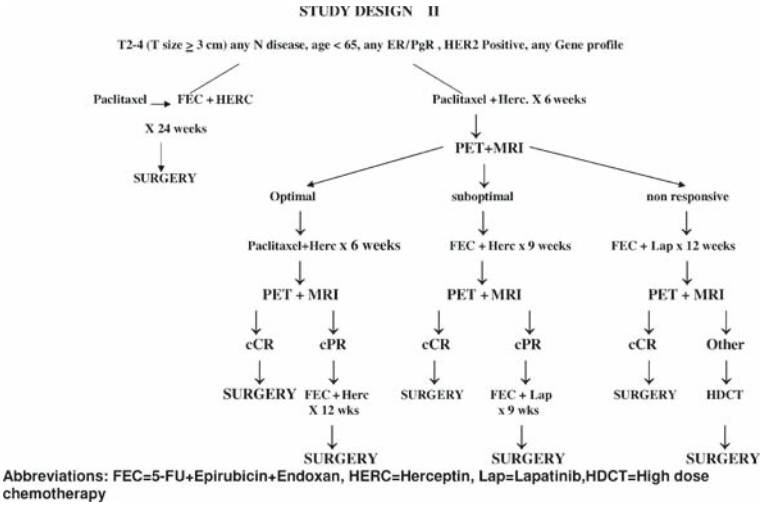

FIGURE 25.2. Model of study design for HER2/neu positive patients

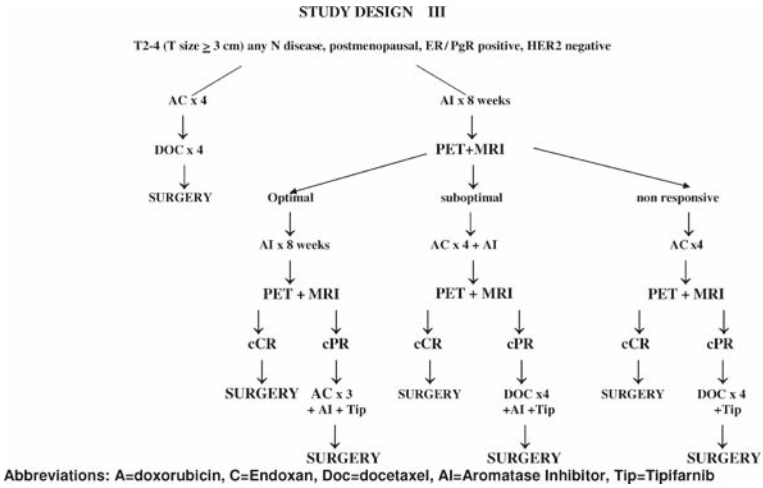

FIGURE 25.3. Model of study design for ER- and/or PgR-positive patients

tumor is shown at imaging, patients will undergo surgery. If not, something else will be added. In optimal responding, a shift to regimen B should be performed. In suboptimal responding, bevacizumab is added to chemotherapy, whereas in the nonresponding group, a salvage HDCT could be indicated. The rationale of this model is to minimize chemotherapy-related toxicity in highly responsive patients, to use more chemotherapy agents in moderately responsive, to combine chemotherapy with targeted agents in non responsive, and to administer HDCT only as a rescue in a very small subset of patients.

The models for HER-2/neu positive and ER-positive patients follow the same philosophy. In both groups the first step will consist of the minimum effective treatment, which is paclitaxel-trastuzumab in HER-2/

neu positive and hormone treatment alone in ER-positive patients. Chemotherapy will be intensified only in poorly responsive patients (or introduced in ER positive), and combined with a targeted agent, if necessary.

CONCLUSIONS

The main aim of this chapter was to address the role of chemotherapy in improving prognosis of women with locally-advanced breast cancer, and to determine whether the advances in chemotherapy have translated into a substantial prognostic gain. According to some retrospective analyses, a more aggressive chemotherapy approach and the introduction of taxanes have substantially improved both the pCR rate and overall survival (Chang *et al.*, 1998; Panades *et al.*, 2005; Cristofanilli *et al.*, 2004). More recently, some randomized trials, including either operable or LABC patients, have shown that a taxane-including treatment results in a significantly higher pCR rate, but the long-term survival advantage still remains doubtful. The long-term outcome reported with the use of HDCT does not look substantially better then that achievable with a standard chemotherapy. Therefore, this approach should not be considered outside clinical trials.

The best regimen schedule and sequence are not yet clearly identified. However, anthracyclines and taxanes given at full doses for at least 18–24 weeks should be the basic approach. The adoption of a weekly schedule is recommended when paclitaxel is used. The addition of other molecules like alkilating agents (given at higher than standard doses), platin compounds, capecitabine and vinorelbine should also be evaluated in well designed clinical trials. The shortening of the interval of CT administration is more feasible nowadays with haematopoietic growth factors support, and might result in a substantial improvement of antitumor activity, and long-term outcome. The combination of chemotherapy with targeted drugs antagonizing HER2/neu protein like Herceptin, lapatinib, etc. might result in a dramatic prognostic improvement in patients with HER2/neu overexpressing tumors.

An accurate baseline evaluation is crucial in planning the therapeutic strategy. Clinical, immunohistochemical, and genetic features permit the identification of patients who are more likely to benefit from chemotherapy. These patients should be included in clinical trials evaluating the role of more intensive and more complex (multidrug sequential approach) treatments.

For patients who are not predicted to benefit from chemotherapy, and for whom a better prognosis can be hypothesized independently of treatment received, different approaches should be evaluated. Hormone receptor positive, HER-2/neu negative not so young women represent a good example of these patients. The role of endocrine therapy plus the minimum effective chemotherapy plus targeted drugs like bevacizumab and/or pertuzumab and/or tipifarnib should be evaluated.

It is time to modify the philosophy of clinical trials in the neoadjuvant setting. A woman with a large operable or even inoperable tumor has a relevant risk of relapse and death from her tumor. The inclusion of hundreds of these patients in a phase III trial comparing the regimen A with the regimen B, is not the best way to take care of each one of them. An alternative model,

which makes each patient a living laboratory could be more effective. An intensive adoption of both diagnostic and therapeutic tools is mandatory for each patient. Starting from the expected less toxic among the likely effective treatments, early assessments of response must be performed, in order to change the treatment in presence of unsatisfactory response parameters. Different chemotherapy combinations, as well as combinations of chemotherapy and targeted agents should be available for each patient if required.

REFERENCES

Bear, H.D., Anderson, S., Brown, A., Smith, R., Mamounas, E.P., Fisher, B., Margolese, R., Theoret, H., and Soran, A., Wickerham, D.L., and Wolmark, N.;National Surgical Adjuvant Breast and Bowel Project Protocol B-27. (2003) The effect on tumor response of adding sequential preoperative docetaxel to preoperative doxorubicin and cyclophosphamide: preliminary results from National Surgical Adjuvant Breast and Bowel Project Protocol B-27. *J. Clin. Oncol. 21*: 4165–4174.

Bear, H.D., Anderson, S., Smith, R.E., Geyer, C.E. Jr., Mamounas, E.P., Fisher, B., Brown, A.M., Robidoux, A., Margolese, R., Kahlenberg, M.S., Paik, S., Soran, A., Wickerham, D.L., and Wolmark N. (2006) Sequential preoperative or postoperative docetaxel added to preoperative doxorubicin plus cyclophosphamide for operable breast cancer:National Surgical Adjuvant Breast and Bowel Project Protocol B-27. *J. Clin. Oncol. 24*: 2019–2027.

Braud, A.C., Levy, E., Feuilhade, F., Otmezguine, Y., Calitchi, E., Kirova, Y., and Le Bourgeois, J.P. (2002) Combination of vinorelbine, epirubicin, and cyclophosphamide as neoadjuvant chemotherapy for locally advanced breast cancer: phase II study. *Am. J. Clin. Oncol. 25*: 303–307.

Burstein, H.J., Harris, L.N., Marcom, P.K., Lambert-Falls, R., Havlin, K., Overmoyer, B., Friedlander, R.J. Jr., Gargiulo, J., Strenger, R., Vogel, C.L., Ryan, P.D., Ellis, M.J., Nunes, R.A., Bunnell, C.A., Campos, S.M., Hallor, M., Gelman, R., and Winer, E.P. (2003) Trastuzumab and vinorelbine as first-line therapy for HER2-overexpressing metastatic breast cancer: multicenter phase II trial with clinical outcomes, analysis of serum tumor markers as predictive factors, and cardiac surveillance algorithm. *J. Clin. Oncol. 21*: 2889–2895.

Buzdar, A.U., Valero, V., Ibrahim, N.K., Francis, D., Broglio, K.R., Theriault, R.L., Pusztai, L., Green, M.C., Singletary, S.E., Hunt, K.K., Sahin, A.A., Esteva, F., Symmans, W.F., Ewer, M.S., Buchholz, T.A., and Hortobagyi, G.N. (2007) Neoadjuvant therapy with paclitaxel followed by 5-fluorouracil, epirubicin, and cyclophosphamide chemotherapy and concurrent trastuzumab in human epidermal growth factor receptor 2-positive operable breast cancer: an update of the initial randomized study population and data of additional patients treated with the same regimen. *Clin. Cancer Res. 13*: 228–233.

Chang, S., Parker, S.L.,Pham, T.,Buzdar, A.U., and Hursting, S.D. (1998) Inflammatory breast carcinoma incidence and survival: the surveillance, epidemiology, and end results program of the National Cancer Institute, 1975–1992. *Cancer 82*: 2366–2372.

Citron, M.L., Berry, D.A., Cirrincione, C., Hudis, C., Winer, E.P., Gradishar, W.J., Davidson, N.E., Martino, S., Livingston, R., Ingle, J.N., Perez, E.A., Carpenter, J., Hurd, D., Holland, J.F., Smith, B.L., Sartor, C.I., Leung, E.H., Abrams, J., Schilsky, R.L., Muss, H.B., and Norton, L. (2003) Randomized trial of dose-dense versus conventionally scheduled and sequential versus concurrent combination chemotherapy as postoperative adjuvant treatment of node-positive primary breast cancer: first report of Intergroup Trial C9741/Cancer and Leukemia Group B Trial 9741. *J. Clin. Oncol. 21*: 1431–1439.

Colleoni, M., Viale, G., Zahrieh, D., Pruneri, G., Gentilini, O., Veronesi, P., Gelber, R.D., Curigliano, G., Torrisi, R., Luini, A., Intra, M., Galimberti, V., Renne, G., Nole, F., Peruzzotti, G., and Goldhirsch, A. (2004) Chemotherapy is more effective in patients with breast cancer not expressing steroid hormone receptors: a study of preoperative treatment. *Clin. Cancer Res.. 10*: 6622–6628.

Coudert, B.P., Arnould, L., Moreau, L., Chollet, P., Weber, B., Vanlemmens, L., Molucon, C.,

Tubiana, N., Causeret, S., Misset, J.L., Feutray, S., Mery-Mignard, D., Garnier, J., and Fumoleau, P. (2006) Pre-operative systemic (neo-adjuvant) therapy with trastuzumab and docetaxel for HER2-overexpressing stage II or III breast cancer: results of a multicenter phase II trial. *Ann. Oncol. 17*: 409–414.

Cristofanilli, M., Gonzalez-Angulo, A.M., Buzdar, A.U., Kau, S.W., Frye, D.K., and Hortobagyi, G.N.. (2004) Paclitaxel improves the prognosis in estrogen receptor negative inflammatory breast cancer: the M. D. Anderson Cancer Center experience. *Clin. Breast Cancer 4*: 415–419.

Cristofanilli, M., Boussen, H., Baselga, J., Lluch, A., Ben Ayed, F., Friaha, M., Ben Ahmed, S., Hurley, J., Johnston, S., Kaufman, B., Findlay, M., Olopade, O., Shannon, C., Harris, J., Stein, S., and Spector, N. (2006) A phase II combination study of lapatinib and paclitaxel as neoadjuvant chemotherapy in patients with newly diagnosed inflammatory breast cancer. *Breast Cancer Res. Treat. 100 (suppl 1)*: S5 (abstr. 1).

Evans, T.R., Yellowlees, A., Foster, E., Earl, H., Cameron, D.A., Hutcheon, A.W., Coleman, R.E., Perren, T., Gallagher, C.J., Quigley, M., Crown, J., Jones, A.L., Highley, M., Leonard, R.C., and Mansi, J.L. (2005) Phase III randomized trial of doxorubicin and docetaxel versus doxorubicin and cyclophosphamide as primary medical therapy in women with breast cancer: an anglo-celtic cooperative oncology group study. *J. Clin. Oncol. 23*: 2988–2995.

Ezzat, A.A., Ibrahim, E.M., Ajarim, D.S., Rahal, M.M., Raja, M.A., Tulbah, A.M., Al-Malik, O.A., Al-Shabanah, M., and Sorbris, R. (2004) Phase II study of neoadjuvant paclitaxel and cisplatin for operable and locally advanced breast cancer: analysis of 126 patients. *Br. J. Cancer 90*: 968–974.

Fisher, B., Bryant, J., Wolmark, N., Mamounas, E., Brown, A., Fisher, E.R., Wickerham, D.L., Begovic, M., DeCillis, A., Robidoux, A., Margolese, R.G., Cruz, A.B. Jr., Hoehn, J.L., Lees, A.W., Dimitrov, N.V., and Bear, H.D. (1998) Effect of preoperative chemotherapy on the outcome of women with operable breast cancer. *J. Clin. Oncol. 16*: 2672–2685.

Frasci, G., D'Aiuto, G., Comella, P., Thomas, R., Botti, G., Di Bonito, M., D'Aiuto, M., Romano, G., Rubulotta, M.R., and Comella G. (2005) A 2-month cisplatin-epirubicin-paclitaxel (PET) weekly combination as primary systemic therapy for large operable breast cancer: a phase II study. *Ann. Oncol. 16*: 1268–1275.

Frasci, G., D'Aiuto, G., Comella, P., Thomas, R., Botti, G., Di Bonito, M., De Rosa, V., Iodice, G., Rubulotta, M.R., and Comella G; Southern Italy Cooperative Oncology Group (SICOG).(2006) Weekly cisplatin, epirubicin, and paclitaxel with granulocyte colony-stimulating factor support vs triweekly epirubicin and paclitaxel in locally advanced breast cancer: final analysis of a sicog phase III study. *Br. J. Cancer 95*: 1005–1012.

Gianni, L., Baselga, J., Eiermann, W., Guillem Porta, V., Semiglazov, V., Lluch, A., Zambetti, M., Sabadell, D., Raab, G., Llombart Cussac, A., Bozhok, A., Martinez-Agullo, A., Greco, M., Byakhov, M., Lopez, J.J., Mansutti, M., Valagussa, P., and Bonadonna G; European Cooperative Trial in Operable Breast Cancer Study Group. (2005a) Feasibility and tolerability of sequential doxorubicin/paclitaxel followed by cyclophosphamide, methotrexate, and fluorouracil and its effects on tumor response as preoperative therapy. *Clin. Cancer Res. 11*(24 Pt 1): 8715–8721.

Gianni, L., Zambetti, M., Clark, K., Baker, J., Cronin, M., Wu, J., Mariani, G., Rodriguez, J., Carcangiu, M., Watson, D., Valagussa, P., Rouzier, R., Symmans, W.F., Ross, J.S., Hortobagyi, G.N., Pusztai, L., and Shak, S. (2005b) Gene expression profiles in paraffin-embedded core biopsy tissue predict response to chemotherapy in women with locally advanced breast cancer. *J. Clin. Oncol. 23*: 7265–7277.

Gonzalez-Angulo, A.M., McGuire, S.E., Buchholz, T.A., Tucker, S.L., Kuerer, H.M., Rouzier, R., Kau, S.W., Huang, E.H., Morandi, P., Ocana, A., Cristofanilli, M., Valero, V., Buzdar, A.U., and Hortobagyi, G.N. (2005) Factors predictive of distant metastases in patients with breast cancer who have a pathologic complete response after neoadjuvant chemotherapy. *J. Clin. Oncol. 23*: 7098–7104.

Green, M.C., Buzdar, A.U., Smith, T., Ibrahim, N.K., Valero, V., Rosales, M.F., Cristofanilli, M., Booser, D.J., Pusztai, L., Rivera, E., Theriault, R.L., Carter, C., Frye, D., Hunt, K.K., Symmans, W.F., Strom, E.A., Sahin, A.A., Sikov, W., and Hortobagyi GN. (2005) Weekly paclitaxel improves pathologic complete remission in operable breast cancer when compared with paclitaxel once every 3 weeks. *J. Clin. Oncol. 23*: 5983–5992.

Harris, L.N., You, F., Schnitt, S.J., Witkiewicz, A., Lu, X., Sgroi, D., Ryan, P.D., Come, S.E., Burstein, H.J., Lesnikoski, R., Kamma, M., Friedman, P.N., Gelman, R., Iglehart, J.D., and Winer, E.P. (2007) Predictors of resistance to preoperative trastuzumab and vinorelbine for HER2-positive early breast cancer. *Clin. Cancer Res. 13*: 1198–1207.

Hess, K.R., Anderson, K., Symmans, W.F., Valero, V., Ibrahim, N., Mejia, J.A., Booser, D., Theriault, R.L., Buzdar, A.U., Dempsey, P.J., Rouzier, R., Sneige, N., Ross, J.S., Vidaurre, T., Gomez, H.L., Hortobagyi, G.N., and Pusztai, L. (2006) Pharmacogenomic predictor of sensitivity to preoperative chemotherapy with paclitaxel and fluorouracil, doxorubicin, and cyclophosphamide in breast cancer. *J. Clin. Oncol. 24*: 4236–4244.

Hurley, J., Doliny, P., Reis, I., Silva, O., Gomez-Fernandez, C., Velez, P., Pauletti, G., Powell, J.E., Pegram, M.D., and Slamon, D.J. (2006) Docetaxel, cisplatin, and trastuzumab as primary systemic therapy for human epidermal growth factor receptor 2-positive locally advanced breast cancer. *J. Clin. Oncol. 24*: 1831–1838.

Kuerer, H.M., Newman, L.A., Smith, T.L., Ames, F.C., Hunt, K.K., Dhingra, K., Theriault, R.L., Singh, G., Binkley, S.M., Sneige, N., Buchholz, T.A., Ross, M.I., McNeese, M.D., Buzdar, A.U., Hortobagyi, G.N., and Singletary SE. (1999) Clinical course of breast cancer patients with complete pathologic primary tumor and axillary lymph node response to doxorubicin-based neoadjuvant chemotherapy. *J. Clin. Oncol. 17*: 460–469.

Lebowitz, P.F., Eng-Wong, J., Swain, S.M., Berman, A., Merino, M.J., Chow, C.K., Venzon, D., Zia, F., Danforth, D., Liu, E., and Zujewski, J. (2004) A phase II trial of neoadjuvant docetaxel and capecitabine for locally advanced breast cancer. *Clin. Cancer Res. 10*(20): 6764–6769.

Lee, Y.J., Doliny, P., Gomez-Fernandez, C., Powell, J., Reis, I., and Hurley, J. (2004) Docetaxel and cisplatin as primary chemotherapy for treatment of locally advanced breast cancers. *Clin. Breast Cancer 5*: 371–376.

Limentani, S.A., Brufsky, A.M., Erban, J.K., Jahanzeb, M., and Lewis, D. (2006) Phase II study of neoadjuvant docetaxel/ vinorelbine followed by surgery and adjuvant doxorubicin/cyclophosphamide in women with stage II/III breast cancer. *Clin. Breast Cancer 6*: 511–517.

Limentani, S.A., Brufsky, A.M., Erban, J.K., Jahanzeb, M., and Lewis, D. (2007) Phase II study of neoadjuvant docetaxel, vinorelbine, and trastuzumab followed by surgery and adjuvant doxorubicin plus cyclophosphamide in women with human epidermal growth factor receptor 2-overexpressing locally advanced breast cancer. *J. Clin. Oncol. 25*: 1232–1238.

Mauri, D., Pavlidis, N., and Ioannidis, J.P..(2005) Neoadjuvant versus adjuvant systemic treatment in breast cancer: a meta-analysis. *J. Natl. Cancer Inst. 97*: 188–194.

Mazouni, C., Kau, S.W., Frye, D., Andre, F., Kuerer, H., Buchholz, T., Symmans, W., Anderson, K., Hess, K., Gonzalez-Angulo, A., Hortobagyi, G.N., Buzdar, A., and Pusztai, L. (2007) Inclusion of taxanes, particularly weekly paclitaxel, in preoperative chemotherapy improves pathologic complete response rate in estrogen receptor-positive breast cancers. *Ann. Oncol. 18*: 874–880.

Moore, H.C., Green, S.J., Gralow, J.R., Bearman, S.I., Lew, D., Barlow, W.E., Hudis, C., Wolff, A.C., Ingle, J.N., Chew, H.K., Elias, A.D., Livingston, R.B., and Martino, S. (2007) Intensive dose-dense compared with high-dose adjuvant chemotherapy for high-risk operable breast cancer: Southwest Oncology Group/Intergroup Study 9623. *J. Clin. Oncol. 25*: 1677–1682.

Natoli, C., Cianchetti, E., Tinari, N., Angelucci, D., Grassadonia, A., Zilli, M., Ficorella, C., Ricevuto, E., Grossi, S., De Tursi, M., Carella, C., Rispoli, A., and Iacobelli, S. (2007) A phase II study of dose-dense epirubicin plus cyclophosphamide followed by docetaxel plus capecitabine and pegfilgrastim support as preoperative therapy for patients with stage II, IIIA breast cancer. *Ann. Oncol. Mar 17*: [Epub ahead of print]

Padhani, A.R., Hayes, C., Assersohn, L., Powles, T., Makris, A., Suckling, J., Leach, M.O., and Husband, J.E. (2006) Prediction of clinicopathologic response of breast cancer to primary chemotherapy at contrast-enhanced MR imaging: initial clinical results. *Radiology 239*: 361–374.

Panades, M., Olivotto, I.A., Speers, C.H., Shenkier, T., Olivotto, T.A., Weir, L., Allan, S.J., and Truong, P.T. (2005) Evolving treatment strategies for inflammatory breast cancer: a population-based survival analysis. *J. Clin. Oncol. 23*: 1941–1950.

Rousseau, C., Devillers, A., Sagan, C., Ferrer, L., Bridji, B., Campion, L., Ricaud, M., Bourbouloux,

E., Doutriaux, I., Clouet, M., Berton-Rigaud, D., Bouriel, C., Delecroix, V., Garin, E., Rouquette, S., Resche, I., Kerbrat, P., and Chatal JF, Campone M. (2006) Monitoring of early response to neoadjuvant chemotherapy in stage II and III breast cancer by [18 F]fluorodeoxyglucose positron emission tomography. *J. Clin. Oncol. 24*: 5366–5372.

Scholl, S.M., Fourquet, A., Asselain, B., Pierga, J.Y., Vilcoq, J.R., Durand, J.C., Dorval, T., Palangie, T., Jouve, M., and Beuzeboc, P. (1994) Neoadjuvant versus adjuvant chemotherapy in premenopausal patients with tumours considered too large for breast conserving surgery: preliminary results of a randomised trial: S6. *Eur. J. Cancer 30A*: 645–652.

Schwartzberg, L., Weaver, C., Lewkow, L., McAneny, B., Zhen, B., Birch, R., West, W., Tauer, K., and Buckner, C. (1999) High-dose chemotherapy with peripheral blood stem cell support for stage IIIB inflammatory carcinoma of the breast. *Bone Marrow Transpl. 24*: 981–987.

Semiglazov, V.F., Topuzov, E.E., Bavli, J.L., Moiseyenko, V.M., Ivanova, O.A., Seleznev, I.K., Orlov, A.A., Barash, N.Y., Golubeva, O.M., and Chepic, O.F..(1994) Primary (neoadjuvant) chemotherapy and radiotherapy compared with primary radiotherapy alone in stage IIb-IIIa breast cancer.*Ann. Oncol. 5*: 591–595

Smith, I.C., Heys, S.D., Hutcheon, A.W., Miller, I.D., Payne, S., Gilbert, F.J., Ah-See, A.K., Eremin, O., Walker, L.G., Sarkar, T.K., Eggleton, S.P., and Ogston, K.N. (2002) Neoadjuvant chemotherapy in breast cancer: significantly enhanced response with docetaxel. *J. Clin. Oncol. 20*: 1456–1466.

Sparano, J.A., Moulder, S., Kazi, A., Vahdat, L., Li, T., Pellegrino, C., Munster, P., Malafa, M., Lee, D., Hoschander, S., Hopkins, U., Hershman, D., Wright, J.J., and Sebti, S.M. (2006) Targeted inhibition of farnesyltransferase in locally advanced breast cancer: a phase I and II trial of tipifarnib plus dose-dense doxorubicin and cyclophosphamide. *J. Clin. Oncol. 24*: 3013–3018.

Spector, N.L., Blackwell, K., Hurley, J., Harris, L., Lombardi, D., Bacus, S., Ahmed, S.B., Boussen, H., Frikha, M., and Ayed, F.B. (2006) EGF103009, a phase II trial of lapatinib monotherapy in patients with relapsed/refractory inflammatory breast cancer: clinical activity and biological predictors of response. *J. Clin. Oncol. 24* (18 S): 3 s (abstr. 502).

Stewart, D.A., Paterson, A.H., Ruether, J.D., Russell, J., Craighead, P., Smylie, M., and Mackey, J. (2005) High-dose mitoxantrone-vinblastine-cyclophosphamide and autologous stem cell transplantation for stage III breast cancer: final results of a prospective multicentre study. *Ann. Oncol. 16*: 1463–1468.

Thomas, E., Holmes, F.A., Smith, T.L., Buzdar, A.U., Frye, D.K., Fraschini, G., Singletary, S.E., Theriault, R.L., McNeese, M.D., Ames, F., Walters, R., and Hortobagyi, G.N. (2004) The use of alternate, non-cross-resistant adjuvant chemotherapy on the basis of pathologic response to a neoadjuvant doxorubicin-based regimen in women with operable breast cancer: long-term results from a prospective randomized trial. *J. Clin. Oncol. 22*: 2294–2302.

Torrisi, R., Magni, E., Della pasqua, S., D'Alessandro, C., Veronesi, P., Luini, A., Minchella, I., Ghiaini, R., Nolè, F., Viale, G., Goldhirsh, A., and Colleoni, M. (2006) Trastuzumab and oral vinorelbine (± endocrine therapy) as primari therapy in patients with HER2/neu positive breast cancer. *Breast Cancer Res. Treat. 100* (suppl. 1): S148 (abstr. 3071).

Torrisi, R., Colleoni, M., Veronesi, P., Rocca, A., Peruzzotti, G., Severi, G., Medici, M., Renne, G., Intra, M., Luini, A., Nole, F., Viale, G., and Goldhirsch, A. (2007) Primary therapy with ECF in combination with a GnRH analog in premenopausal women with hormone receptor-positive T2-T4 breast cancer. *Breast 16*: 73–80.

Ueno, N.T., Buzdar, A.U., Singletary, S.E., Ames, F.C., McNeese, M.D., Holmes, F.A., Theriault, R.L., Strom, E.A., Wasaff, B.J., Asmar, L., Frye, D., and Hortobagyi, G.N.(1997) Combined-modality treatment of inflammatory breast carcinoma: twenty years of experience at M. D. Anderson Cancer Center. *Cancer Chemother. Pharmacol. 40*: 321–329.

Untch, M., Konekny, G., and Ditsch, N. (2002) Dose-dense sequential epirubicin-paclitaxel as preoperative treatment of breast cancer: results of a randomised AGO study. *Proc. Am. Soc. Clin. Oncol. 21*: 34a (abstr. 133).

von Minckwitz, G., Raab, G., Caputo, A., Schutte, M., Hilfrich, J., Blohmer, J.U., Gerber, B., Costa, S.D., Merkle, E., Eidtmann, H., Lampe,

D., Jackisch, C., du Bois, A., and Kaufmann, M. (2005) Doxorubicin with cyclophosphamide followed by docetaxel every 21 days compared with doxorubicin and docetaxel every 14 days as preoperative treatment in operable breast cancer: the GEPARDUO study of the German Breast Group. *J. Clin. Oncol. 23*: 2676–2685.

Wedam, S.B., Low, J.A., Yang, S.X., Chow, C.K., Choyke, P., Danforth, D., Hewitt, S.M., Berman, A., Steinberg, S.M., Liewehr, D.J., Plehn, J., Doshi, A., Thomasson, D., McCarthy, N., Koeppen, H., Sherman, M., Zujewski, J., Camphausen, K., Chen, H., and Swain, S.M. (2006) Antiangiogenic and antitumor effects of bevacizumab in patients with inflammatory and locally advanced breast cancer. *Clin. Oncol. 24*: 769–777.

Wolmark, N., Wang, J., Mamounas, E., Bryant, J., and Fisher, B. (2001) Preoperative chemotherapy in patients with operable breast cancer: nine-year results from National Surgical Adjuvant Breast and Bowel Project B-18. *J. Natl. Cancer Inst. Monogr. 30*: 96–102.

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Relevance of Dose-Intensity for Adjuvant Treatment of Breast Cancer

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INTRODUCTION

Despite all progress in the management of primary breast cancer in general and in perioperative systemic treatment in particular, many patients still relapse and eventually die as a consequence of their disease (EBCTCG, 2005) Thus, different approaches have been made to the improvement of preoperative or adjuvant polychemotherapy, including the addition of novel drugs such as taxanes or targeted agents or the establishment of dose-intensive or dose-dense regimens. This chapter outlines the theoretical framework for dose-dense chemotherapy, and reviews recent clinical trials that address this concept to adjuvant breast cancer treatment.

DOSE INTENSITY AND DOSE DENSITY – THEORETICAL FRAMEWORK

When considering the role of dose density, it is useful to revisit the concept of dose intensity. *Dose intensity* describes the ratio of chemotherapy dose and time. It is generally calculated by dividing the body-surface adjusted total dose by the duration of treatment in weeks. Dose intensification can be achieved by increasing the dose of chemotherapy, shortening the interval between treatment cycles, or a combination of both approaches.

The role of increased dose intensity obtained by *increasing the single dose* per cycle or the total dose of cytotoxic drugs has been widely studied in trials of adjuvant breast cancer. The concept was strongly influenced by the Skipper-Schabel-Wilcox model, also known as the log-kill model, which is based on the assumption that tumors grow exponentially with a constant doubling time until they reach a lethal volume. Cytotoxic therapy is believed to induce constant fractional cell-kill irrespective of the initial size of the malignant population with increasing doses resulting in more substantial cytotoxic effects (Skipper *et al*., 1964). Unfortunately, clinical trials in early breast cancer have not been able to confirm these assumptions as randomized trials found no benefit from increased doses of cyclophosphamide or doxorubicin compared with the standard

levels i.e., cyclophosphamide at 600 mg/m^2 and doxorubicin at 60 mg/m^2 (Henderson *et al.*, 2003; Fisher *et al.*, 1997,1999).

The concept of increasing dose density has been less extensively evaluated. *Dose density* is a relative term that refers to the frequency of administration of chemotherapy drugs and regimens compared to standard regimens. Although dose-dense regimens are frequently dose-intensified, the term "dose-dense" does not define the dose intensity. A dose-dense but not dose-intensified regimen can, for example, be obtained by dividing a single dose in three equal doses which are given in weekly instead of 3-weekly intervals. On the other hand, shortening of the interval between treatment cycles from 3 to 2 weeks leads to a dose-dense and dose-intensified regimen, if the same doses are maintained.

The concept of dose-density has been strongly influenced by an alternative model developed by Norton and Simon (1986), which hypothesizes that the logarithmic cell-kill is not constant but proportional to the relative growth-rate. Accordingly, slow-growing collections of tumor cells will tend to regress more slowly in response to a given therapy than will the faster-growing tumor cells treated at the same time. The Norton-Simon model is based on the assumption of a Gompertzian growth pattern which is characterized by an increasing doubling time with increasing tumor size. This growth pattern leads to an exponentially dropping relative growth rate with increasing cell numbers as the mass reaches a plateau phase of very slow actual growth (Norton, 1988). Consequently, smaller tumors should experience greater log kill (cell kill on a logarithmic scale) than larger tumors of the same kinetics, because they grow relatively more rapidly. Unfortunately, the outcome could still be the same because smaller tumors tend to regrow more rapidly according to the Gompertzian model. This conceptual model led to the suggestion that chemotherapy could be made more efficient by giving it at a greater dose rate thus minimizing the regrowth of cancer between cycles of treatment.

The approach was first applied to a clinical trial in breast cancer in the 1985 NCI Milan Study which compared an alternating treatment strategy of 3-week cycles of CMF (Cyclophosphamide, Methotrexate, 5-Fluorouracil) followed by one dose of doxorubicin with a sequential therapy regime consisting of four 3-week cycles of doxorubicin followed by eight cycles of CMF (Bonadonna *et al.*, 1995). Both treatment arms comprised a total of eight cycles of CMF chemotherapy and four cycles of single agent doxorubicin. Although the doses, the interval between cycles and the duration of treatment were the same in both arms, the dose-density and dose-intensity of CMF and Doxorubicin, respectively, were higher for the sequential strategy. Contrary to the initial expectations, the sequential (more dose-dense) strategy was associated with a significantly improved disease-free survival (DFS) and overall survival (OS) providing support to the concept of dose-dense chemotherapy (Bonadonna *et al.*, 2004).

Following this initial work, several randomized trials were conducted to test the feasibility and effectiveness of dose-dense chemotherapy. The Cancer and Leukemia Group B (CALGB) and the Gruppo Oncologico Nord Ovest - Mammella Inter Gruppo (GONO-MIG) designed two randomised trials comparing standard 3-weekly

with dose-dense 2-weekly chemotherapy. In both trials standard and dose-dense treatment arms contained the same chemotherapy regimens at the same doses, providing a relatively "pure study design" that allows conceptual conclusions on dose-dense chemotherapy. In a different approach, the Eastern Cooperative Oncology Group (ECOG) compared weekly and 3-weekly docetaxel or paclitaxel after four cycles of standard doxorubicin and cyclophosphamide providing further insight in the value of dose-density for single agent taxanes. Other trials have been more difficult to interpret as the treatment groups were not equal in terms of the number of cycles or total drug dose, or even different drugs delivered.

CANCER AND LEUKEMIA GROUP B TRIAL 9741 AND GRUPPO ONCOLOGICA GONO-MIG STUDY

The CALGB trial 9741 was set-up to evaluate the concepts of dose-dense therapy and sequential therapy in patients with node-positive primary breast cancer. Using a 2 × 2 factorial design, the trial compared concurrent and sequential chemotherapy regimens and dose-dense 2-weekly and standard 3-weekly application. Concurrent chemotherapy consisted of four cycles of doxorubicin (60 mg/m^2) and cyclophosphamide (600 mg/m^2) followed by four cycles paclitaxel (175 mg/m^2). Sequential treatment comprised four cycles doxorubicin (60 mg/m^2) followed by four cycles paclitaxel (175 mg/m^2) and four cycles cyclophosphamide (600 mg/m^2). Granulocyte colony stimulating factor (G-CSF) support was used in dose-dense schedules at fixed doses on days 3–11 of each cycle. The trial was designed to have a 90% power to detect a 33% difference in hazard ratios (HR) for DFS and OS (Citron *et al*., 2003). Although chemotherapy doses were identical in all four arms, the number of cycles, the interval between cycles, and the total duration of treatment varied, resulting in a 50% increase in dose-intensity for the dose-dense arms. Nevertheless, all four treatment schedules proved to be safe and feasible. With the possible exception of anemia, which was eminently treatable with erythropoietins, the dose-dense approach was associated with a small but notably lower incidence of common toxicities including neutropenia and neutropenic complications. Long-term follow up showed no relevant late toxicities or increased risk of secondary malignancies.

With a median follow-up of 72 months, 508 out of 2005 patients have relapsed and 370 patients have died (Hudis *et al*., 2005). Dose-dense treatment significantly improved DFS (HR 1.25, 95% confidence interval (CI) 1.05–1.49; p = 0.012) and OS (p = 0.049), whereas no differences were observed between sequential and concurrent schedules (HR 1.04, 95% CI 0.88–1.24; p = 0.65). An unplanned retrospective subset analysis suggested a larger absolute benefit for dose-dense therapy in patients with ER-negative disease (HR 0.76, p = 0.014) but no significant benefit for patients with ER-positive disease.

In a similar concept, the GONO-MIG trial compared six cycles of fluorouracil (600 mg/m^2), epirubicin (60 mg/m^2), and cyclophosphamide (600 mg/m^2) administered every 3 weeks (FEC_{21}) versus the same regimen administered every 2 weeks with filgrastim support (FEC_{14}) in patients with

lymph node-positive or high-risk lymph node-negative breast cancer (Venturini *et al*., 2005). The trial was designed to detect a 20% relative reduction in the hazard of death with a power of 80%, corresponding to a 5–6% absolute increase in 5-year OS.

Both treatments were well tolerated. No incident cases of acute myelogenous leukemia or myelodysplastic syndrome were observed. Only one case of grade 2 cardiac toxicity was reported on each arm. Despite a 48% increase in the dose-intensity in the FEC_{14} arm compared with that in the FEC_{21} arm, fewer dose reductions were required among patients in the FEC_{14} arm and there were no differences in the total doses delivered between arms.

There was a trend toward improved event-free survival and OS for women who received the dose-dense regimen, but the difference between the two arms did not reach statistical significance for either recurrence (HR 0.88, 95% CI 0.71–1.08) or death (HR 0.87, 95% CI 0.67–1.13). Exploratory subgroup analyses showed a trend towards a larger benefit for dose-dense therapy in patients younger than 50 years (HR for DFS 0.66, 95% CI 0.46–0.94) and in patients with ER-negative disease (HR 0.79, 95% CI 0.59–1.06). Subgroup analyses suggested furthermore that the benefit of dose-dense treatment may be confined to HER-2-overexpressing patients, even though differences in outcome did not reach statistical significance. In the HER-2-positive cohort, the relative risks of failure for FEC_{14}-treated patients as compared to FEC_{21} were 0.54 (95% CI 0.27–1.11; $p = 0.092$) for DFS and 0.59 (95% CI 0.26–1.37; $p = 0.22$) for OS accounting for a 15.2% and 14.8% absolute increase in 5-year DFS and OS, respectively, as compared to HER-2-negative patients receiving FEC_{21}.

On comparing the two randomized studies several differences emerge, including patient selection (e.g., 100% node-positive patients in CALGB trial versus 36% node-negative in the GONO-MIG study), chemotherapy regimen (in particular the absence of a taxane and the use of a relatively low anthracycline dose in the Italian study) and sample size (1,214 versus 2,005 patients, respectively). The GONO-MIG study was also relatively underpowered to detect the planned 20% risk reduction due to an unexpected low event rate (assumed 5-year OS 65–70% versus actual OS of 89% in the control group).

Nevertheless, despite the fact that on the surface the results appear to be discordant, the findings from CALGB trial 9741 and the GONG-MIG study are not entirely inconsistent on closer examination. Both trials support the concept that dose density has a modest impact on the outcome, particularly among younger patients, HER-2-positive patients, and ER-negative patients. Although the subgroup analyses should be viewed as hypothesis generating only, they contribute to the mounting evidence that the benefits of chemotherapy are strongly influenced by the hormone receptor status and the HER-2 status of the tumor, as well as other biologic factors that are yet to be defined.

ECOG 9911 Study

ECOG 9911 was a 2 × 2 randomized trial comparing 3-weekly doxorubicin (60 mg/m^2) and cyclophosphamide (600 mg/m^2) followed by either paclitaxel or docetaxel, given either weekly (80 mg/m^2 or 35 mg/m^2, respectively) or every 3 weeks (175 mg/m^2

or 100 mg/m^2, respectively). The study accrued 4,950 eligible women with node-positive or high-risk node-negative breast, and final data were presented with 5 years of follow-up. Interestingly, the primary study comparisons demonstrated equivalence. Neither paclitaxel nor docetaxel emerged as superior with respect to DFS (HR 1.02, p = 0.73), nor was weekly therapy different from every-3-weeks therapy in the aggregate (HR 1.07, p = 0.30) (Sparano *et al.*, 2007). Preplanned subgroup analyses suggest, however, a potential DFS-benefit of dose-dense therapy for paclitaxel (HR 1.24; p = 0.02) but not for docetaxel (HR 1.09; p = 0.33). When interpreting these results, it has to be considered that the planned dose density and cumulative dose were 37% higher for weekly paclitaxel compared to 3-weekly therapy, whereas dose-density and cumulative dose were similar for weekly and 3-weekly docetaxel (5% increase for the weekly application). The relevance of these differences is unclear, and further analyses are awaited.

Evidence from other adjuvant trials

Several other randomized trials have included dose-dense regimens which will be reviewed below. In contrast to the CALGB 9741, GONO-MIG and ECOG 9911 studies; however, these studies are difficult to interpret, as treatment groups differ not only in terms of dose-density, but also in several other relevant parameters including the use of higher single or cumulative doses, different drugs or different number of cycles, or the application of sequential strategies. Some of the trials are furthermore limited by their relatively small size, reducing the statistical power to detect smaller differences. Consequently, conclusions can only be drawn for the specific treatment regimen evaluated in the study whereas the relative benefit of the increase in dose-density can not be assessed.

The Arbeitsgemeinschaft Gynaekologische Onkologie (AGO) compared a dose-dense regimen consisting of sequential 2-weekly cycles of single agent epirubicin (150 mg/m^2), paclitaxel (225 mg/m^2) and cyclophosphamide (2,500 mg/m^2), with conventional 3-weekly treatment with four cycles of epirubicin (90 mg/m^2) and cyclophosphamide (600 mg/m^2) followed by four cycles of paclitaxel (175 mg/m^2). The trial encompassed 1,284 patients with four or more affected lymph nodes. The dose-intensified regimen was safe and feasible but was associated with substantially increased haematologic and non-haematologic toxicity. At a median follow-up of 62 months, an interim analysis showed a significant benefit in recurrence-free survival (HR 0.72; 95% CI 0.59–0.87, p = 0.0008) and OS (HR 0.76; 95% CI 0.59–0.97, p = 0.03) for the dose-dense arm (Moebus *et al.*, 2006). There was no substantial heterogeneity in the relative treatment effects among subgroups defined by menopausal status, number of affected lymph nodes, HER-status or hormone-receptor status. The results are certainly intriguing, but they have to be interpreted with caution due a relatively low number of events and the lack of long-term toxicity data.

The National Cancer Institute of Canada (NCIC) performed a three-arm randomized phase III trial comparing six 4-weekly cycles of cyclophosphamide, epirubicin and fluorouracil (CEF) with four 3-weekly cycles of standard doxorubicin and cyclophosphamide

followed by four cycles of paclitaxel (AC/T) or six cycles of dose-dense epirubicin (120 mg/m^2) and cyclophosphamide (830 mg/m^2) in 2-weeks intervals followed by four cycles of paclitaxel (EC/T). The study accrued 2,104 patients node-positive or high-risk node-negative breast, and preliminary data were presented with a median follow-up of 30.4 months. Interestingly, AC/T given every 3 weeks was significantly inferior to CEF (HR 1.49, 95% CI, 1.12–1.99, p = 0.005) or EC/T (HR 1.68, 95% CI, 1.25–2.27, p = 0.0006) in terms of relapse free survival, whereas the trial failed to show a significant difference between dose dense EC/T and CEF at this time-point (HR 0.89, 95% CI, 0.64–1.22, p = 0.46). Survival data are not available at the present time and the results have to be interpreted with caution due a relatively short follow-up associated with a low number of events (Burnell *et al*., 2006).

The European Organization for Research and Treatment of Cancer (EORTC), National Cancer Institute of Canada (NCIC), and Swiss Group for Clinical and Epidemiological Cancer Research (SAKK) jointly set-up a trial to compare six 2-weekly cycles of epirubicin (120 mg/m^2) and cyclophosphamide (830 mg/m^2) with six 4-weekly cycles of cyclophosphamide, epirubicin and fluorouracil (CEF) in 448 patients with locally advanced breast cancer. Both regimens proved to be safe and feasible. After a median follow-up of 5.5 years, the study showed no therapeutic advantage for the dose-intensified regimen in comparison with CEF (Therasse *et al*., 2003). Treatment effects were not different according to hormonal receptor status.

The Southwest Oncology Group designed a study to compare modern dose-dense and dose-escalated anthracycline- and taxane-based chemotherapy with a (near)-standard anthracycline-based adjuvant chemotherapy regimen followed by high-dose chemotherapy (HDC) with autologous hematopoietic progenitor cell support (Moore *et al*., 2007). The dose dense regimen consisted of sequential administration of three cycles each of doxorubicin (80 mg/m^2), paclitaxel (200 mg/m^2) and cyclophosphamide (3 g/m^2) in 2-week intervals. Patients in the HDC-arm received four cycles of doxorubicin 80 mg/m^2) and cyclophosphamide 600 mg/mg^2 in combination repeated every 3 weeks followed by HDC according to the STAMP I or V protocol. This study included 536 women with four or more axillary lymph nodes, and had a median follow-up of 70 months. Despite a trend in favor of the dose-dense regimen, there was no significant difference between the two arms for DFS or overall survival. Estimated 5-year DFS was 80% (95% CI, 76–85%) for dose-dense therapy and 75% (95% CI, 69–80%) for transplantation. Estimated 5-year OS was 88% (95% CI, 84–92%) for dose-dense therapy and 84% (95% CI, 79–88%) for transplantation. Thus, dose-dense and dose-intensified adjuvant therapy is at least equivalent to standard chemotherapy followed by HDC but results are difficult to interpret since the role of HDC in early breast cancer remains yet to be defined.

The Nordostdeutsche Gesellschaft für gynäkologische Onkologie (NOGGO) compared a taxane-based dose-dense regimen in 2-week intervals with a taxane-free 3-weekly regimen (Kümmel *et al*., 2006). Patients with four or more positive lymph nodes were randomized to four cycles of dose-dense epirubicin (90 mg/m^2) and paclitaxel (175 mg/m^2) followed by three cycles of dose-dense CMF, or to four

cycles of standard epirubicin (90 mg/m^2) and cyclophosphamide (600 mg/m^2) followed by three 3-weekly CMF cycles. Both treatments were safe and feasible and the frequency of treatment modifications, premature discontinuation and adverse events were similar in both groups. After a median follow-up of 78 months, 101 out of 216 patients had relapsed. DFS (61% vs. 49% at 6 years) and OS analysis (77% vs. 69% at 6 years) suggested a benefit for dose-dense therapy, although results were not statistically significant, a result which has to be interpreted in the context of the size of the difference the study was powered to detect.

In contrast to the trials outlined above, which applied a dose-dense strategy to only one of the treatment arms, the Hellenic Cooperative Oncology Group (HeCOG) trial was the first study to directly compare two different dose-dense sequential regimens. The trial included 604 patients with node-positive or high-risk node-negative breast cancer. All patients received chemotherapy in 2-week intervals. Patients were randomized to three cycles of epirubicin (110 mg/m^2) followed by three cycles of paclitaxel (250 mg/m^2) and three cycles of a dose-intensified CMF regimen, or to four cycles of epirubicin followed by four cycles of CMF. The study failed to show a significant difference in DFS or OS between both treatment groups but suggested a potential benefit for the paclitaxel-treated patients in hormone-receptor negative patients. It remains to be seen whether these findings, based on a small number of events, will hold with longer follow-up.

Evidence from neoadjuvant trials

Several trials have tried to apply the concept of dose-dense therapy to primary chemotherapy of early breast cancer. The AGO compared a dose-dense sequential neoadjuvant regimen with three cycles each of epirubicin (150 mg/m^2) and paclitaxel (250 mg/m^2) in 2-week intervals, with conventional 3-weekly treatment with four cycles of epirubicin (90 mg/m^2) and paclitaxel (175 mg/m^2). Early results of the trial showed a higher breast conversation rate and a significantly higher rate of pathological complete remission (pCR) for the dose-dense regimen (18% vs. 10%), but data on DFS or OS are not yet available (Untch *et al.*, 2002). Similar results have been reported for a randomized trial comparing neoadjuvant weekly and 3-weekly paclitaxel therapy followed by surgery and four cycles of adjuvant fluorouracil, doxorubicin and cyclophosphamide chemotherapy (Green *et al.*, 2005). Dose-dense therapy was again associated with a higher rate of breast-conserving surgery and a significantly higher pCR rate (28.2% vs. 15.7%, $p = 0.02$), but DFS or OS have not yet been reported. It is, therefore, currently unclear, whether the improved antitumor response with dose-dense regimens, which was demonstrated in both trials, will translate into an advantage in DFS or OS. Furthermore, dose-dense therapy was associated with a higher dose intensity and cumulative total dose in both trials, limiting the conclusions that can be drawn with respect to the value of dose-dense therapy.

In contrast to the two trials, outlined above, the Geparduo Study failed to demonstrate a benefit for neoadjuvant, dose-dense therapy. This study compared four cycles of standard doxorubicin and cyclophosphamide followed by four cycles of docetaxel with a dose-dense regimen consisting of four cycles of doxorubicin (50 mg/m^2) and docetaxel (75 mg/m^2) in 2-week intervals.

The two regimens did not only have different dose-densities, but also differed in terms of dose-intensity, cumulative dose, number of chemotherapy drugs and overall duration of treatment (8 vs. 24 weeks). It is, therefore, not entirely surprising that the short but dose-dense therapy was associated with a lower pCR rate (7% vs. 14%) and a higher mastectomy rate compared to the conventional regimen. Overall, the value of dose-dense therapy in the neoadjuvant setting is currently unclear. Some trials suggest a modest effect of dose-dense therapy on antitumor response, but it remains to be shown whether or not this translates into a survival benefit.

In conclusion, several trials have demonstrated that dose-dense strategies with G-CSF support are safe and feasible and have a modest impact on disease recurrence and overall survival of unselected patients with early-stage breast cancer. Other important issues such as the optimal dose or the optimal sequence of chemotherapy drugs, and the importance of dose intensity, cumulative dose or duration of treatment, remain to be elucidated by future randomized trials. There is increasing reason to believe that the benefits of dose-dense therapy will be greater for specific tumor subtypes such as hormone receptor-negative, highly proliferative or HER-2 overexpressing tumors. Recent studies suggest furthermore, that topoisomerase IIα might be an even better molecular predictor of the efficacy of anthracycline-based adjuvant therapy, but there is as yet no evidence of the value of topoisomerase IIα in predicting a differential benefit of dose-dense treatment over conventional regimens. Future studies are needed to better define the patient population that will receive the greatest benefit from dose-dense therapy and it is imperative that we incorporate the increasing knowledge on breast cancer biology prospectively into the design of innovative, adequately powered clinical trials that test therapeutic principles in major biologic subtypes of breast cancer.

REFERENCES

Bonadonna, G., Zambetti, M., and Valagussa, P. 1995. Sequential or alternative doxorubicin and CMF regimes in breast cancer with more than three positive nodes. Ten year results. *J. Am. Med. Assoc. 273*: 542–547.

Bonadonna, G., Zambetti, M., Moliterni, A., Gianni, L., and Valagussa, P. 2004 Clinical relevance of different sequencing of doxorubicin and cyclophosphamide,methotrexate and fluorouracil in operable breast cancer. *J. Clin. Oncol. 22*: 1614–1620.

Burnell,M.,Levine,M.,Chapman,J.A.,Bramwell,V., Gelmon, K., Walley, B., Whelan, T., Albain, K., Perez, E., Rugo, H., Ding, Z., O'Brien, P., Shepherd, L., and Pritchard, K. 2006. A randomized trial of CEF versus dose dense EC followed by paclitaxel versus AC followed by paclitaxel in women with node positive high-risk node negative breast cancer, NCIC CTG MA.21: results of an interim analysis. *Proc. San Antonio Breast Cancer Symposium*, abstr.53.

Citorn, M.l., Berry, D.A., Cirrincione, C., Hudis, C., Winer, E.P., Gradishar, W.J., Davidson, N.E., Martino, S., Livingston, R., Ingle, J.N., Perez, E.A., Carpenter, J., Hurd, D., Holland, J.F., Smith, B.L., Sartor, C.l., Leung, E.H., Abrams, J., Schilsky, R.L., Muss, H.B., and Norton, L. 2003. Randomized trial of dose-dense versus conventionally sheduled and sequential versus concurrent combination chemotherapy as postoperative adjuvant treatment of node positive primary breast cancer. First report of intergroup trial C9741/cancer and leukemia group B trial 9741. *J. Clin. Oncol. 21*: 1431–1439.

Early Breast Cancer Trialists Collaborative Group (EBCTCG, 2005). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomized trials. *Lancet 365*: 1687–1717.

Fisher, B., Anderson, S., Wickerham, D.L., DeCillis, A., Dimitrov, N., Mamounas, E., Wolmark, N., Pugh, R., Atkins, J.N., Meyers, F.J., Abramson, N., Wolter, J., Bornstein, R.S., Levy, L., Romond, E.H., Caggiano, V., Grimaldi, M., Jochimsen, P., and Deckers, P. 1997. Increased intensification and total dose of cyclophosphamide in a doxorubicin-cyclophosphamide regimen for the treatment of primary breast cancer: findings from National Surgical Adjuvant Breast and Bowel Project B22. *J. Clin. Oncol. 15*: 1858–1869.

Fisher, B., Anderson, S., DeCillis, A., Dimitrov, N., Atkins, J.N., Fehrenbacher, L., Henry, P.H., Romond, E.H., Lanier, K.S., Davila, E., Kardinal, C.G., Laufmann, L., Pierce, H.I., Abramson, N., Keller, A.M., Hamm, J.T., Wickerham, D.L., Begovic, M.,Tan-Chiu, E., Tian, W., and Wolmark, N. 1999. Further evaluation of Intensified and increased total dose of cyclophosphamide for the treatment of primary breast cancer. National Surgical Adjuvant Breast and Bowel Project B-25. *J. Clin. Oncol. 17*: 3374–3388.

Green, M.C., Buzdar, A.U., Smith, T., Ibrahim, N.K., Valero, V., Rosales, M.F., Cristofanilli, M., Booser, D.J., Pusztai, L., Rivera, E., Theriault, R.L., Carter, C., Frye, D., Hunt, K.K., Symmans, W.F., Strom, E.A., Sahin, A.A., Sikov, W., and Hortobagyi, GN. 2005. Weekly paclitaxel improves pathologic complete remission in operable breast cancer when compared with paclitaxel once every 3 weeks. *J. Clin. Oncol. 23*: 5983–5992.

Henderson, I.C., Berry, D.A., Demetri, G.D., Cirrincione, C.T., Goldstein, L.J., Martino, S., Ingle, J.N., Cooper, M.R., Hayes, D.F., Tkaczuk, K.H., Fleming, G., Holland, J.F., Duggan, D.B., Carpenter, J.T., Frei, E., Schilsky, R.L., Wood, W.C, Muss, H.B., and Norton, L. 2003. Improved outcomes from adding sequential paclitaxel but not from escalating doxorubicin dose in an adjuvant chemotherapy regimen for patients with node-positive primary breast cancer. *J. Clin. Oncol. 21*: 976–983.

Hudis, C., Citron, M., and Berry, D. 2005. Five-year follow-up of INT C9741: dose-dense (DD) chemotherapy (CRx) is safe and effective. *Proc. San Antonio Breast Cancer Symp*. abstr. 41.

Kümmel, S., Krocker, J., Kohls, A., Breitbach, G.P., Morack, G., Budner, M., Blohmer, J.U., and Elling, D. 2006. Randomized trial: survival benefit and safety of adjuvant dose-dense chemotherapy for node-positive breast cancer. *Br. J. Cancer 94*: 1237–1244.

Moebus, V.J., Lueck, H.J., Thomssen, C., Kuhn, W., Kurbacher, C., Nitz, U., Kreienberg, R., Untch, M., Jackisch, C., Schneeweiss, A., Huober, J., and du Bois, A. 2006. Dose-dense sequential chemotherapy with epirubicin (E), paclitaxel (T) and cyclophosphamide (C) (ETC) in comparison to conventional dosed chemotherapy in high-risk breast cancer patients (> 4+LN). Mature results of an AGO-trial. *Proc. San Antonio Breast Cancer Symp*. abstr.43.

Moore, H.C., Green, S.J., Gralow, J.R., Bearman, S.I., Lew, D., Barlow, W.E., Hudis, C., Wolff, A.C., Ingle, J.N., Chew, H.K., Elias, A.D., Livingston, R.B., and Martino, S. 2007. Southwest Oncology Group/ Intergroup Study 9623. Intensive dose-dense compared with high-dose adjuvant chemotherapy for high-risk operable breast cancer: Southwest Oncology Group/ Intergroup study 9623. *J. Clin. Oncol. 25*: 1677–1682.

Norton, L. 1988. Gompertzian model of human breast cancer growth. *Cancer Res. 48*: 7067–7071.

Norton, L., and Simon, R. 1986. The Norton-Simon hypothesis revisited. *Cancer Treat. Rep. 70*: 163–169.

Skipper, H.E., Schabel, F.M., and Wilcox, W.S. 1964. Experimental evaluation of potential anticancer agents XIII. On the criteria and kinetics associated with "curability" of experimental leukemia. *Cancer Chemother. Rep. 35*: 1.

Sparano, J.A., Wang, M., Martino, S., Jones, V., Perez, E., Saphner, T., Wolff, A.C., Sledge, G.W., Wood, W.C., and Davidson, N.E. 2007. Phase III study of doxorubicin-cyclophosphamide followed by paclitaxel or docetaxel given every 3 weeks or weekly in operable breast cancer: results of Intergroup Trial E 1199. *J. Clin. Oncol. 25*: 516.

Therasse, P., Mausiac, L., Welnicka-Jaskiewicz, M., Bruning, P., Cufer, T., Bonnefoi, H., Tomiak, E., Pritchard, K.I., Hamilton, A., and Piccart, M.J. 2003. EORTC: final results of a randomized phase III trial comparing cyclophosphamide, epirubicin and fluorouracil with a dose-intensified epirubicin and cyclophosphamide + filgrastim as neoadjuvant treatment in locally advanced

breast cancer: an EORTC-NCIC-SAKK multicenter study. *J. Clin. Oncol. 21*: 843–850.

Untch M., Konecny G., and Ditsch N. 2002. Dose-dense sequential epirubicin/paclitaxel as preoperative treatment of breast cancer: results of a randomized AGO study. *Proc. Am. Soc. Clin. Oncol. 21*: 133.

Venturini, M., Del Mastro, L., Aitini, E., Baldini, E., Caroti, C., Contu, A., Testore, F., Brema, F., Pronzato, P., Cavazzini, G., Sertoli, M.R., Canavese, G., Rosso, R., and Bruzzi, P. 2005. Dose-dense adjuvant chemotherapy in early breast cancer patients: results from a randomized trial. *J. Natl. Cancer Inst. 97*: 1724–1733.

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Advanced Breast Cancer: Treatment with Docetaxel/Epirubicin

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INTRODUCTION

Metastatic breast cancer (MBC) remains a fatal disease despite the great amount of research performed in recent years and the progress achieved. The median survival of patients with MBC is ~ 18–24 months after the initial diagnosis of metastases (Dickson *et al*., 2005). Hence, the principal goals of treatment are palliation of symptoms and prolongation of survival while maintaining or improving the quality of life. Many drugs have been approved for the treatment of MBC and among them the taxanes and anthracyclines represent the two major chemotherapy classes commonly used in daily practice.

EPIRUBICIN

Anthracyclines are considered to be the most active drugs in the treatment of MBC. Single agent doxorubicin has significant activity against MBC, with overall response rates (ORR) ranging from 35% to 50%, in chemotherapy-naïve patients (Ellis *et al*., 2000). Until recently, doxorubicin was one of the most active single agents available against MBC and doxorubicin-containing regimens were considered the "standard" of care in first and second line treatments (A'Hern *et al*., 1993). Epirubicin, another anthracycline agent, has similar single agent activity with doxorubicin as first-line treatment in MBC with response rates of 25–62% (Launchbury and Habboubi, 1993); but its toxicity profile is more favorable, especially in terms of cardiotoxicity, compared to doxorubicin (Ganzina, 1983; Launchbury and Habboubi, 1993). Due to its faster elimination leading to a reduced area under the curve than equimolar doses of doxorubicin, epirubicin can be given at higher doses (120–180 mg/m^2 every 3 weeks) (Feld *et al*., 1992) and with a dose-response curve favoring dose intensification (Bastholt *et al*., 1996; Brufman *et al*., 1997).

DOCETAXEL

The taxanes, paclitaxel, and docetaxel, exert their antineoplastic properties by promoting the *in vitro* assembly of stable

microtubules in the absence of guanosine triphosphate and thus, inducing microtubule-bundle formation inside the cells (Rowinsky, 1997). Paclitaxel was the first taxane proven to be active against MBC (Holmes *et al*., 1991; Nabholtz *et al*., 1996). Docetaxel is a semi-synthetic taxane derived from 10-deacetyl bacatin III (Gueritte-Voegelein *et al*., 1991) and was developed later. Several clinical phase II and III trials reported significant activity as 1st- and 2nd-line therapy in MBC, as well as in patients previously exposed or resistant to anthracyclines (Chan *et al*., 1999; Crown, 2001; Nabholtz *et al*., 1999). As a single agent, docetaxel has achieved response rates of 54–68% in previously untreated patients (Cortes and Pazdur, 1995) and 41% in patients with anthracycline-resistant disease (Ravdin, 1997), suggesting that there is no significant cross resistance with anthracyclines. Moreover, docetaxel is active even in patients with paclitaxel-refractory disease (Valero *et al*., 1998). In randomized phase III studies, docetaxel, but not paclitaxel, was more active than doxorubicin in patients with MBC (Chan *et al*., 1999; Paridaens *et al*., 2000). Furthermore, docetaxel seems to be less cardiotoxic than paclitaxel (Verweij *et al*., 1994). Taken together, these data indicate that docetaxel is a highly active chemotherapeutic agent for the treatment of MBC.

ANTHRACYCLINE-TAXANE COMBINATION

Given that both taxanes and anthracyclines lack cross resistance and are both highly active agents against MBC, their combined use is a logical step. A randomized phase III Intergroup trial, evaluating doxorubicin (60 mg/m^2) versus paclitaxel (175 mg/m^2) versus doxorubicin/paclitaxel combination (50/150 mg/m^2), reported a higher response rate in favor of the doxorubicin/paclitaxel doublet. Response rate was 36% for doxorubicin, 34% for paclitaxel, and 47% for the combination ($p = 0.84$ for doxorubicin vs paclitaxel, $p = 0.007$ for doxorubicin vs doxorubicin/paclitaxel, $p = 0.004$ for paclitaxel vs doxorubicin/paclitaxel combination). However, no significant difference regarding overall survival and quality of life was reported. Patients receiving single agent doxorubicin or paclitaxel crossed over to the other agent, and this may have diluted any survival benefit (Sledge *et al*., 2003). Furthermore, the paclitaxel/doxorubicin combination has been associated with a high incidence of cardiotoxicity (Gehl *et al*., 1996).

On the contrary, docetaxel has not been associated with cardiotoxicity (Verweij *et al*., 1994). Doxorubicin has also been combined with docetaxel in the setting of randomized phase III trials. TAX 306 randomized 429 patients to doxorubicin/docetaxel (50/75 mg/m^2) versus doxorubicin/cyclophosphamide (50/600 mg/m^2) combinations (Nabholtz *et al*., 2003). Time to tumor progression (TTP) was the primary end point. Doxorubicin/docetaxel doublet was more active than doxorubicin/cyclophosphamide in terms of response rate (59% vs 47%, $p = 0.009$) and TTP (median TTP, 37.3 v 31.9 weeks; log-rank $p = 0.014$); however, overall survival (OS) was similar in the two arms. Given the significant activity of the docetaxel/doxorubicin combination in MBC and the fact that epirubicin has similar single agent activity with doxorubicin although

with a more favorable toxicity profile, the docetaxel/epirubicin combination is a logical doublet to study.

Docetaxel-Epirubicin Combination

Phase I studies

Several phase I studies have evaluated the maximum tolerated doses (MTD) and the dose limiting toxicities (DLT) of docetaxel in combination with epirubicin. First, the Greek Breast Cancer Cooperative group (GBCCG) enrolled 47 chemotherapy-naïve patients in a phase I study, to determine the MTD and the DLT of the Docetaxel/Epirubicin doublet (Kouroussis *et al*., 1999). Docetaxel was given as a 1-h infusion after appropriate premedication on either day 1 or 2 in escalated doses with increments of 10 mg/m^2. Epirubicin was given first as a 5-min bolus i.v., infusion on day 1 in escalated doses with increments of 10 mg/m^2. When the two drugs were given on the same day, the MTD was reached at the doses of Epirubicin 60 mg/m^2 and Docetaxel 80 mg/m^2; administration of prophylactic G-CSF could not result in further dose intensification. When the drugs were given on two consecutive days, the MTD2 was reached at the doses of Epirubicin 80 mg/m^2 (d1) and Docetaxel 90 mg/m^2 (d2). The dose-limiting events were febrile neutropenia and grade 4 neutropenia, which developed in 30 (64%) patients during the study; among 227 delivered cycles, grade 3–4 neutropenia occurred in 64 (28%) cycles but only 22 (10%) of them were complicated by fever. There were no septic deaths. Grade 1–2 neurosensory toxicity occurred in nine (19%) patients. Four (9%) patients presented a greater than 10% decrease of LVEF and treatment discontinuation was required in two of them. However, none of the patients developed congestive heart failure. Nevertheless, one patient suddenly died 10 days after treatment initiation of myocardial ischemia, and this death was considered treatment-related. Regarding efficacy, five (14.7%) complete and thirteen (38.2%) partial responses (ORR: 53.9%; 95% confidence interval: 36.1–69.7%) were observed in 34 evaluable patients.

At the same time an Italian group (Pagani *et al*., 1999) conducted a dose-finding study to determine the MTD of the combination with or without granulocyte-colony stimulating factor (G-CSF) support. Forty-two patients who had previously received neither palliative chemotherapy nor adjuvant anthracyclines, were treated on four dose escalating levels with Epirubicin 75–120mg/m^2 and Docetaxel 75–85 mg/m^2 given on the same day with epirubicin administered first. Cardiac toxicity was monitored at baseline and after every second course by echocardiography. Febrile neutropenia and prolonged, severe neutropenia (absolute neutrophil count (ANC) $< 0.1 \times 10^9$/l for more than 3 days) were the DLT. The MTD of the combination without G-CSF support was Epirubicin 90 mg/m^2 and Docetaxel 75 mg/m^2. With the subsequent administration of G-CSF, the MTD was established at Epirubicin 120 mg/m^2 and Docetaxel 85 mg/m^2. No severe neurotoxicity, mucositis, or fluid retention were observed and there were no clinical signs of cardiotoxicity. The overall response rate in 40 evaluable patients was 60% (95% CI: 43–75%) with no apparent dose-response effect.

A third study published later by Venturini *et al*. recommended for future

phase II studies the Epirubicin 75 mg/m^2 and docetaxel 80 mg/m^2 combination (Venturini *et al*., 2001). Fifty-eight women with locally advanced or metastatic breast cancer were included in that study. Docetaxel administration was started at 60 mg/m^2 with escalated increments of 10 mg/m^2, in association with two fixed doses of epirubicin (90 mg/m^2, and 75 mg/m^2). The authors also studied a third group with prophylactic G-CSF support in order to determine the MTD of docetaxel in combination with a fixed dose of 90 mg/m^2 of epirubicin. In the first group, the MTD was docetaxel 60 mg/m^2 and epirubicin 90 mg/m^2. Dose limiting toxicities were neutropenia, febrile neutropenia, while there was one toxic death. In the second group (75 mg/m^2 of epirubicin) the MTD for docetaxel was 80 mg/m^2. Neutropenia and febrile neutropenia were again the DLTs, while one patient developed grade III mucositis. In the third group (epirubicin 90 mg/m^2) with G-CSF administration, docetaxel was escalated up to 90 mg/m^2. DLTs were febrile neutropenia and grade III myalgia. Most frequent non-hematological adverse effects were asthenia (45%), nausea (39%) and mucositis (36%). No patient developed congestive heart failure. Two toxic deaths occurred. Overall response rate was 73% (42 out of 58 patients) with no apparent epirubicin dose-response effect.

Finally, a study by Viens *et al*. (2001) included 27 women with MBC having measurable and/or evaluable disease. Epirubicin was escalated from 60 to 110 mg/m^2 according to five different dose levels, in combination with a fixed dose of 75 mg/m^2 docetaxel. Dose-limiting toxicities consisted of grade III asthenia and febrile neutropenia (epirubicin 75 mg/m^2), grade IV thrombocytopenia and grade III asthenia (epirubicin 90 mg/m^2), grade IV stomatitis and grade III diarrhea (epirubicin 100 mg/m^2), and grade III diarrhea (epirubicin 110 mg/m^2). In three patients a decrease of left ventricular ejection was observed, which normalized during follow-up. Based on the above data, the recommended doses were epirubicin 100 mg/m^2 epirubicin and 75 mg/m^2 docetaxel.

TOXICITY OF THE DOCETAXEL-EPIRUBICIN COMBINATION

In terms of toxicity and safety, the phase I studies cited above (Kouroussis *et al*., 1999; Pagani *et al*., 1999; Venturini *et al*., 2001; Viens *et al*., 2001) indicated that the major toxicity of the combination was haematological. Neutropenia and its consequences were the main toxicities associated with the combination. Approximately, 28–87% of chemotherapy cycles were complicated with grade III–IV neutropenia. However, febrile neutropenia was less frequent and septic deaths were rare. Secondly, non-hematological toxicities were relatively mild. The most common non-hematological toxicities were asthenia, mucositis, and diarrhea. Thirdly, and perhaps most importantly, the docetaxel/epirubicin combination did not result in any significant increase in anthracycline cardiotoxicity.

This was further confirmed by a Finnish study (Salminen *et al*., 2003). The aim of that study was to evaluate clinical and subclinical cardiac toxicity of docetaxel/epirubicin combination. Previously untreated breast cancer patients were given epirubicin (75 mg/m^2 for 15 min), followed

1 h later by a 1-h infusion of docetaxel (75 mg/m^2). Cardiac function was monitored using a 24-h ambulatory electrocardiogram (ECG), left ventricular ejection fraction (LVEF), physical examination, and chest radiography. The median LVEF did not decrease during the course of the treatment: pretreatment median LVEF was 64% prior to treatment and 68% after cycle 8, while the 24-h ECG did not reveal any considerable changes in heart rate variability. Furthermore, the number of extrasystoles or cardiac arrhythmias did not increase with the epirubicin-docetaxel treatment. No patient experienced congestive heart failure during treatment or after a mean follow-up of 34 months.

PHARMACOKINETIC DATA

In a phase I/II study of the paclitaxel with epirubicin combination, it was observed that the pharmacokinetics of paclitaxel were not modified by the administration of epirubicin; on the contrary, the metabolism of epirubicin was affected, with a reduction of epirubicinol levels as the paclitaxel dose increased (Conte *et al*., 1997). This observation justified the evaluation of pharmacokinetic interactions in the docetaxel/epirubicin combination. In order to study these interactions, Ceruti *et al*. (1999) administered epirubicin (75 mg/m^2) and docetaxel (75 mg/m^2) to 16 patients with MBC according to two different schedules: (1) docetaxel as infusion given 1 h after epirubicin administration (schedule A); and (2) docetaxel as infusion given immediately (10 min) after the end of epirubicin i.v., bolus administration (schedule B). The conclusion was that a significant increase in epirubicin clearance was seen when moving from schedule A to schedule B. The difference in docetaxel clearance was less evident and statistically non significant.

1. Phase II studies of the Docetaxel/Epirubicin combination

Based on the encouraging results of phase I studies, the docetaxel/epirubicin combination was evaluated in phase II studies. The phase I study by the GBCCG, was further expanded into a multicenter phase II study. Fifty four women with advanced breast cancer (stage IIIB/IV) were treated with epirubicin (70 mg/m^2, day 1) and docetaxel (90 mg/m^2, day 2), as first line treatment (Mavroudis *et al*., 2000). The median age of patients was 55 years, while the vast majority (91%) had performance status of 0–1. In an intent to treat analysis, the overall response rate (ORR) was 66% (95% confidence interval 54–79%), with five patients (9%) achieving complete response (CR) and 31 (57%) partial response (PR). Stable disease (SD) was observed in nine (17%) and progressive disease (PD) in nine (17%) patients. After a median follow-up period of 11.5 months, the authors reported a median duration of response of 8 months, a median TTP of 11.5 months, while the median overall survival (OS) had not been reached at the time of publication of that study. The probability of 1-year survival was calculated at 65%. The major haematological toxicity was grade III/IV neutropenia, which was observed in eight (15%) and 31 (57%) patients, respectively. Febrile neutropenia, was also common, occurring in 19 (35%) patients; however, it was always successfully treated with intravenous antibiotics.

Prophylactic G-CSF was used in 45 (83%) patients, or 226 (74%) cycles. The major non-hematological toxicity was grade III and IV diarrhea, occurring in four (7%) and one (2%) patients, respectively. All other toxicities were generally mild. Five patients (9%) presented a more than 10% decrease of LVEF during treatment; however, none of the patients developed congestive heart failure or had to stop therapy due to cardiotoxicity. During treatment there were two deaths, due to respiratory insufficiency, without associated neutropenia. The authors considered those deaths possibly treatment-related either due to the immunosuppressive properties of the regimen, or due to a probable pulmonary toxicity of the combination (Mavroudis *et al*., 2000).

A second phase II study of the combination was reported by Milla-Santos *et al*. (2001). They used high dose epirubicin (130 mg/m^2, day 1) with docetaxel administered 1 h following epirubicin at a dose of 100 mg/m^2, with prophylactic administration of G-CSF on days 4–13. A total of 32 patients were included in the study and 236 chemotherapy cycles were administered. The ORR was 87.5% (95% confidence interval 77–98) with 11 (34.4%) patients achieving a CR and 17 (53.1%) patients with PR. The major toxicity was neutropenia (2.9% of cycles were delayed 3–6 days because of neutropenia) despite the prophylactic administration of G-CSF. After a median follow-up of 490 days, the authors reported a median TTP of 490 days and a median OS of 604 days. The significantly higher response rate yielded in this study, compared with the above mentioned Greek study, could be attributed to the higher epirubicin dose used. A clear dose-response relationship for single agent epirubicin (up to a dose of 90 mg/m^2) has been shown in postmenopausal women with MBC (Bastholt *et al*., 1996). Furthermore, doubling of the epirubicin dose intensity (100 mg/m^2 versus 50 mg/m^2) in the FEC regimen, significantly increased the complete and overall response rates but not the overall survival, especially in patients with visceral metastases or multiple metastatic organ sites (Brufman *et al*., 1997).

In an Italian phase I–II, the docetaxel/epirubicin combination was administered (in the phase II part) at the doses of 75 mg/m^2 and 90 mg/m^2, respectively. A total of 70 patients were included in both parts of the study (Pagani *et al*., 2000). The ORR in 68 evaluable patients was 66% (95% confidence interval: 54–73%). After a median follow-up time of 22 months (range 4–39+), the median TTP was 4.5 months and the median duration of response was 8 months (range 3–16).

Another phase II study including 38 women with MBC was reported by a Finnish group (Salminen *et al*., 2002). This study used a regimen of epirubicin (75 mg/m^2) and docetaxel (75 mg/m^2), both drugs administered on day 1. The ORR reported 54% (95% confidence interval 37–71), with a median duration of response of 14.8 months (95% confidence intervals 8.8–27.8). Median TTP was 12 months and median OS 26 months. Neutropenia grade IV was observed in 113 (39%) of the 285 chemotherapy cycles administered; 21 patients were hospitalized due to febrile neutropenia. The authors' conclusion was that epirubicin/docetaxel regimen needed further dose reduction and tailoring in order to avoid the high incidence of grade IV neutropenia.

The same regimen (epirubicin 75 mg/m^2 and docetaxel 75 mg/m^2) was used in a

large phase II study reported by Morales *et al*. (2004), which included 133 patients with MBC. This study also reported a high ORR of 67%, with an impressive CR rate of 23%. The median TTP was 10.8 months (95% confidence interval: 9.7–12.6) and the median OS was 19.5 months. The major toxicity was grade III/IV neutropenia which occurred in 35%, while febrile neutropenia was observed in 19% of patients. Granulocyte colony-stimulating factor support was administered to 32% of patients and in 22% of cycles. The most frequent grade 3/4 non-hematological toxicities were asthenia (6%), vomiting (5%) and nausea (5%). No patients developed congestive heart failure.

An Italian group used higher doses of both drugs in a small phase II study of 25 patients with MBC (Fabi *et al*., 2004). Patients were treated with the combination of epirubicin 90 mg/m^2 plus docetaxel 90 mg/m^2, with prophylactic G-CSF administration. Overall response rate was 79%, with 21% of these patients achieving CR. The median response duration was 10 months (range: 3–16). The main toxicity was grade III/IV neutropenia (41% of cycles) regardless of the use of G-CSF; while febrile neutropenia was observed in 14% of cycles necessitating a dose reduction of both drugs in 30% of patients. The median TTP was 11 months and the overall 3-year survival was 49.7%. Despite the use of higher doses, the ORR observed in this series was comparable with that seen in other studies of epirubicin/docetaxel combination. However, as the authors comment, the degree of myelosuppression was severe, despite the prophylactic administration of G-CSF, and therefore, they recommend a lower dose of both drugs.

Finally, the Minnie Pearl Cancer Research Network reported a small phase II study (Hainsworth *et al*., 2006). Thirty patients with MBC were treated with docetaxel 60 mg/m^2 and epirubicin 90 mg/m^2 as first line treatment; both drugs were repeated at 21-day intervals. Objective responses were observed in 50%; an additional 20% of patients had stable disease of more than 6 months duration. The median and 2-year progression-free survival (PFS) was 12 months and the 2-year PFS rate 34%. The median survival was 18 months and the 2-year overall survival rate 42%. Myelosuppression was the most common grade III/IV toxicity, with two (6%) treatment-related deaths due to sepsis.

Based on the high activity of the docetaxel/epirubicin combination reported in the above mentioned studies, Bonneterre *et al*. (2004) conducted a multicenter randomized phase II study in order to compare the efficacy and safety of docetaxel plus epirubicin (ET) combination versus the 5-fluorouracil plus epirubicin and cyclophosphamide (FEC) regimen as first-line chemotherapy for MBC. A total of 142 patients were randomised to receive either docetaxel 75 mg/m^2 plus epirubicin 75 mg/m^2 or 5-fluorouracil 500 mg/m^2 plus epirubicin 75 mg/m^2 and cyclophosphamide 500 mg/m^2, once every 3 weeks for up to eight cycles. Prophylactic granulocyte-colony-stimulating factor was only permitted after the first cycle, if required. In an intent-to-treat analysis, the ORR for docetaxel plus epirubicin combination was 59% (95% CI, 47–70%) and for FEC 32% (95% CI, 21–43%) after a median of seven and six cycles, respectively. The median response duration for ET was 8.6 months (95% CI, 7.2–9.6 months) and for FEC 7.8 months (95% CI, 6.5–10.4 months).

The median TTP for docetaxel plus epirubicin combination was 7.8 months (95% CI, 5.8–9.6 months) and for FEC 5.9 months (95% CI, 4.6–7.8 months). After a median follow-up period of 23.8 months, median OS for docetaxel plus epirubicin and FEC combinations were 34 and 28 months, respectively. Nonhaematologic grade 3–4 toxicities were infrequent in both arms. Hematologic toxicity was more common with ET combination and febrile neutropenia was reported in 13 patients (18.6%) in that group. Two deaths in the docetaxel plus epirubicin group were possibly related to study treatment. The authors concluded that the toxicity of both arms was acceptable, while the taxane/anthracycline combination was significantly more active.

The above mentioned studies confirmed that the docetaxel/epirubicin combination is a highly active regimen as first line treatment of patients with MBC, with observed RR from 50% to 87.5% (Fabi *et al*., 2004; Hainsworth *et al*., 2006; Mavroudis *et al*., 2000; Milla-Santos *et al*., 2001; Morales *et al*., 2004; Pagani *et al*., 2000; Salminen *et al*., 2002). The doses used ranged from 60 mg/m^2 (Hainsworth *et al*., 2006) to 100 mg/m^2 (Milla-Santos *et al*., 2001) for docetaxel and from 70 mg/m^2 (Mavroudis *et al*., 2000) to 130 mg/m^2 (Milla-Santos *et al*., 2001) for epirubicin. Despite the previously reported dose-response relationship for single agent epirubicin (up to a dose of 90 mg/m^2) (Bastholt *et al*., 1996), and the significantly increased ORR by doubling the epirubicin dose (100 mg/m^2 versus 50 mg/m^2) in the FEC regimen (Brufman *et al*., 1997), there were no major differences regarding ORR in the above mentioned studies, using different dose intensities for both drugs. The only exception was the Spanish study, which used the higher doses of both drugs (docetaxel 100 mg/m^2 and epirubicin 130 mg/m^2) (Milla-Santos *et al*., 2001), and reported a high ORR of 87.5%, with 34.3% CR.

The most frequently reported toxicity for the docetaxel-epirubicin combination was neutropenia, as observed in both phase I and II studies. However, febrile neutropenia was much less frequent and septic deaths were rare. All other toxicities were, in general, mild and easily manageable. An interesting observation regarding a higher incidence of central nervous system (CNS) involvement in patients treated with docetaxel/epirubicin was reported by an Italian group, based on a pooled analysis of their phase I and II studies (Pagani *et al*., 1999, 2000). A total of 92 patients were included in these two studies and the authors reported that 28 (30%) of the 92 patients treated with this combination developed CNS metastases; 25 patients developed cerebral metastasis, two leptomeningeal, and one both (Crivellari *et al*., 2001). Median time for the development of CNS metastases from the start of chemotherapy was 15 months (range 5–42), when the six patients presenting CNS progression within 3 months from start of treatment were excluded. It is noteworthy that 11 patients (39%) had disease progression only in the CNS. Although, this observation could be easily explained by the sanctuary site 'hypothesis', as a consequence of an intact blood-brain barrier, this is not proven and the exact explanation remains to be elucidated. The authors conclude that as anthracycline- and taxane-containing regimens are increasingly used both in the metastatic and in the adjuvant setting, a careful monitoring of any neurological symptoms should be advisable.

Taken together, the results of all aforementioned reports clearly indicate that the docetaxel/epirubicin combination is very effective and with manageable toxicity and therefore merits further evaluation in the context of phase III randomized studies. These studies should compare this regimen with other taxane-anthracycline combinations or "standard" anthracycline-based therapies.

REFERENCES

A'Hern, R.P., Smith, I.E., and Ebbs, S.R. 1993. Chemotherapy and survival in advanced breast cancer: the inclusion of doxorubicin in Cooper type regimens. *Br. J. Cancer 67*: 801–805.

Bastholt, L., Dalmark, M., Gjedde, S.B., Pfeiffer, P., Pedersen, D., Sandberg, E., Kjaer, M., Mouridsen, H.T., Rose, C., Nielsen, O.S., Jakobsen, P., and Bentzen, S.M. 1996. Dose-response relationship of epirubicin in the treatment of postmenopausal patients with metastatic breast cancer: a randomized study of epirubicin at four different dose levels performed by the Danish Breast Cancer Cooperative Group. *J. Clin. Oncol. 14*: 1146–1155.

Bonneterre, J., Dieras, V., Tubiana-Hulin, M., Bougnoux, P., Bonneterre, M.E., Delozier, T., Mayer, F., Culine, S., Dohoulou, N., and Bendahmane, B. 2004. Phase II multicentre randomised study of docetaxel plus epirubicin vs 5-fluorouracil plus epirubicin and cyclophosphamide in metastatic breast cancer. *Br. J. Cancer 91*: 1466–1471.

Brufman, G., Colajori, E., Ghilezan, N., Lassus, M., Martoni, A., Perevodchikova, N., Tosello, C., Viaro, D., and Zielinski, C. 1997. Doubling epirubicin dose intensity (100 mg/m2 versus 50 mg/m2) in the FEC regimen significantly increases response rates. An international randomised phase III study in metastatic breast cancer. The Epirubicin High Dose (HEPI 010) Study Group. *Ann. Oncol. 8*: 155–162.

Ceruti, M., Tagini, V., Recalenda, V., Arpicco, S., Cattel, L., Airoldi, M., and Bumma, C. 1999. Docetaxel in combination with epirubicin in metastatic breast cancer: pharmacokinetic interactions. *Farmaco 54*: 733–739.

Chan, S., Friedrichs, K., Noel, D., Pinter, T., VanBelle, S., Vorobiof, D., Duarte, R., Gil, G.M., Bodrogi, I., Murray, E., Yelle, L., von Minckwitz, G., Korec, S., Simmonds, P., Buzzi, F., Gonzalez, M.R., Richardson, G., Walpole, E., Ronzoni, M., Murawsky, M., Alakl, M., Riva, A., and Crown, J. 1999. Prospective randomized trial of docetaxel versus doxorubicin in patients with metastatic breast cancer. *J. Clin. Oncol. 17*: 2341–2354.

Conte, P.F., Baldini, E., Gennari, A., Michelotti, A., Salvadori, B., Tibaldi, C., Danesi, R., Innocenti, F., Gentile, A., Dell'Anna, R., Biadi, O., Mariani, M., and DelTacca, M. 1997. Dose-finding study and pharmacokinetics of epirubicin and paclitaxel over 3 hours: a regimen with high activity and low cardiotoxicity in advanced breast cancer. *J. Clin. Oncol. 15*: 2510–2517.

Cortes, J.E., and Pazdur, R. 1995. Docetaxel. *J. Clin. Oncol. 13*: 2643–2655.

Crivellari, D., Pagani, O., Veronesi, A., Lombardi, D., Nole, F., Thurlimann, B., Hess, D., Borner, M., Bauer, J., Martinelli, G., Graffeo, R., Sessa, C., and Goldhirsch, A. 2001. High incidence of central nervous system involvement in patients with metastatic or locally advanced breast cancer treated with epirubicin and docetaxel. *Ann. Oncol. 12*: 353–356.

Crown, J. 2001. Docetaxel: overview of an active drug for breast cancer. *Oncologist 6*: 1–4.

Dickson, R.B., Pestell, R.G., and Lippman, M.E. 2005. Cancer of the Breast. in: Principles and Practice of Oncology, DeVita, V.T., Hellman, S., and Rosenberg, S.A. Eds Lippincott Williams & Wilkins, Philadelphia, 7th Edition: 1399–1487.

Ellis, M.J., Hayes, D.F., and Lippman, M.E. 2000. Treatment of metastatic breast cancer. in: Diseases of the Breast, Lippman, M.E., Morrow, M., and Osborne, C.K., Eds Lippincott, Williams & Willkins, Philadelphia: 749–797.

Fabi, A., Papaldo, P., Pino, M.S., Ferretti, G., Carlini, P., Pacetti, U., Di Cosimo, S., Nardoni, C., Giannarelli, D., Sacchi, I., and Cognetti, F. 2004. Epirubicin plus docetaxel in metastatic breast cancer: escalating dose does not improve efficacy. A phase II study. *Anticancer Res. 24*: 1963–1967.

Feld, R., Wierzbicki, R., Walde, P.L., Shepherd, F.A., Evans, W.K., Gupta, S., Shannon, P., and Lassus, M. 1992. Phase I-II study of high-dose epirubicin

in advanced non-small-cell lung cancer. *J. Clin. Oncol. 10*: 297–303.

Ganzina, F. 1983. 4´-epi-doxorubicin, a new analogue of doxorubicin: a preliminary overview of preclinical and clinical data. *Cancer Treat. Rev. 10*: 1–22.

Gehl, J., Boesgaard, M., Paaske, T., Vittrup, J.B., and Dombernowsky, P. 1996. Combined doxorubicin and paclitaxel in advanced breast cancer: effective and cardiotoxic. *Ann. Oncol. 7*: 687–693.

Gueritte-Voegelein, F., Guenard, D., Lavelle, F., Le Goff, M.T., Mangatal, L., and Potier, P. 1991. Relationships between the structure of taxol analogues and their antimitotic activity. *J. Med. Chem. 34*: 992–998.

Hainsworth, J.D., Yardley, D.A., Spigel, D.R., Meluch, A.A., Rinaldi, D., Schnell, F.M., and Greco, F.A. 2006. Docetaxel and epirubicin as first-line treatment for patients with metastatic breast cancer: a Minnie Pearl Cancer Research Network Phase II trial. *Cancer Invest. 24*: 469–473.

Holmes, F.A., Walters, R.S., Theriault, R.L., Forman, A.D., Newton, L.K., Raber, M.N., Buzdar, A.U., Frye, D.K., and Hortobagyi, G.N. 1991. Phase II trial of taxol, an active drug in the treatment of metastatic breast cancer. *J. Natl. Cancer Inst. 83*: 1797–1805.

Kouroussis, C., Xydakis, E., Potamianou, A., Giannakakis, T., Kakolyris, S., Agelaki, S., Sara, E., Malamos, N., Alexopoulos, A., Mavroudis, D., Samonis, G., Papadouris, S., Georgoulias, V., and Panagos, G. 1999. Front-line treatment of metastatic breast cancer with docetaxel and epirubicin: a multicenter dose-escalation study. The Greek Breast Cancer Cooperative Group (GBCCG). *Ann. Oncol. 10*: 547–552.

Launchbury, A.P., and Habboubi, N. 1993. Epirubicin and doxorubicin: a comparison of their characteristics, therapeutic activity and toxicity. *Cancer Treat. Rev. 19*: 197–228.

Mavroudis, D., Alexopoulos, A., Ziras, N., Malamos, N., Kouroussis, C., Kakolyris, S., Agelaki, S., Kalbakis, K., Tsavaris, N., Potamianou, A., Rigatos, G., and Georgoulias, V. 2000. Front-line treatment of advanced breast cancer with docetaxel and epirubicin: a multicenter phase II study. *Ann. Oncol. 11*: 1249–1254.

Milla-Santos, A., Milla, L., Rallo, L., and Solano, V. 2001. High-dose epirubicin plus docetaxel at standard dose with lenograstim support as first-line therapy in advanced breast cancer. *Am. J. Clin. Oncol. 24*: 138–142.

Morales, S., Lorenzo, A., Ramos, M., Ballesteros, P., Mendez, M., Almanza, C., Castellanos, J., Moreno-Nogueira, J.A., Casal, J., Lizon, J., Oltra, A., Frau, A., Machengs, I., Galan, A., Belon, J., and Llorca, C. 2004. Docetaxel plus epirubicin is a highly active, well-tolerated, first-line chemotherapy for metastatic breast cancer: results of a large, multicentre phase II study. *Cancer Chemother. Pharmacol. 53*: 75–81.

Nabholtz, J.M., Gelmon, K., Bontenbal, M., Spielmann, M., Catimel, G., Conte, P., Klaassen, U., Namer, M., Bonneterre, J., Fumoleau, P., and Winograd, B. 1996. Multicenter, randomized comparative study of two doses of paclitaxel in patients with metastatic breast cancer. *J. Clin. Oncol. 14*: 1858–1867.

Nabholtz, J.M., Senn, H.J., Bezwoda, W.R., Melnychuk, D., Deschenes, L., Douma, J., Vandenberg, T.A., Rapoport, B., Rosso, R., Trillet-Lenoir, V., Drbal, J., Molino, A., Nortier, J.W., Richel, D.J., Nagykalnai, T., Siedlecki, P., Wilking, N., Genot, J.Y., Hupperets, P.S., Pannuti, F., Skarlos, D., Tomiak, E.M., Murawsky, M., Alakl, M., and Aapro, M. 1999. Prospective randomized trial of docetaxel versus mitomycin plus vinblastine in patients with metastatic breast cancer progressing despite previous anthracycline-containing chemotherapy. 304 Study Group. *J. Clin. Oncol. 17*: 1413–1424.

Nabholtz, J.M., Falkson, C., Campos, D., Szanto, J., Martin, M., Chan, S., Pienkowski, T., Zaluski, J., Pinter, T., Krzakowski, M., Vorobiof, D., Leonard, R., Kennedy, I., Azli, N., Murawsky, M., Riva, A., and Pouillart, P. 2003. Docetaxel and doxorubicin compared with doxorubicin and cyclophosphamide as first-line chemotherapy for metastatic breast cancer: results of a randomized, multicenter, phase III trial. *J. Clin. Oncol. 21*: 968–975.

Pagani, O., Sessa, C., Martinelli, G., Crivellari, D., Buonadonna, A., Thurlimann, B., Hess, D., Borner, M., Bauer, J., Zampino, G., Zimatore, M., Graffeo, R., Riva, A., and Goldhirsch, A. 1999. Dose-finding study of epidoxorubicin and docetaxel as first-line chemotherapy in patients with advanced breast cancer. *Ann. Oncol. 10*: 539–545.

Pagani, O., Sessa, C., Nole, F., Crivellari, D., Lombardi, D., Thurlimann, B., Hess, D., Borner, M., Bauer, J., Martinelli, G., Graffeo, R., Zucchetti, M., D'Incalci, M., and Goldhirsch, A. 2000. Epidoxorubicin and docetaxel as first-line chemotherapy in patients with advanced breast cancer: a multicentric phase I-II study. *Ann. Oncol. 11*: 985–991.

Paridaens, R., Biganzoli, L., Bruning, P., Klijn, J.G., Gamucci, T., Houston, S., Coleman, R., Schachter, J., Van Vreckem, A., Sylvester, R., Awada, A., Wildiers, J., and Piccart, M. 2000. Paclitaxel versus doxorubicin as first-line single-agent chemotherapy for metastatic breast cancer: a European Organization for Research and Treatment of Cancer Randomized Study with cross-over. *J. Clin. Oncol. 18*: 724–733.

Ravdin, P.M. 1997. Docetaxel (Taxotere) for the treatment of anthracycline-resistant breast cancer. *Semin. Oncol. 24*: 10–17.

Rowinsky, E.K. 1997. The development and clinical utility of the taxane class of antimicrotubule chemotherapy agents. *Ann. Rev. Med. 48*: 353–374.

Salminen, E., Korpela, J., Varpula, M., Asola, R., Varjo, P., Pyrhonen, S., Mali, P., Hinkka, S., and Ekholm, E. 2002. Epirubicin/docetaxel regimen in progressive breast cancer-a phase II study. *Anticancer Drugs 13*: 925–929.

Salminen, E., Syvanen, K., Korpela, J., Varpula, M., Antila, K., Varjo, P., and Ekholm, E. 2003. Docetaxel with epirubicin–investigations on cardiac safety. *Anticancer Drugs 14*: 73–77.

Sledge, G.W., Neuberg, D., Bernardo, P., Ingle, J.N., Martino, S., Rowinsky, E.K., and Wood, W.C. 2003. Phase III trial of doxorubicin, paclitaxel, and the combination of doxorubicin and paclitaxel as front-line chemotherapy for metastatic breast cancer: an intergroup trial (E1193). *J. Clin. Oncol. 21*: 588–592.

Valero, V., Jones, S.E., Von Hoff, D.D., Booser, D.J., Mennel, R.G., Ravdin, P.M., Holmes, F.A., Rahman, Z., Schottstaedt, M.W., Erban, J.K., Esparza-Guerra, L., Earhart, R.H., Hortobagyi, G.N., and Burris, H.A. III. 1998. A phase II study of docetaxel in patients with paclitaxel-resistant metastatic breast cancer. *J. Clin. Oncol. 16*: 3362–3368.

Venturini, M., Michelotti, A., Papaldo, P., Del Mastro, L., Bergaglio, M., Lionetto, R., Lunardi, G., Sguotti, C., Frevola, L., Donati, S., Rosso, R., and Cognetti, F. 2001. Identification of the highest dose of docetaxel associable with active doses of epirubicin. Results from a dose-finding study in advanced breast cancer patients. *Ann. Oncol. 12*: 1097–1106.

Verweij, J., Clavel, M., and Chevalier, B. 1994. Paclitaxel (Taxol) and docetaxel (Taxotere): not simply two of a kind. *Ann. Oncol. 5*: 495–505.

Viens, P., Roche, H., Kerbrat, P., Fumoleau, P., Guastalla, J.P., and Delozier, T. 2001. Epirubicin–docetaxel combination in first-line chemotherapy for patients with metastatic breast cancer: final results of a dose-finding and efficacy study. *Am. J. Clin. Oncol. 24*: 328–335.

28
Systemic Therapy for Breast Cancer: Using Toxicity Data to Inform Decisions

John K. Erban and Joseph Lau

INTRODUCTION

Systemic therapy for breast cancer is effective in delaying or preventing morbidity and mortality from this prevalent disease. Its history spans an unusually broad range of modalities and indications. No other area of oncology has explored so many different categories of agents or applications in the prevention and treatment of one disease. Originating with insights by surgeons that oophorectomy, adrenalectomy and hypophysectomy benefited some patients with metastatic breast cancer through hormonal mechanisms, it is now appreciated that systemic agents can treat active disease, prevent recurrence, and decrease the likelihood of ever developing breast cancer.

Initial application of systemic treatment to breast cancer involved relatively nontoxic therapies (hormonal) to patients with relatively shorter life spans, limiting the risk of harm. In the 1970s, with the advent of effective systemic chemotherapy, single agent and ultimately combination chemotherapy was introduced largely applied in unselected fashion to patients who were refractory or resistant to hormonal agents. The development of the CMF regimen (Bonadonna *et al*., 2005) was a landmark event in the treatment of breast cancer. Combination chemotherapy was proven to be effective in treatment of advanced disease. Subsequently, it was recognized that the application of effective agents to early stage disease could prevent recurrence and improve survival. The number of chemotherapeutic agents available for use to prevent recurrence (adjuvant indication) has since greatly expanded. In demonstrating an undisputed role for adjuvant treatment, the paradigm shifted from one where treatment was only applied to patients without alternatives and limited life span to a population of patients who (1) would have a significant likelihood of being cured of breast cancer, and (2) be unlikely to *personally* benefit from the intervention, even though the outcome of the population so treated would improve. Thus, balancing the potential benefits against the risk of harms is increasingly important to clinicians advising patients on treatment options in the adjuvant setting, in the prevention setting, and even

in the metastatic setting. The increasingly effective use of chemotherapeutics, hormonal therapies, and targeted molecular therapeutics has made it commonplace for patients to live years, and even decades, with metastatic breast cancer. The median survival for patients with metastatic breast cancer has improved over the past several decades from 23 months in the period from 1987 to 1993, to 29 months in the period from 1994 to 2000 as seen in an analysis conducted by Andre *et al*. (2004), and will likely improve further with the use of increasingly effective agents. Thus, it is important to recognize and characterize the potential harms of systemic therapies that are offered to patients with breast cancer.

Acute harms such as hyperemesis, febrile neutropenia and mucositis are discrete events with potentially serious consequences. However, with the introduction of effective agents to prevent nausea and vomiting, as well as growth factors to support neutrophil and red blood cell production, the important harms that influence risk/benefit analyses are those that are subacute, chronic or genotoxic. Acute harms, therefore, are not the major focus of this chapter. Rather, toxicities that are potentially irreversible, progressive, life altering or life threatening will be discussed as they often determine whether to use a particular treatment.

A discussion of therapeutic harms includes not only the toxicities of a particular therapy but also the characteristics of the patient and cointerventions (Figure 28.1). Topics considered in the following sections include: (1) types of systemic therapy for breast cancer, (2) measuring harms of systemic therapy, (3) risks of acute toxicities, and (4) long term sequelae of systemic therapy. The final two sections discuss specific examples of harms and recommendations for future investigations.

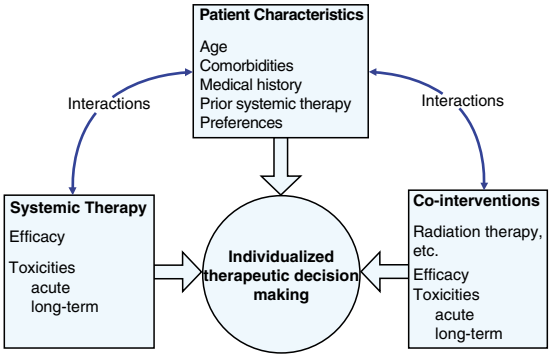

FIGURE 28.1. Factors to consider in individualizing therapeutic decision-making for patients undergoing systemic therapy for breast cancer

CATEGORIES OF SYSTEMIC THERAPY FOR BREAST CANCER

Systemic treatment for breast cancer includes several broad types that exploit a variety of cellular mechanisms that define cancer as distinct from normal tissues. In addition, systemic therapy more recently has included treatments that do not directly target the cancer cell but instead protect normal tissues from the effects of the disease. The number of treatment categories is perhaps greater than for any other type of cancer.

Hormonal Therapy

Medicine has recently entered the era of targeted and rationally designed therapy, but it is of historical interest that the origins of targeted therapy are much older.

Following the observation around 1900 that oophorectomy benefited premenopausal breast cancer patients through estrogen deprivation and the identification of estrogen receptor (ER) in the 1960s, tamoxifen ultimately became the first targeted therapy for the treatment of breast cancer. Hormonal therapy for breast cancer has been an enormous advance for over two-thirds of patients who express estrogen or progesterone receptor (PR). Although benefits of tamoxifen outweigh its toxicities, serious long term toxicities including thromboembolic disease and uterine cancer, as well as bone loss in premenopausal women exist. These are real harms that must be factored into decisions to use tamoxifen with or without ovarian ablation in the adjuvant setting and tamoxifen in the prevention setting. Despite a history of use dating to the 1970s, and a history of ovarian ablation spanning >100 years, the evaluation of the efficacy and toxicity of tamoxifen versus its combination with ovarian suppression remains unfinished. Moreover, the relative "toxicity" of estrogen deprivation at varying times in a woman's life on overall health remains uncertain. Hormonal therapies have historically included not only ER binding agents like tamoxifen or toremifene, but also progestational agents, androgens and even estrogen. More recently, the development of steroidal and nonsteroidal inhibitors of aromatase has led to clinically active agents that effectively improve disease free survival and overall survival in a variety of settings. In addition to blocking estrogen binding and prevention of its synthesis, the down regulation of estrogen receptor expression on tumor cells has produced clinical benefit. Because hormonal agents have relatively little acute toxicity, important questions remain as to how these agents may affect major processes such as cardiovascular health, bone density, and neuro-cognitive function in the long term.

Chemotherapy

The classes of chemotherapeutic agents used to treat breast disease are broad and include alkylating agents, topoisomerase II inhibitors, microtubule polymerization stabilizing or destabilizing agents, antimetabolites and others. Chemotherapeutics, for the purposes of this review, are defined as agents that may work through a variety of nonspecific mechanisms through inhibition of cell division or proliferation, DNA synthesis, or mitosis. These agents are directed at tumor cells as opposed to stromal or host cells, and there is a relative antiproliferative or proapoptotic effect on tumor cells compared to normal tissues. Thus, as a class, these agents have less mechanistic specificity. Whether this translates into greater potential for toxicity depends upon cointerventions and the nature of the particular agent.

The use of chemotherapy has included regimens with agents used singly or in combination, often with additive acute toxicities, such as neutropenia and nausea, and toxicities are increasingly expected as therapeutic agents are combined. The experience of the National Surgical Adjuvant Breast and Bowel Project in trials B22 and B25 highlights the difficulty, making predictions from prior studies or preclinical models. In two sequential trials, Fisher *et al*. (1999) escalated doses of alkylating agents to two- and fourfold the typically administered doses of the alkylating

agent cyclophosphamide (600 mg/m^2) in combination with the topoisomerase II inhibitor doxorubicin, administered at 60 mg/m^2. While both agents are recognized as having low but finite risks of inducing leukemia based upon dose and method of administration, leukemias characteristic of alkylating agents (5q-, 7q-) as well as those characteristic of topoisomerase II administration (11q23) and mixed chromosomal abnormalities were seen with increased frequency, approaching a cumulative risk of 0.87% at 4 years in one analysis, without a corresponding increase in disease free survival or overall survival (DeCillis *et al*., 1997; Smith *et al*., 2003). The problem of assessing toxicity from combination chemotherapy, used either sequentially or in parallel, has not been adequately addressed by the design of oncology clinical trials to date. Unlike most other disease models, the use of combinations of agents in varying schedules and doses remains a fundamental part of the clinical practice of oncology.

Biological and Targeted Therapeutics

Recent understanding of molecular mechanisms important in breast cancer progression and metastasis has led to the development of a number of agents that are biologically based (e.g., angiogenesis inhibitors, immunomodulating agents, signal transduction inhibitors). Based on the recent understanding of tumor initiation, growth, and metastasis, agents of this type, unlike chemotherapy, may selectively target tumor cells or host processes that participate in tumor progression. In a short time these agents have established themselves as important, meaningful, and effective therapies, as Slamon *et al*. (2001) have demonstrated in human studies of HER-2/neu over-expressing breast cancer. The addition of trastuzumab to chemotherapy improves disease free and overall survival in HER-2/neu over-expressing breast cancer. By targeting the HER-2/neu oncogene product, a transmembrane kinase whose over-expression is both prognostic for worse outcome and predictive for relative resistance to certain chemotherapeutic agents, drug resistance can be avoided. Neutralization of HER-2/neu signaling may occur via antibody therapy or by use of small molecule inhibitors such as lapatinib that target the kinase active site.

Despite binding to the same target, these two approaches may not be equally effective or toxic. Of general interest to the question of toxicity and harms, targeted therapeutics may also be relatively but not absolutely selective for tumor pathways, and the trastuzumab experience has been instructive in highlighting how difficult it has been to predict relative specificity. While Slamon *et al*. (2001) demonstrated that trastuzumab is effective in treating HER-2/neu amplified breast cancer is largely ineffective for HER-2/neu nonamplified tumors and nontoxic to normal breast tissue, trastuzumab in the adjuvant setting has demonstrated a 1–3% attributable risk of cardiac toxicity from binding to normal cardiac cells. In patients with metastatic breast cancer, trastuzumab increased cardiac toxicity when combined with anthracycline containing chemotherapy from the 8% to 27% of patients, and, when combined with paclitaxel from 1% to 13% (Slamon *et al*., 2001).

Other biological agents have specifically targeted pathways that support normal development and physiologic processes such as wound healing or embryogenesis.

The broad category of angiogenesis inhibition is one such approach based upon the relative importance of angiogenesis in tumor biology and the relative lack of angiogenic activity in normal tissues. Antiangiogenic strategies have been shown to be effective by Miller *et al*. (2005) in breast cancer, Hurwitz *et al*. (2004) in colorectal cancer, in phase III trials in advanced non-small lung cancer, and other settings. However, the requirement for some level of angiogenic activity and endothelial cell regeneration in normal tissues during the aging process suggests that chronic maintenance use in combination with several chemotherapeutic agents in stable metastatic patients, and routine use in the adjuvant setting will require careful monitoring to ultimately determine any potential for long term toxicity.

As related to toxicity monitoring, targeted therapy presents a number of fundamental problems that have not been well anticipated in current clinical trial design. First, as with tamoxifen's use in the adjuvant setting, static agents are designed for indefinite or prolonged use. Second, in the case of agents that target tumor pathways such as EGFR, mTOR or HDAC pathways, agents have been selected for their relative ability to inhibit kinase or other enzyme processes that contribute to tumor growth, yet these pathways also play important roles in normal tissue biology. Thus, chronic inhibition may induce toxicity of an indolent type. Increases in absolute specificity for one kinase in theory may diminish the risk of unintended consequences, but may be associated with diminished efficacy and utility as an anticancer drug. In the case of angiogenesis inhibition, broader inhibition of a number of VEGF and other targets to completeness may alter the balance toward more toxicity as survival pathways for normal vascular cells become increasingly affected.

Supportive Agents as Anticancer Therapy

Recent advances in the understanding of bone remodeling and the biology of metastasis have led to the investigation of bisphosphonates in metastatic and early breast cancer. Use of repeated doses of bisphosphonates has decreased pain and skeletal fracture rates in women with metastatic breast cancer (Hortobagyi *et al*., 1996). More recent studies have evaluated the role of bisphosphonates in preventing both skeletal and nonskeletal metastases as part of adjuvant treatment (Diel *et al*., 1998). While data are conflicting, the potential for prevention of both types of metastases raises the possibility that early use may become routine. Because of the extremely long terminal half life of these agents, even short term exposure requires long term toxicity monitoring relative to bone health, sequential use of anticancer therapies, and younger women who may chose to consider pregnancy following breast cancer therapy.

More recently, the worth of bisphosphonates has been evaluated as a potential solution for toxicity incurred from use of antiestrogen therapy. Both tamoxifen and aromatase inhibitors may accelerate bone mineral loss in specific circumstances. Gnant *et al*. (2007) have shown that infrequent (twice/year) bisphosphonate use can eliminate skeletal bone loss attributable to tamoxifen and aromatase inhibitors administered to premenopausal women. Optimal frequency and duration

of use remain unknown. Without studies defining such endpoints for bisphosphonates, their duration of use remains open ended. Often, protocol defined therapy for breast cancer merges with the use of similar agents for indications other than cancer such as prevention of osteoporosis. Bisphosphonate use has been associated with renal toxicity depending on the rate and mode of administration. More recently the serious complication of osteonecrosis of the jaw has caused many to examine critically optimal dosing of bisphosphonates and duration of use (Woo *et al*., 2006). Studies leading to approval of these agents for general use have not been helpful in this regard, as the agents were administered in trials monthly for up to 1–2 years in the metastatic setting, mainly intravenously. As median survival of metastatic breast cancer exceeds 2 years and includes a sizable cohort of patients that will live 5 years and a meaningful number will live 10 years or more, the potential for late toxicity is significant. Prolonged chronic use in the adjuvant setting or metastatic setting at any schedule has not been fully evaluated in controlled clinical trials.

Osteonecrosis of the jaw as a rare consequence of bisphosphonate therapy highlights the complexity of modern breast cancer study as new agents, for example, those that inhibit angiogenesis and wound healing, simultaneously advance as anticancer therapeutics. Bevacizumab, a humanized monoclonal antibody directed at VEGF-A has been shown to prolong time to progression significantly when combined with paclitaxel in advanced breast cancer, a population for whom bisphosphonates are routinely prescribed (Miller *et al*., 2005). How these agents might interact, if at all, in bone healing and remodeling is difficult to predict without properly designed studies or other methods of tracking data on patients dually treated. The interactions of antiangiogenic agents or other biological agents that may interfere with normal healing processes is difficult to test prospectively due to the structure of current trial design, the number of permutations and combinations of agents that are permissible post marketing, the lack of focus on minimal (as opposed to possible) duration of needed use, and the eligibility criteria for most registration trials. Clinical trial design and the requirements for FDA approval do not currently include a rigorous mechanism to track and measure such interactions post-marketing, and depend heavily on voluntary reporting by physicians and others once an agent enters general use.

MEASURING HARMS OF SYSTEMIC THERAPY

Traditional trial design in breast cancer has focused on sequential phase I–IV studies that historically begin by finding a maximally tolerated dose, determined exclusively by acute toxicities. Promising agents entering phase II studies are administered to a relatively small numbers of subjects for the purpose of determining activity in an advanced disease model. The conclusions of the study may strain the initial statistical considerations for the trial (Ioannidis, 2005). Neither phase I nor phase II trials are designed to assess the toxicities of agents used in oncology because of sample size.

Phase III trials of cancer therapeutics have traditionally been powered for primary endpoints such as rates of disease free survival, time to progression, response rates or overall survival. Randomized trials are heavily relied upon to influence practice and provide one source of data to estimate harms, but do not provide a complete or whole picture (Chou and Helfand, 2005). To compound the problem, the recognition that survival and longevity are increasing following systemic therapy for breast cancer has led to several important considerations:

1. The time points at which primary endpoints and toxicities are measurable are likely to be different.
2. Primary endpoints assessing disease response predictably occur earlier than late/long term toxicity.
3. The difference in time between attainment of the primary endpoint and the potential to experience toxicity is lengthening as patients live longer.
4. Primary endpoints such as disease free survival and overall survival are often reported at 2–5 years from treatment in adjuvant studies and at 6–12 months for metastatic studies given the number of subjects in a trial and the expected rate of anticipated events.
5. Studies of systemic treatment of breast cancer often enroll patients who are younger than the general population for whom therapy will be prescribed. Thus, the population studied is not always the population for whom treatment is recommended.
6. The number of subjects needed to determine small differences between treatments is often measured in the thousands for primary endpoints, and assumes events will occur within a relatively narrow time point based upon prior knowledge of the biology of disease. There are often no such estimates prospectively available for the rates expected for harm.
7. Reporting of trials is often truncated at the reporting of the primary endpoint for a clinical trial. Indeed, except for certain endpoints such as second malignancy and cardiac failure, large sponsored trials often truncate reporting of adverse events arbitrarily at 5 years.
8. Reporting of death from disease or relapse are discrete, unambiguous events, while toxicity reporting often is by grade, with variable consistency of reporting likely for lesser grades, or as a result of lack of investigator tracking or inadequate attribution.

In consideration of infrequent toxicities, studies with 250 to 1,000 patients per treatment cohort may be powered to detect recurrence free survival differences of 3–5% among treatments, while not observing differences in serious adverse effects. A 95% confidence estimate of the potential frequency of an adverse effect that might not be detected in a trial is $3n$, where n is the number of subjects at risk (Hanley and Lippman-Hand, 1983). Thus, a cohort with 600 individuals might still not detect a specific harm as frequent as 0.5% within the conduct of a trial, assuming there is a strict definition of the harm.

To highlight the difficulty comparing risks, Hutchins *et al.* (2005) reported small disease free and overall survival benefits of doxorubicin containing chemotherapy (planned cumulative dose 360 mg/m^2, actual dose less) for women with node negative breast cancer at the first two

analyses of the trial. At the third report at 10.8 years, there was a minimally significant difference in favor of doxorubicin containing therapy for overall survival but not disease-free survival. In their analysis, total cardiac events were similar among the anthracycline/no anthracycline groups, as the 1-year cardiac mortality was 0.075% vs. 0%, but the cumulative mortality at 10.8 years was not reported. Rather, cumulative rates of cardiomyopathy, cardiomegaly, and congestive heart failure were reported as events/100 patient years in a subset of patients, and were reassuringly similar. In contrast, Zambetti *et al*. (2001) summarized the experience of 1,000 patients, comparing those who received doxorubicin (n = 637, median dose 294 mg/m^2) to those who did not, in three Italian trials. At 11 years, the cumulative rate of cardiac death was reported as 0.6% in the anthracycline group, and 0% in the no anthracycline group. The lack of uniform methods of reporting hazards in this example makes it difficult to draw precise conclusions relative to the qualitative impact of harms in the decision of whether to utilize anthracyclines.

Once the primary endpoint of a breast cancer clinical trial (often recurrence free survival) is met, vigilant reporting of all further adverse events may cease. While the primary endpoint result may not change significantly after a 5-year endpoint, the cumulative risk of an event such as cardiomyopathy may not be known for 5–15 years or more. At year 1, Hutchins *et al*. (2005) reported the incidence of grade 1–5 cardiac toxicity events in the anthracycline cohorts to be ~ 1.5%, and 1% in the no-anthracycline group. As the cumulative risk of cardiac death at 10.8 years was not reported, it could not be directly compared to the 0.6% excess risk of death reported at approximately the same time point by Zambetti *et al*. (2001). Similarly, the cumulative grades 1–5 cardiac toxicity event rate at 10.8 years reported by Hutchins *et al*. (2005) could not be directly compared to the data of Zambetti *et al*. (2001) to help inform the decision on use.

The example of anthracycline toxicity is instructive for other reasons. Historically, one of the most active agents for breast cancer, this class of drugs was developed in the 1960s and doxorubicin was approved by the FDA in the 1974 for use in the United States. When first introduced, its significant single agent activity led to chronic use at moderate to high cumulative doses of 400–500 mg/m^2 and higher. The combination of significant therapeutic activity and lack of overriding acute toxicity permitted administration of repeated cycles. Despite the recognition of a relationship of cumulative dose and peak dose with cardiotoxicity, there remains significant uncertainty over patient specific factors and cointerventions that may influence cumulative toxicity of doxorubicin even 30 years into its use, independent of more recent investigations combining it with other agents such as taxanes and kinase inhibitors (trastuzumab) that may modulate both its effectiveness and its toxicity. At the 2007 meeting of the American Society of Clinical Oncologists, the vigilance needed to assess harms from combination therapies was demonstrated by Perez *et al*. (2007). Three years following enrollment in a trial combining moderate doses of anthracycline (240 mg/m^2 doxorubicin in four cycles) with taxane

and trastuzumab, 2.5% of patients sustained clinical cardiac events, compared with 0.2% of patients who did not receive trastuzumab. It is reassuring that the rate appeared to plateau by the time of reporting (36 months), but additional toxicity cannot be excluded at such an early time point as suggested by the studies cited above and others.

In contrast to chronic toxicities, acute toxicities such as neutropenia and nausea are relatively well predicted, manageable, and even now preventable, rather than lifestyle altering or limiting (Hortobagyi, 2001). Nonetheless, as both Hassett *et al*. (2006) and Chou and Helfand (2005) have discussed, the reporting of harm in clinical trials is but one source of data and may underestimate the true incidence when applied in the general population for many reasons (patient selection, inadequate sample size to assess the toxicity endpoint, underreporting, inability to anticipate combinations with other agents or predisposing factors).

RISKS OF ACUTE TOXICITIES

While individual trials have presented varying estimates of harm, in none of these trials has toxicity reporting been the primary endpoint. Shapiro and Recht (2001) summarized the frequency of a number of acute and chronic toxicities of adjuvant therapy as reported in National Cancer Institutes Clinical trials. More recently, Hassett *et al*. (2006) performed an analysis of patients who received chemotherapy for breast cancer deriving information from insurance claims and found a significantly higher incidence of acute toxicities and costs than would be suggested from clinical trials data. The study included only insured populations in the analysis, but this would not likely have biased the data in favor of a higher risk of adverse effects. Rather, the study pointed out that a number of factors might influence the underreporting of acute harms and costs in clinical trials, emphasizing that those trials are but a measure of harm and not a precise estimate (Erban and Lau, 2006). Hassett *et al*. (2006) demonstrated that the experience reported in randomized controlled clinical trials might underestimate the true frequency of even relatively early adverse effects.

Acute toxicities of importance include allergic reactions, nausea, vomiting, neutropenic fever, infections, alopecia, and mucosa related adverse effects (mouth sores, diarrhea, abdominal discomfort), not to mention the issues of economic and time cost. The successes of novel agents to control nausea (5HT-3 and NK-1 receptor antagonists) have largely made nausea and vomiting less relevant, but have replaced certain adverse effects (nausea, vomiting) with others (e.g., constipation). More recently, Citron *et al*. (2003) evaluated the administration of certain chemotherapeutic agents in accelerated schedules (every 3 week vs. every 2 week administration of adjuvant therapy) as a means of improving efficacy. Sequential analyses have reported diminishing estimates of the difference in efficacy for ER+ patients, while certain toxicities (e.g., anemia, fatigue, neuropathy) and cost were increased by the accelerated schedule. Thus, the relative estimates of acute and chronic harms become increasingly important for estimating the overall worth of treatment.

An important consideration in selecting therapy for patients, particularly in the

adjuvant setting, is the potential for immunosuppression and viral reactivation in chronic active hepatitis or HIV infection. Hepatitis B reactivation has been reported during adjuvant chemotherapy for breast and other malignancies, and in certain populations, the risk of chronic hepatitis B infection may approach 10%. Thus, use of chemotherapy and support measures must be weighed carefully in this population. Available literature suggests that antiviral prophylaxis should be administered prior to and in the early post chemotherapy period for these patients (Kohrt *et al*., 2006). The influence of hematopoietic growth factors on viral reactivation cannot be assessed. It is reasonable for all patients with chronic viral infection to have prechemotherapy assessments for prophylactic measures.

Acute adverse effects of hormonal therapy may not infrequently be the cause of symptoms but are infrequently reported as limiting of their use. Ovarian suppression may be accomplished through surgical means, pituitary suppression or radiation of the ovaries, yet the three modalities have not been formally compared in randomized fashion, either for effectiveness or toxicity. The ATAC Trialists' Group (2002) reported excellent tolerance of both anastrazole and tamoxifen as adjuvant treatments for post-menopausal women. Investigators evaluating other aromatase inhibitors have reported similar results. It is possible that the true estimate of acute adverse effects, however, is more accurately assessed from nontrial data. Allergic reactions and serious acute toxicities are extremely uncommon. Tamoxifen historically has been reported to have an extremely low incidence of acute reactions/toxicities that require interruption or disruption of treatment. True allergic reactions are rare and thromboembolic and cerebrovascular events depend upon age, comorbidities and duration of use. Uterine cancer occurs with a frequency of 1% or less and appears to be associated with older age at time of use.

The development of biologically targeted agents has raised fundamental questions regarding the worth of traditional phase I–II testing to arrive at the proper dose and schedule of an agent. For example, dose limiting toxicities in phase I testing such as GI toxicity and marrow suppression, attributed to effects on rapidly dividing cells often has no meaning in the context of an immunotherapeutic like trastuzumab or bevacizumab. Thus, for most promising agents to come, acute toxicities and dose limiting toxicity will likely recede in importance. Instead, biomarkers designed to measure desired effects will determine treatment schedules, making the reporting of long term or chronic harms equivalent to or even more important than the principal endpoint.

LATER SEQUELAE OF SYSTEMIC THERAPY AND CHOICE OF TREATMENT

Much attention has been paid recently to the area of chronic toxicity reporting. Reasons for this increased attention include the following important developments:

1. Median survival of patients with metastatic breast cancer has improved, and patients are living significantly longer with disease. Most of these patients will be on continually changing therapy

for their entire life in an attempt to control the disease. Unlike other chronic disease models such as diabetes and hypertension, frequent changes in use of breast cancer agents are expected. Thus, cointerventions will be increasingly important (see below).

2. The number of agents available for use, either in combination or sequence has dramatically increased. While the use of chemotherapy from the 1970s through the early 1990s was largely limited to cyclophosphamide in combination with doxorubicin or methotrexate and 5FU, the numbers of agents that can be used sequentially or in combination now include a wide array of chemotherapies with the "retirement" of virtually none. Hormonally active agents included tamoxifen, progestins, and androgens, and now include several steroidal and nonsteroidal aromatase inhibitors, and estrogen receptor down-regulators. Finally, biological agents that interfere with signaling, such as trastuzumab and lapatinib, or other generic biological processes such as angiogenesis, have recently been developed.
3. The use of these agents has increasingly been applied to younger women who are expected to have longer lifespan, as the agents increasingly are effective at curing the disease. Thus, toxicity must be measured over decades. Patient characteristics will shift as older patients are increasingly offered less acutely toxic treatments.
4. As acute toxicities have become less common in the use of newer agents, the freedom to test them for longer periods has increased. Initial trials of trastuzumab in the United States included use of the agent for 1 or 2 years compared with no use, despite a chronic risk of cardiac toxicity. Neither acute toxicities nor intolerance indicated a need to design trials of shorter duration. This is analogous to the experience with tamoxifen, a well tolerated agent with minimal acute toxicity, initially postulated to be a static and not a cidal drug. In early trials, its minimum duration of obligated use was not clearly defined and thus, the standard of care for many women was indefinite therapy. Sequential trials have demonstrated that for many women, 5 years of tamoxifen may be adequate treatment.
5. Increasingly, as agents become more numerous, sequential use of agents in different classes, for example, the use of a biologically cardiotoxic agent after a chemotherapeutically toxic agent, either immediately or delayed, will be unavoidable.
6. Long term toxicities may not become clinically relevant for years, or even decades, but once apparent, they may be serious or life threatening, and irreversible. Consider the risk of hip fractures that occur in the 7th or 8th decade at a rate as high as 300,000 per year in the United States (1991 data) with a mortality rate of 20–30% in the first year. The benefit of a commonly applied treatment at age 40 that decreased the mortality from breast cancer by 3% with little acute morbidity might ultimately decrease survival if the risk of hip fractures in the elderly patient is increased by 10%, let alone the influence on morbidity. Whether this could occur as a result of currently effective therapies is unknown.
7. Long term toxicities include those that may be meaningful in terms of quality

of life or in terms of major morbidity. Examples of the former may include vasomotor symptoms and chronic sleep disturbance, ovarian failure resulting in premature menopause, teratogenicity and childbearing risks, and musculoskeletal pain-limiting mobility. Examples of the latter include chronic cardiac disease, cardiovascular, cerebrovascular or thromboembolic disease, neurological disease including late cognitive effects, skeletal effects such as progressive bone loss, hematological malignancies as a result of therapy, and secondary malignancies of another nature.

8. Compounding the risk assessment of toxicities of individual agents is the certainty that outside of trials, as patients live longer, these agents will be used sequentially or in combination, with or without typically used modalities such as chemotherapies or radiation therapy. The ability to predict toxicity in general use will be difficult at best or in some cases impossible based upon current trial design.
9. Most interventions will either impart a small amount of benefit to the entire population to which it is applied, or a relatively large amount of benefit to a select few. Tamoxifen and trastuzumab are examples of the latter. Chemotherapy, however, could be an example of either, and the distribution of effect of biological agents like bevacizumab remains uncertain. The current lack of patient specific predictive instruments to direct therapy to the relatively small number of patients who will benefit from the intervention makes it crucial to understand the potential for universally likely harms that may occur 10 or more years from therapy.

HARMS RELATED TO EFFECTIVE TREATMENT MODALITIES

In this section, several clinically important side effects of therapy are discussed as examples of potential for late toxicity that may significantly alter the decision to utilize agents effective against breast cancer. All may have varying intensity as well as times of first presentation.

Cardiac and Cardiovascular Toxicity

Beginning in the 1970s adjuvant chemotherapy for breast cancer became accepted as effective in improving survival. By the mid-1980s, anthracyclines were increasingly utilized as agents in a number of regimens and schedules of agents as efficacy was appreciated. Regimens such as AC, CAF (oral or IV based), FAC, VATH,[1] were utilized in a variety of trials for both node-positive and node-negative breast cancers. Within such trials individual doses ranged from 30 to 90 mg/m^2 and dose intensity ranged from 10 to 30 mg/m^2/week. Modern cumulative totals for adjuvant treatment range from 240 to 360 mg/m^2. Trials designed to demonstrate improvement in outcome in using anthracyclines have varied in design with the following parameters:

[1] A: doxorubicin; C:cyclophosphamide; F: 5-fluorouracil; V: vinblastine; T: thiotepa; H: halotestin.

1. Individual doses ranged in individual trials from 30 to 90 mg/m^2 per dose.
2. Peak serum levels as well as cumulative dose have influenced risks of cardiomyopathy.
3. The risk of cardiomyopathy is nonlinear. That is, as one approaches a cumulative dose of 500–550 mg/m^2 there is nonlinear accelerating increase in risk of cardiomyopathy. The time point at which one assesses risk as well as dose interacts in a complex function with dose to determine the ultimate risk of cardiomyopathy at 10, 15, or 20 years.
4. More recent trials that have included other agents, such as radiation and biologic agents, may modify risk.

Specific investigations have demonstrated that with regard to efficacy:

1. There is a dose threshold effect in preventing recurrence of breast cancer for anthracyclines in node-positive patients (Wood *et al*., 1994).
2. Anthracycline containing regimens are more effective in preventing recurrence for both node-negative and node-positive patients. Relative advantages are shifting and new regimens may durably equal or surpass anthracyclines in the near future, both in terms of efficacy and toxicity. It is likely that toxicity and cost will be as or more important than efficacy in determining the use as time passes.
3. Not all anthracycline containing regimens are the same, and the ideal anthracycline containing regimen is not defined.
4. Keeping total dose constant, the sequencing of anthracycline dose in and around other treatment may influence effectiveness as well as toxicity (Bonadonna *et al*., 2004).
5. Increasing single doses by 25–50% above typically utilized doses (60 mg/m^2) did not improve disease free or overall survival while acute toxicities did change (Henderson *et al*., 2003).
6. The use of agents that have potential for cardiotoxicity may alter the relative short term risk of cardiac toxicity, as demonstrated by Rastogi *et al*. (2007). In this report, the use of trastuzumab for 1 year following the completion of anthracycline and in combination with paclitaxel significantly increased the risk of cardiotoxicity at 3 years. The estimates of cardiac toxicity as defined by the study did not change appreciably between the first and second analyses 24 months apart.
7. Of trials that demonstrated efficacy in favor of anthracyline, there is no uniformity of reporting of cardiac harms relative to interval from treatment, classification (total events vs. grade 5) or annual incidence.

Cardiac toxicities often may present as continuous variables that require instrumentation or other tools for measurement. For example, a decline in ejection fraction may occur over time and not present clinically for years or decades to be measurably detected early on. In the asymptomatic patient, repeated measurements of ejection fraction would assess toxicity yet require specific studies on asymptomatic patients over decades, adding significant cost. Few trials have proven that at 10–20 years the absolute incidence of toxicity has reached a plateau, and the formal process to track events other than recurrence, death or second malignancy has

not been established. The Breast Cancer International Research Group (BCIRG) amended a large prospective randomized trial comparing cyclophosphamide, doxorubicin and docetaxel with cyclophosphamide, doxorubicin and 5-FU to include annual reporting of numerical changes in ejection fraction as assessed by formal noninvasive testing. The general standards for monitoring trials of this type or others that use anthracyclines and criteria for both funding and duration of reporting have not been uniformly adopted (Kremer *et al*., 2002).

Hormonal therapy may influence cardiovascular health and mortality through several mechanisms. Thromboembolic risk in women over the age of 50 is well known from tamoxifen, who have a risk of venous thrombosis and or embolism of 1%, similar to estrogen replacement. Large scale trials of tamoxifen in healthy women under age 50 as a preventative measure did not demonstrate significantly increased risk of either stroke or heart attack. Thus, the influence on long term mortality and health appears to be relatively well characterized in this population, emphasizing the role of patient characteristics in influencing risk of harms for the same intervention.

Less well known is the influence of aromatase inhibitors on cardiovascular mortality. Part of the difficulty is due to the variation in initial conditions prior to use of aromatase inhibitors. Patients have (1) received aromatase inhibitor following 4.5–6 years of tamoxifen, (2) received aromatase inhibitor or tamoxifen with no prior therapy, or (3) received aromatase inhibitor for 2–3 years as initial adjuvant therapy before or following 2–3 years of tamoxifen. In each situation, the prior treatment of the patient the duration and drug differed slightly. While no evidence of excessive aggregated cardiovascular risk from letrozole appeared following 5 years of tamoxifen (Goss *et al*., 2003) uncertainty remains regarding the attributable long term risks of aromatase inhibitor use relative to tamoxifen when used as initial therapy. The Breast International Group (BIG) 1–98 Collaborative Group (2005) demonstrated no difference in aggregated grade 3–5 cardiovascular toxicity comparing letrozole to tamoxifen as initial adjuvant therapy, but estimates of cardiovascular grade 5 toxicity remains the subject of investigation. Thus, in the choice of aromatase inhibitor, timing, schedule, patient characteristics at time of use remain important variables that require independent assessment for harms, especially given the number of agents within a class and the opportunity to use agents in multiple orders. As the duration of aromatase inhibitor use is further refined, yet another variable will be introduced.

The use of signal transduction molecules and antiangiogenic agents has presented several challenges in assessing cardiovascular risk. The potential for trastuzumab-containing regimens to cause cardiac dysfunction is well known, and has been estimated by Slamon *et al*. (2001) to be sixfold over regimens that do not include trastuzumab, although the absolute risk remains low. Concern for agents that inhibit specific kinase pathways remains nonetheless, particularly when coupled sequentially or concurrently with anthracycline. Kerkela *et al*. (2006) for example, have demonstrated the potential for the kinase inhibitor STI-571 (imatinib) to induce cardiomy-

opathy. Antiangiogenic agents have been associated with systemic hypertension and more recently pulmonary hypertension. It remains uncertain whether novel agents such as kinase inhibitors or angiogenesis inhibitors will influence late cardiovascular events in any meaningful way.

Neurological Effects of Therapy

Recent concerns on the long term effects of treatment on neurological function have centered on three aspects of treatment: neuropathy, cognitive effects of hormonal therapy, and cognitive effects of systemic chemotherapy and radiation.

These toxicities are of great concern to patients in the adjuvant setting but also have affected patients with metastatic breast cancer who have lived with their disease as a result of increasingly effective and sequentially applied therapies. In this realm, studies provide no data that may help to guide clinicians and current trial design, and follow up precludes making conclusions. Nonetheless, surviving long term with breast cancer has become so common that risk of memory loss and dementia have become increasingly important.

Neuropathy remains an important but manageable complication of certain classes of agents, namely vinca alkaloids, taxanes, and platinum salts. The risk of neuropathy is dose and cycle number-dependent, predictable and usually nonprogressive once treatment is over. The overt presence of symptoms and the grading based on such reporting makes it usual that assessments are complete and effectively incorporated into future treatment plans prior to continued use of offending agents.

More difficult have been assessments of hormonal therapy on cognitive function. Yaffe *et al*. (1998) have shown that use of estrogen in postmenopausal women is associated with induction of menopause, which has been associated with improved cognitive function. Studies that focused on the epidemiology of Alzheimer's and early dementia have been unable to agree on the role of estrogen and hormones. Tang *et al*. (1996) have demonstrated a relationship between postmenopausal estrogen use and decreased risk of dementia. By contrast, there did not appear to be increased cognitive dysfunction after 3 years of raloxifene use (Yaffe *et al*., 2001).

The effects of systemic chemotherapy on neurocognitive function are more problematic. The term "chemo-brain" implies that there are neurocognitive effects of chemotherapy. While many studies have included quality of life components into large scale randomized trials, no large randomized controlled trials have prospectively reported formal longitudinal neurocognitive testing as an outcome related to treatment. There are few data on short or long term use of agents, which are selected for activity in the metastatic setting when applied in the adjuvant setting to a population where long term survival is expected. Evidence of harm or potential for harm arises from models of anticancer treatments applied to other situations, such as whole brain radiation for metastatic disease or high dose methotrexate as treatment for malignant lymphoma. In both cases, predictable increases in frequency of long-term effects are known.

The recent application of agents with modest cumulative toxicity, such as capecitabine to the treatment of breast

cancer coupled with biological agents such as trastuzumab, has made it possible that patients live years following first central nervous system metastasis/use of CNS directed radiation. How they will potentiate the effective toxicity of other agents is not well understood. In addition, agents that interfere with processes known to be formally involved in healing/reparative processes, such as antiangiogenesis factors, when applied to long term use, may affect normal organ/brain function in ways that current trial design is incapable of measuring. As agents are increasingly applied to patients with an expectation of survival, quantitative measurement of neurocognitive decline will be increasingly vital to monitor and prevent major increases in cognitive impairment in later years of life.

Musculoskeletal Complications

Neuromuscular complaints have not prominently influenced the use of antineoplastic chemotherapy in either the adjuvant or the metastatic setting. Neuropathies with taxanes, ever more frequently utilized in the adjuvant setting, are dose and schedule-dependent, related to the type (docetaxel vs. paclitaxel) and the method of formulation (paclitaxel vs. nab-paclitaxel). Effects, while cumulative, are usually nonprogressive once the drug is discontinued. This makes use of taxanes relatively predictable. In a large adjuvant trial of docetaxel combined with cyclophosphamide and doxorubicin (TAC) for six cycles, the incidence of grade 3–4 neuropathy was reported to be 0% (Martin *et al*., 2005), while the incidence of adverse events that interfered with function reported by Henderson *et al*. (2003) following four cycles of paclitaxel was 3%. The vast majority of patients do not report disabling neuropathy months or years from treatment.

Hormonal therapy, by contrast, has led to a number of adverse effects that include symptomatic joint discomfort, arthritis, and fractures requiring symptomatic treatment or discontinuation of therapy compared with tamoxifen which induces this adverse effect less often. There is a significant relationship of use of aromatase inhibitors with two chronic musculoskeletal adverse events: arthralgias and arthritis, and bone demineralization.

Grade 3–4 arthralgias were roughly twice as common in patients treated with letrozole, compared to patients treated similarly with tamoxifen in the Breast International Group (BIG) 1–98 trial (2005). Both anastrazole and exemestane also cause arthralgia, and a formal comparison among the aromatase inhibitors has not been completed. Of more concern is the silent complication of bone mineral loss. While measurable, quantifying the medical cost of aromatase remains difficult. New fractures attributable to changes in bone density have been quantified in clinical trials and in several studies increased fracture risk in short term follow up has been noted. The actuarial risk of hip fractures, one of the most serious types of bone fractures, escalates significantly as one ages. Thus, introduction of aromatase inhibitors either means that bone density of the population on average will fall, or that agents to prevent bone loss with potential for adverse effects of their own will be introduced. While Brufsky *et al*. (2007) have shown that the use of bisphosphonates, even as infrequently as once or twice per year, can reverse the loss of bone mineralization introduced by

aromatase inhibitors, the worth of such an intervention in terms of preventing fractures and not introducing complications such as osteonecrosis of the jaw in the population as a whole remains uncertain. The example of bone loss illustrates the difficulty in using intermediate endpoints such as bone mineralization as surrogates for discrete endpoints such as hip fractures that may be a cause of mortality 25% of the time. Attempts to model risk of such complications may be the only way to guide therapy when the endpoint may not occur for decades and the need to intervene is immediate.

Secondary Malignancy

The introduction of modern chemotherapy and hormonal therapy has been a major success story in curing patients of breast cancer. Mortality statistics published over the past few years affirm the worth of systemic treatment in reducing mortality from breast cancer. As more patients have been cured of disease, the risk of malignancy resulting from therapy has become more important. Three decades of tamoxifen use has provided an estimated risk of uterine cancer of 1% in postmenopausal women. The risk of second breast cancer attributable to tamoxifen is difficult to assess but likely to be very small after 5 years of tamoxifen use. In the adjuvant setting, the risk of contralateral breast cancer was decreased for both anastrozole and letrozole compared with tamoxifen in the adjuvant setting. Thus, one cannot eliminate a possible slight agonist effect of tamoxifen as a potential initiator or promoter of second breast cancers. Other malignancies attributable to tamoxifen have not been reported in meaningful numbers. Thus, with regard to secondary neoplasia, the risks of tamoxifen are small, discrete, and unlikely to change.

Regarding chemotherapy, the uses of alkalyating agents and anthracyclines have dated to the 1970s and 1980s and thus, 30-year data are available. Risks of leukemia have been identified that peak around 5–7 years and then recede, and risk of secondary solid tumors has varied but is thought to be quite low. Taxanes have not been utilized for nearly as long but are not thought to have a meaningful risk of secondary cancers. Other agents have not been routinely used in the adjuvant setting where secondary risk of cancer is a consideration.

The use of trastuzumab in clinical trials has not been associated with increased risk of malignancy. In the combined US adjuvant trastuzumab trials, Romond *et al*. (2005) reported a lower incidence of second cancers in women treated with trastuzumab for 1 year. The significance of this finding is unknown. There are not enough patients at long enough follow-up to know if there are any meaningful concerns related to second malignancies. Other agents that may affect signaling (lapatinib) or angiogenesis (bevacizumab) have not been associated with malignancy, but definitive studies have not been done and no conclusions can be made for the patient cured of disease.

A new and important area that will require long-term attention is the risk of hematopoietic growth factors and other medications used for supportive care relative to disease progression. In particular, where small differences in breast cancer response or survival exist between regimens that utilize growth factors and those that do not, this effect will require close

observation. Leyland-Jones *et al*. (2005) demonstrated decreased survival among patients who received erythropoietin support during treatment for metastatic breast cancer compared with those who did not. Others have reported decreased survival in adjuvant or curative models. The role of functional erythropoietin receptors on tumor and stromal cells in affecting cancer outcomes is controversial but relates to the issue that supportive interventions should be used with caution in the absence of controlled trials demonstrating safety. In particular, maintaining transfusion independence during intensive chemotherapy should not only be viewed as supportive, but also as potentially tumor promoting unless proven otherwise by rigorous study.

Fertility, Teratogenicity, and *in utero* Effects on the Fetus

Once rare, women treated for breast cancer now have chosen to conceive after completing therapy. Ongoing use of tamoxifen is contraindicated, although it is not likely to increase the risk of permanent menopause, and there are no data on conception following use of aromatase inhibitors and ovarian suppression, as is being done in the current clinical trials. There are no data on the use of biological agents, and it is unlikely that there will ever be data to support the use of many of these agents during pregnancy. Whether they will increase the risk of infertility in women is unknown. The intensity of exposure to alkylating agents and anthracyclines determines whether infertility will occur following adjuvant therapy.

Alkalating agents and anthracyclines have been used during pregnancy with small studies suggesting that they are not harmful to the developing fetus if utilized later (second and third trimester) in pregnancy. Because these agents have not been in routine use for much more than 2 decades, there are simply no data on what effects might emerge later in life for persons exposed *in utero* to these agents. The experience with diethylstilbestrol has shown that relative to vaginal and breast cancers, adverse effects may not be evident for many years.

CONCLUSIONS AND RECOMMENDATIONS

The introduction of ever more effective agents in the treatment of breast cancer has led to more women living long after completion of therapy. Yet, very little is known regarding the long-term quality of health and survival following successful treatment. In part, the difficulty reflects complex interactions of treatments with factors that are inherently independent of the treatment itself.

Patient characteristics, cointerventions, and the systemic therapy should be considered in evaluating the potential for overall toxicity. Factors of importance include primary characteristic of the patients (age, obesity, for example) or concurrent, prior or subsequent treatments. However, an important reason for uncertainty over estimate of harms is the lack of consensus among investigators on standardized follow up of patients cured of disease, or living longitudinally with metastatic breast cancer that is carefully controlled but not eradicated. The sequential use of multiple agents has become commonplace, and limitations on trial design preclude detection of infrequent harms that may occur

following coincident use of agents. Thus, careful tracking and post marketing reporting of harms will remain critical to fully inform the choice of best therapy at 5, 10, or 20 years beyond treatment. It will be useful for investigators to agree on a specific limited set of adverse event reporting guidelines that should be followed in all future studies (Ioannidis *et al*., 2006; Trotti *et al*., 2003). These should include:

1. Universal standards of collection and reporting of data on agreed upon endpoints to facilitate the comparison of harms among trials
2. Indefinite follow-up of patients treated with agents that are newly introduced into practice or in novel combinations

While survival benefits measured at 5 and 10 years from treatment are crucial milestones, it is important that interest in long term survivorship be translated into research on, for example, cognitive effects of therapy, bone fractures, and cardiac events that may be attributable to treatment years earlier. Only with precise long term monitoring of such events will it be possible to guide future therapies and the use of sequential agents.

REFERENCES

Andre, F., Slimane, K., Bachelot, T., Dunant, A., Namer, M., Barrelier, A., Kabbaj, O., Spano, J., Marsiglia, H., Rouzier, R., Delaloge, S., and Spielmann, M. 2004. Breast cancer with synchronous metastases: trends in survival during a 14-year period. *J. Clin. Oncol. 22*: 3302–3308.

ATAC Trialists' Group. 2002. Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early breast cancer: first results of the ATAC randomised trial. *Lancet 359*: 2131–2139.

Bonadonna, G., Zambetti, M., Moliterni, A., Gianni, L., and Valagussa, P. 2004. Clinical relevance of different sequencing of doxorubicin and cyclophosphamide, methotrexate, and fluorouracil in operable breast cancer. *J. Clin. Oncol. 22*: 1614–1620.

Bonadonna, G., Moliterni, A., Zambetti, M., Daidone, M., Pilotti, A., Gianni, L., and Valagussa, P. 2005. 30 years' follow up of randomized studies of adjuvant CMF in operable breast cancer: cohort study. *Brit. Med. J. 330*: 217–222.

Breast International Group (BIG) 1–98 Collaborative Group. 2005. A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. *N. Engl. J. Med. 353*: 2747–2757.

Brufsky, A., Harker, W., Beck, J., Carroll, R., Tan-Chiu, E., Seidler, C., Hohneker, J., Lacerna, L., Petrone S., and Perez E. 2007. Zoledronic acid inhibits adjuvant letrozole-induced bone loss in postmenopausal women with early breast cancer. *J. Clin. Oncol. 25*: 829–836.

Chou, R., and Helfand, M. 2005. Challenges in systematic reviews that assess treatment harms. *Ann. Intern. Med. 142*: 1090–1099.

Citron, M., Berry, D., Cirrincione, C., Hudis, C., Winer, E., Gradishar, W., Davidson, N., Martino, S., Livingston, R., Ingle, J., Perez, E., Carpenter, J., Hurd, D., Holland, J., Smith, B., Sartor, C., Leung, E., Abrams, J., Schilsky, R., Muss, H., and Norton, L. 2003. Randomized trial of dose-dense versus conventionally scheduled and sequential versus concurrent combination chemotherapy as postoperative adjuvant treatment of node-positive primary breast cancer: first report of Intergroup Trial C9741/Cancer and Leukemia Group B Trial 9741. *J. Clin. Oncol. 21*: 1431–1439.

DeCillis, A., Anderson, S., Bryant, J., Wickerham, D., Fisher, B., and contributing investigators. 1997. Acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) on NSABP B-25: an update. *J. Clin. Oncol. Proc. Am. Soc. Clin. Oncol. 16*: 130a (abstract 459).

Diel, I., Solomayer, E., Costa, S., Gollan, C., Goerner, R., Wallwiener, D., Kaufmann, M., and Bastert G. 1998. Reduction in new metastases in breast cancer with adjuvant clodronate treatment. *N. Engl. J. Med. 339*: 357–363.

Erban, J., and Lau, J. 2006. On the toxicity of chemotherapy for breast cancer–the need for

vigilance. *J. Natl. Cancer Inst. 98*: 1096–1097.

Fisher, B., Anderson, S., DeCillis, A., Dimitrov, N., Atkins, J., Fehrenbacher, L., Henry, P., Romond, E., Lanier, K., Davila, E., Kardinal, C., Laufman, L., Pierce, H., Abramson, N., Keller, A., Hamm, J., Wickerham, D., Begovic, M., Tan-Chiu, E., Tian, W., and Wolmark, N. 1999. Further evaluation of intensified and increased total dose of cyclophosphamide for the treatment of primary breast cancer: findings from national surgical adjuvant breast and bowel project B-25. *J. Clin. Oncol. 17*: 3374–3388.

Gnant, M., Mlineritsch, B., Luschin-Ebengreuth, G., Grampp, S., Kaessmann, H., Schmid, M., Menzel, C., Piswanger-Soelkner, J., Galid, A., Mittlboeck, M., Hausmaninger, H., and Jakesz, R. 2007. Zoledronic acid prevents cancer treatment-induced bone loss in premenopausal women receiving adjuvant endocrine therapy for hormone-responsive breast cancer: a report from the austrian breast and colorectal cancer study group. *J. Clin. Oncol. 25*: 820–828.

Goss, P., Ingle, J., Martino, S., Robert, N., Muss, H., Piccart, M., Castiglione, M., Tu, D., Shepherd, L., Pritchard, K., Livingston, R., Davidson, N., Norton, L., Perez, E., Abrams, J., Therasse, P., Palmer, M., and Pater, J. 2003. A randomized trial of letrozole in postmenopausal women after five years of tamoxifen therapy for early-stage breast cancer. *N. Engl. J. Med. 349*: 1793–1802.

Hanley, J., and Lippman-Hand, A. 1983. If nothing goes wrong, is everything alright? *J. A. M. A. 259*: 1743–1745.

Hassett, M., O'Malley, A., Pakes, J., Newhouse, J., and Earle, C. 2006. Frequency and cost of chemotherapy-related serious adverse events in a population sample of women with breast cancer. *J. Natl. Cancer Inst. 98*: 1108–1117.

Henderson, I., Berry, D., Demetri, G., Cirrincione, C., Goldstein, L., Martino, S., Ingle, J., Cooper, M., Hayes, D., Tkaczuk, K., Fleming, G., Holland, J., Duggan, D., Carpenter, J., Frei, E., Schilsky, R., Wood, W., Muss, H., and Norton, L. 2003. Improved outcomes from adding sequential paclitaxel but not from escalating doxorubicin dose in an adjuvant chemotherapy regimen for patients with node-positive primary breast cancer. *J. Clin. Oncol. 21*: 976–983.

Hortobagyi, G. 2001. Adjuvant systemic therapy for early breast cancer: progress and controversies. *Clin. Cancer Res. 7*: 1839–1842.

Hortobagyi, G., Theriault, R., Porter, L., Blayney, D., Lipton, A., Sinoff, C., Wheeler, H., Simeone, J., Seaman, J., and Knight, R. 1996. Efficacy of pamidronate in reducing skeletal complications in patients with breast cancer and lytic bone metastases. *N. Engl. J. Med. 335*: 1785–1791.

Hurwitz, H., Fehrenbacher, L., Novotny, W., Cartwright, T., Hainsworth, J., Heim, W., Berlin, J., Baron, A., Griffing, S., Holmgren, E., Ferrara, N., Fyfe, G., Rogers, B., Ross, R., and Kabbinavar, F. 2004. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N. Engl. J. Med. 350*: 2335–2342.

Hutchins, L., Green, S., Ravdin, P., Lew, D., Martino, S., Abeloff, M., Lyss, A., Allred, C., Rivkin, S., and Osborne, C. 2005. Randomized, controlled trial of cyclophosphamide, methotrexate, and fluorouracil versus cyclophosphamide, doxorubicin, and fluorouracil with and without tamoxifen for high-risk, node-negative breast cancer: treatment results of intergroup protocol INT-0102. *J. Clin. Oncol. 23*: 8313–8321.

Ioannidis, J. 2005. Why most published research findings are false. *PLoS Med. 2*: e124.

Ioannidis, J., Mulrow, C., and Goodman, S. 2006. Adverse events: the more you search, the more you find. *Ann. Intern. Med. 144*: 298–300.

Kerkela, R., Grazette, L., Yacobi, R., Iliescu, C., Patten, R., Beahm, C., Walters, B., Shevtsov, S., Pesant, S., Clubb, F. J., Rosenzweig, A., Salomon, R. N., Van Etten, R. A., Alroy, J., Durand, J. B., and Force, T. 2006. Cardiotoxicity of the cancer therapeutic agent imatinib mesylate. *Nat. Med. 12*: 908–916.

Kohrt, H., Ouyang, D., and Keeffe, E. 2006. Systematic review: lamivudine prophylaxis for chemotherapy-induced reactivation of chronic hepatitis B virus infection. *Aliment Pharmacol. Ther. 24*: 1003–1016.

Kremer, C., van der Pal, H., Offringa, M., van Dalen, E., and Voûte P. 2002. Frequency and risk factors of subclinical cardiotoxicity after anthracycline therapy in children: a systematic review. *Ann. Oncol. 13*: 819–829.

Leyland-Jones, B., Semiglazov, V., Pawlicki, M., Pienkowski, T., Tjulandin, S., Manikhas, G., Makhson, A., Roth, A., Dodwell, D., Baselga, J., Biakhov, M., Valuckas, K., Voznyi, E., Liu, X., and Vercammen, E. 2005. Maintaining normal hemoglobin levels with epoetin alfa in mainly nonanemic patients with metastatic breast cancer receiving first-line chemotherapy: a survival study. *J. Clin. Oncol. 23*: 5960–5972.

Martin, M., Pienkowski, T., Mackey, J., Pawlicki, M., Guastalla, J., Weaver, C., Tomiak, E., Al-Tweigeri, T., Chap, L., Juhos, E., Guevin, R., Howell, A., Fornander, T., Hainsworth, J., Coleman, R., Vinholes, J., Modiano, M., Pinter, T., Tang, S., Colwell, B., Prady, C., Provencher, L., Walde, D., Rodriguez-Lescure, A., Hugh, J., Loret, C., Rupin, M., Blitz, S., Jacobs, P., Murawsky, M., Riva, A., Vogel, C., and the Breast Cancer International Research Group 001 Investigators. 2005. Adjuvant docetaxel for node-positive breast cancer. *N. Engl. J. Med. 352*: 2302–2313.

Miller, K., Wang, M., Gralow, J., Dickler, M., Cobleigh, M., Perez, E., Shenkier, T., and Davidson, N. 2005. A randomized phase III trial of paclitaxel versus paclitaxel plus bevacizumab as first-line therapy for locally recurrent metastatic breast cancer: a trial coordinated by the Eastern Cooperative Oncology Group. *Breast Cancer Res. Treat. 94* (supplement 1): S6 (abstr 3).

Perez, E., Romond, E., Suman, V., Jeong, J., Davidson, N., Geyer, C., Martino, S., Mamounas, E., Kauffman, P., Wolmark, N., and NCCTG/NSABP. 2007. Updated results of the combined analysis of NCCTG N9831 and NSABP B-31 adjuvant chemotherapy with/without trastuzumab in patients with HER2-positive breast cancer. *J. Clin. Oncol. Proc. Am. Soc. Clin. Oncol. 25* No. 18 S (June 20 supplement): abstract #512.

Rastogi, P., Jeong, J., Geyer, C., Costantino, J., Romond, E., Ewer, M., Keefe, D., Levine, T., Swain, S., and Wolmark, N. 2007. Five year update of cardiac dysfunction on NSABP B-31, a randomized trial of sequential doxorubicin/cyclophosphamide (AC)→paclitaxel (T) vs. AC→T with trastuzumab (H). *J. Clin. Oncol. Proc. Am. Soc. Clin. Oncol. 25* No. 18 S (June 20 supplement): LBA #513.

Romond, E., Perez, E., Bryant, J., Suman, V., Geyer, C., Davidson, N., Tan-Chiu, E., Martino, S., Paik, S., Kaufman, P., Swain, S., Pisansky, T., Fehrenbacher, L., Utteh, L., Vogel, V., Visscher, D., Yothers, G., Jenkins, R., Brown, A., Dakhil, S., Mamounas, E., Lingle, W., Klein, P., Ingle, J., and Wolmark, N. 2005. Trastuzumab plus adjuvant chemotherapy for operable HER-2-positive breast cancer. *N. Engl. J. Med. 353*: 1673–1684.

Shapiro, C., and Recht, A. 2001. Side effects of adjuvant treatment of breast cancer. *N. Engl. J. Med. 344*: 1997–2008.

Slamon, D., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., Fleming, T., Eiermann, W., Wolter, J., Pegram, M., Baselga, J., and Norton, L. 2001. Use of chemotherapy plus a monoclonal antibody against HER-2 for metastatic breast cancer that over expresses HER-2. *N. Engl. J. Med. 344*: 783–792.

Smith, R., Bryant, J., DeCillis, A., and Anderson, S. 2003. Acute myeloid leukemia and myelodysplastic syndrome following doxorubicin-cyclophosphamide adjuvant therapy for operable breast cancer: the NSABP experience. *J. Clin. Oncol. 21*: 1195–1204.

Tang, M., Jacobs, D., Stern, Y., Marder, K., Schofield, P., Gurland, B., Andrews, H., and Mayeux, R. 1996. Effect of oestrogen during menopause on risk and age at onset of Alzheimer's disease. *Lancet 348*: 429–432.

Trotti, A., Colevas, A., Setser, A., Rusch, V., Jaques, D., Budach, V., Langer, C., Murphy, B., Cumberlin, R., Coleman, C., and Rubin, P. 2003. CTCAE v3.0: development of a comprehensive grading system for the adverse effects of cancer treatment. *Semin. Radiat. Oncol. 13*: 176–181.

Woo, S., Hellstein, J., and Kalmar, J. 2006. Systematic review: bisphosphonates and osteonecrosis of the jaws. *Ann. Intern. Med. 144*: 753–761.

Wood, W., Budman, D., Korzun, A., Cooper, M., Younger, J., Hart, R., Moore, A., Ellerton, J., Norton, L., Ferree, C., Ballow, A., Frei, E., and Henderson, I. 1994. Dose and dose intensity of adjuvant chemotherapy for stage II, node-positive breast carcinoma. *N. Engl. J. Med. 330*: 1253–1259.

Yaffe, K., Sawaya, G., Lieberburg, I., and Grady, D. 1998. Estrogen therapy in postmenopausal

women: effects on cognitive function and dementia. *J. A. M. A. 279*: 688–695.

Yaffe, K., Krueger, K., Sarkar, S., Grady, D., Barrett-Connor, E., Cox, D., and Nickelsen, T. 2001. Cognitive function in postmenopausal women treated with raloxifene. *N. Engl. J. Med. 344*: 1207–1213.

Zambetti, M., Moliterni, A., Materazzo, C., Stefanelli, M., Cipriani, S., Valagussa, P., Bonadonna, G., and Gianni, L. 2001. Long-term cardiac sequelae in operable breast cancer patients given adjuvant chemotherapy with or without doxorubicin and breast irradiation. *J. Clin. Oncol. 19*: 37–43.

29
Chemotherapy for Metastatic Breast Cancer Patients Who Received Adjuvant Anthracyclines (An Overview)

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INTRODUCTION

Anthracyclines are among the most active agents in the treatment of metastatic breast cancer (MBC), and are components of many regimens that have been shown to be more effective than regimens based on CMF (cyclophosphamide, methotrexate, fluorouracil). In addition, the metanalysis of Early Breast Cancer Trialists Collaborative Group demonstrated that anthracycline-based adjuvant polychemotherapy (e.g., with FAC [fluorouracil, doxorubicin, cyclophosphamide] or FEC [fluorouracil, epirubicin, cyclophosphamide]) reduces the annual breast cancer death rate by ~ 38% for women younger than 50 years and by ~ 20% for those of age 50–69 years. Despite this success, relapse after adjuvant chemotherapy remains a critical problem, and the rate of MBC cancer patients who have already received anthracyclines as adjuvant or neoadjuvant will increase. The extensive use of anthracyclines in the adjuvant setting increases the possibility of selecting cellular clones with intrinsic resistance to anthracyclines or of inducing development of acquired multidrug resistance. Moreover, the cumulative dose-related risk of cardiotoxicity could be a major limitation to the use of conventional anthracyclines in patients with MBC who received an adjuvant treatment with anthracyclines. Both phenomena could act against the opportunity of retreatment with antracyclines when metastatic disease is diagnosed. Published reports show conflicting results and leave open the question of whether an anthracycline-containing regimen is worthy as first-line treatment for MBC patients pretreated with adjuvant or neoadjuvant anthracyclines. This issue is particularly relevant for HER-2-negative MBC patients, because for women with MBC whose tumors are HER-2-positive the concurrent administration of anthracycline with trastuzumab is limited by a high rate of cardiotoxicity. In this overview, we summarize the results observed with first-line anthracycline-containing regimens in patients with MBC treated

with adjuvant anthracyclines and discuss the current options of first-line therapy for these patients.

FIRST-LINE ANTHRACYCLINE-CONTAINING REGIMENS

The activity of first line anthracycline-containing regimens on the basis of prior adjuvant therapy has been described in several retrospective studies and in one prospective phase 3 trial. Some authors found that previous adjuvant chemotherapy adversely affected the prognosis of MBC patients treated with an anthracycline-based first-line chemotherapy, but this effect was independent of the type of chemotherapy, CMF-like or anthracycline-based. Pierga *et al*. (2001), in a retrospective analysis on 1,430 MBC patients accrued in eight prospective trials of anthracycline-based first-line chemotherapy conducted at the Institut Curie between 1977 and 1992, reported that patients who had not received adjuvant chemotherapy had a higher response rate than pretreated patients (66 vs. 56%, respectively; $p < 0.0001$). Moreover, they had a median time to progression and overall survival (14 and 26 months, respectively) significantly longer than those treated with previous adjuvant chemotherapy (10 and 19 months, respectively). However, anthracycline-based adjuvant therapy had the same adverse effects on overall survival as the other CMF-like regimens. Venturini *et al*. (1996) evaluated the effect of previous adjuvant chemotherapy with or without anthracyclines on overall survival, progression-free survival and objective response rates of MBC patients treated with cyclophosphamide, epidoxorubicin, and fluorouracil (CEF) as first-line chemotherapy in Gruppo Oncologico del Nord Ovest (GONO) cooperative centers from December 1983 to June 1994. Of the 326 assessable patients entered into the analysis, 144 (44%) had not been treated with adjuvant chemotherapy, 143 (44%) had received CMF-based chemotherapy and 39 (12%) had received anthracycline-based adjuvant chemotherapy. A response to chemotherapy was observed in 161 patients (49.4%). At univariate analysis, patients who had received prior adjuvant chemotherapy had a significantly lower probability of response than patients who did not (43 vs. 58%, respectively; $p = 0.02$). Moreover, when a logistic regression model was fitted to the data, adjuvant chemotherapy was among the strongest predictive factors for poor response. However, no difference in response rate between CMF-based and anthracycline-based adjuvant chemotherapy was observed (43% vs. 44%, respectively). The median progression-free survival was 11.4 months in patients who had not received previous adjuvant chemotherapy, while it was 8.8 and 6.6 months in those treated with CMF-based and anthracycline-based adjuvant chemotherapy, respectively. Patients who did not receive adjuvant chemotherapy had a longer survival time (21.1 months) compared with patients previously treated with CMF-based or anthracycline-based adjuvant chemotherapy (15.3 and 15.8 months, respectively). On the basis of these results, the conclusions of the authors were that an anthracycline should not be denied to patients who relapse after adjuvant chemotherapy, regardless of the type of adjuvant chemotherapy received. However, given the non randomized allocation of adjuvant chemotherapy, these findings could represent a selection bias, because patients

with worse baseline prognosis could have been preferentially selected for adjuvant chemotherapy.

Other studies did not demonstrate a poorer outcome in metastatic breast cancer patients previously treated with adjuvant chemotherapy. Kardinal *et al*. (1988) evaluated the response to cyclophosphamide, doxorubicin, fluorouracil (CAF) regimen as first line therapy in 46 patients with MBC previously treated with adjuvant chemotherapy, who relapsed 6 months or more after completion of adjuvant chemotherapy, and the results were compared with those observed in 379 patients of the same study who were not treated with adjuvant chemotherapy. The response rate of patients previously treated with adjuvant chemotherapy was not significantly different from the response rate of patients who did not receive adjuvant chemotherapy (50% vs. 59%, p = 0.22). The type of prior adjuvant chemotherapy did not significantly influence the response to subsequent CAF (p = 0.68). Furthermore, there was no significant difference in the duration of response, time to treatment failure or survival between the two groups of patients. However, with the small sample of patients who had received previous chemotherapy in this study, only large effects were likely to be detected.

Finally, Gennari *et al*. (2004) evaluated the prognostic impact of adjuvant chemotherapy with anthracyclines on response rate, progression free survival, and overall survival in 291 metastatic breast cancer patients treated in five consecutive studies with epirubicin and paclitaxel as first line therapy: 101 (35%) were chemonaive, 109 (37%) had received adjuvant CMF and 81 (28%) adjuvant anthracyclines. No difference in response rate was observed in patients treated with adjuvant CMF, anthracyclines or chemonaive (63% vs. 67% vs. 68%, respectively, p = 0.5). The median progression-free survival was 11 months for prior CMF, 10.2 months for anthracyclines and 12.5 months in chemonaive patients (p = 0.33). Finally, the median overall survival was 23.8 months for CMF, 20.2 months for anthracyclines and 27.5 months in chemonaive patients (p = 0.61). Therefore, the authors did not find any negative influence of adjuvant anthracyclines on the activity of first-line epirubicin and paclitaxel, confirming that modern chemotherapy regimens, including anthracyclines and taxanes, provide satisfactory results in metastatic breast cancer patients, regardless of previous adjuvant chemotherapy. However, symptomatic cardiac heart failure (CHF) was observed in 13 patients (4.5%) of this study: five of them developed CHF after a cumulative epirubicin dose of 1,080 mg/m^2 and one after 990 mg/m^2. In conclusion, this study does not provide direct evidence on the efficacy of anthracyclines in advanced breast cancer patients after adjuvant anthracyclines. The possibility that different regimens could produce the same results without cardiac toxicity cannot be ruled out.

The question whether metastatic breast cancer patients who have relapsed after an adjuvant or neoadjuvant anthracycline-containing regimen should be retreated with an anthracycline-based schedule of chemotherapy was answered by Pacilio *et al*. (2006) in a prospective, randomized phase 3 trial. The aim of the study was to evaluate the efficacy of the combination of epirubicin and docetaxel (ED) versus docetaxel (D) alone as first-line chemotherapy of metastatic breast cancer patients

pretreated with epirubicin in adjuvant or neoadjuvant setting. A similar antitumor activity was observed with both therapeutic regimens and no significant differences were found in terms of progression-free survival and overall survival. Epirubicin and docetaxel showed significantly worse toxicity in terms of leukopenia, nausea and stomatitis. Due to an early stopping of the trial, a Bayesian analysis for the efficacy of epirubicin and docetaxel was performed and showed that the predictive probability that the response rate of the epirubicin and docetaxel group would be significantly better than that of the docetaxel group, if the trial were brought to its completion, was equal to 0.0334, strengthening the conclusion of no greater activity of the epirubicin and docetaxel treatment. Therefore, the conclusion of this phase III study was that the addition of epirubicin to docetaxel does not improve outcomes, but increases toxicity, as compared to single-agent docetaxel, in the first-line treatment of metastatic breast cancer patients, who had previously received epirubicin.

CURRENT OPTIONS OF TREATMENT FOR HER-2 NEGATIVE METASTATIC BREAST CANCER PATIENTS

What should be the first-line chemotherapy of choice for HER-2-negative metastatic breast cancer patients, who need to be treated with chemotherapy, but have already received anthracycline-containing adjuvant regimens? Retreatment with anthracyclines in combination with taxanes should be avoided in this group of patients, also taking into account the availability of new active, non-cross-resistant drugs, such as capecitabine, gemcitabine, and vinorelbine. Reasonable options of treatment in this setting may include: taxanes as single agents or in combination with non-cross-resistant drugs, combinations without anthracyclines and taxanes or combinations with liposomal anthracyclines (Table 29.1).

Taxanes as single agents or in combination with non-cross-resistant drugs

Nabholtz *et al*. (1999) and Sjöström *et al*. (1999) demonstrated that docetaxel as single agent gives survival benefits in anthracycline-resistant patients, as compared to mitomycin plus vinblastine or methotrexate and 5-fluorouracil. Until recent years, docetaxel as single agent was considered a standard treatment for advanced breast cancer patients who received anthracyclines as adjuvant treatment, with an objective response rate of 40–68% in previously untreated metastatic breast cancer patients and 30–57% in patients resistant to a first-line anthracycline-containing chemotherapy. Moreover, Jones *et al*. (2005) conducted a randomized phase III trial comparing docetaxel and paclitaxel at approved doses and schedules which demonstrated the superiority of docetaxel to paclitaxel in terms of overall survival (15.4 vs. 12.7 months, $p = 0.03$) and time to progression (5.7 vs. 3.6 months, $p < 0.0001$), supporting the choice of docetaxel as compared to paclitaxel, when a single agent chemotherapy is indicated.

Several randomized phase 3 clinical trials have recently demonstrated that taxane-containing combinations of two drugs provide superior benefit compared

TABLE 29.1. Randomized phase 3 trials evaluating treatments for anthracycline pretreated metastatic breast cancer patients.

Option of treatment	Author, year	Pts (no.)	Treatment line	Treatment arms	OS (mos)	TTP (mos)	RR (%)
Taxanes as single agents	Nabholtz *et al*., 1999	392	First and second	Docetaxel	11.4*	4.7*	30*
				vs Vinblastine + Mitomycin	8.7	2.7	11.6
	Sjostrom *et al*., 1999	283	First and second	Docetaxel	10.4	6.3*	42*
				vs Methotrexate + Fluorouracil	11.1	3.0	21
	Jones *et al*., 2005	449	First and second	Paclitaxel	12.7	3.6	25
				vs Docetaxel	15.4*	5.7*	31
	Pacilio *et al*., 2006	51	First	Docetaxel	21	11	79
				vs Epirubicin + Docetaxel	18	9	72
Combinations with taxanes	O'Shaughnessy *et al*., 2002	511	First, second and third	Capecitabine + Docetaxel	14.5*	6.1*	42*
				vs Docetaxel	11.5	4.2	30
	Albain *et al*., 2004	529	First	Gemcitabine +	18.5*	5.4*	39*
				Paclitaxel vs Paclitaxel	15.8	3.5	25
	Fountzilas *et al*., 2004	327	First	Paclitaxel + Carboplatin	27.8	10.8	41
				vs Epirubicin + Paclitaxel	22.4	8.1	47
	Chan *et al*., 2005	305	First and second	Gemcitabine + Docetaxel	nr	8.1	32
				vs Capecitabine + Docetaxel		8.1	32
	Miller *et al*., 2005b	682	First	Paclitaxel + Bevacizumab	HR 0.64*	11*	28.2*
				vs Paclitaxel		6.1	14.2
	Beslija *et al*., 2006	100	First	Capecitabine + Docetaxel	22*	9.3*	68*
				vs Docetaxel → Capecitabine	19	7.7	40
	Soto *et al*., 2006	368	First and second	Capecitabine → Taxanes	24+	8.4	46
				vs Capecitabine + Paclitaxel	24+	6.7	65*
				or Capecitabine + Docetaxel	24+	8.1	74*
Combinations without taxanes	Mavroudis *et al*., 2006	114	"Salvage"	Gemcitabine + Vinorelbine	nr	3.7	25.8
				vs Capecitabine		5.8	24.1
	Martin *et al*., 2007	252	First, second and third	Gemcitabine + Vinorelbine	nr	6.3*	37*
				vs Vinorelbine		4.1	25
	Vahdat *et al*., 2007	752	Second and third	Ixabepilone + Capecitabine	nr	5.8*	35*
				vs Capecitabine		4.2	14

* statistically significant differences; nr: not reported

with a single agent or the sequence of each single agent in anthracycline-pretreated metastatic breast cancer patients. O'Shaughnessy *et al.* (2002) demonstrated a significantly superior time to progression (6.1 vs. 4.2 months, p = 0.0001) and overall survival (14.5 vs. 11.5 months, p = 0.0126) with the addition of capecitabine (1,250 mg/m^2 twice daily on days 1–14) to docetaxel (75 mg/m^2 on day 1), indicating that this combination provides clear benefit over docetaxel as single-agent at 100 mg/m^2. Moreover, the early separation of the survival curves of patients suggested that the combination chemotherapy could prevent early death in a subset of patients who have been heavily pretreated and have a significant tumor burden. This result was reinforced by the recent demonstration of the superiority of the combination of docetaxel and capecitabine over the sequential administration of the two drugs, with an advantage in terms of objective response (68% vs. 40%, p = 0.004), time to progression (9.3 vs. 7.7 months, p = 0.001) and overall survival (22 vs. 19 months, p = 0.006) (Beslija *et al.*, 2006). However, in both studies, there was a higher incidence of gastrointestinal side effects and hand-foot syndrome with the combination therapy. Therefore, Leonard *et al.* (2006) conducted a detailed analysis of the safety profile of the capecitabine/docetaxel combination arm of the former study, evaluating the effect of dose reduction on tolerability and efficacy. This retrospective analysis demonstrated that early capecitabine and docetaxel dose reduction is associated with improved tolerability and fewer treatment interruptions with no evidence of loss of the efficacy advantage over docetaxel as single agent. On this basis, the combined administration of capecitabine at the reduced starting dose of 950 mg/m^2 twice daily and docetaxel at 60 mg/m^2 should be considered a reasonable strategy of treatment for fit patients, with anthracycline-pretreated aggressive MBC. The Mexican Oncology Study Group evaluated the efficacy of the inverse sequence of capecitabine followed by taxanes versus capecitabine/taxane combination in a randomized phase III trial in patients with anthracycline-pretreated MBC. At the interim analysis presented by Soto *et al.* (2006), the sequential therapy was as effective as the combination therapy in terms of progression-free survival and overall survival. However, the objective response rate was significantly higher in the combination arm than the sequential arm ($p \leq 0.01$). The authors concluded that when rapid response is the primary goal, patients should receive a combination treatment with docetaxel or paclitaxel and capecitabine. Albain *et al.* (2004) demonstrated the superiority of the combination of gemcitabine (1,250 mg/m^2 on days 1 and 8) and paclitaxel (175 mg/m^2 on day 1) as compared with paclitaxel alone at the same dose, in terms of time to progression (5.4 vs. 3.5 months, p = 0.0013) and overall survival (18.5 vs. 15.8 months, respectively, p = 0.018). The combination therapy was associated with a more pronounced grade 4 hematological toxicity, while non-hematological toxicity was manageable in both arms. Finally, a European phase III study presented by Chan *et al.* (2005) recently demonstrated that the combination of gemcitabine and docetaxel (GD arm) is an active regimen in anthracycline-pretreated MBC, with similar efficacy to capecitabine plus docetaxel (CD arm) and with a better safety profile. Median progression-free survival was 35

weeks for both treatments (p = 0.28) and the response rate was 32% (p = 0.93). However, gemcitabine plus docetaxel had a better risk-benefit profile in terms of less drug-related discontinuation (13% vs. 28%), less grade 3 hand-foot syndrome (0% vs. 26%), less grade 3–4 diarrhea (7% vs. 18%) and less grade 3–4 mucositis (4% vs. 17%).

Several phase II studies have shown that combination therapies with platinum salts and taxanes in first line treatment of patients with advanced breast cancer are active and well tolerated. In a phase III study, Fountzilas *et al*. (2004a) demonstrated that the combination of paclitaxel and carboplatin shows no significant difference in overall survival as compared with paclitaxel and epirubicin. Interestingly, only 25% of patients in this study had received adjuvant anthracyclines. Therefore, paclitaxel and carboplatin can be safely prescribed to patients previously exposed to anthracyclines in the adjuvant setting or in which anthracyclines have the potential to be harmful. Variable results in terms of response rate have been observed with the combination of cisplatin and taxanes, according to the different schedules used and percentages of anthracycline-resistant patients, with a response rate slightly higher for patients treated with higher doses of cisplatin and docetaxel (ranging from 36% to 56%).

Combinations without anthracyclines and taxanes

Front-line treatments with combinations not containing anthracyclines and taxanes should be considered for those patients who are ineligible or resistant to anthracyclines and taxanes. In this population, no standard chemotherapy has been defined. The combination of gemcitabine and vinorelbine represents an attractive schedule, due to the favorable toxicity profile of the two drugs, and the activity shown in patients with metastatic breast cancer pretreated with anthracyclines and taxanes, as reported by Morabito *et al*. (2003). Interestingly, Martín *et al*. (2007) have recently demonstrated in a randomized phase III study significant efficacy advantages with the combination of the two drugs (gemcitabine at 1,200 mg/m^2 plus vinorelbine 30 mg/m^2 on days 1 and 8) over vinorelbine alone (30 mg/m^2) as first, second, or third line therapy of patients with MBC previously treated with anthracyclines and taxanes: objective response rate was 37% for the combination and 25% for vinorelbine alone (p = 0.035), median progression-free survival was 6.3 months and 4.1 months, respectively (p = 0.0011). Hematological toxicity was significantly higher with the combination of the two drugs, while non-hematological toxicity was low and manageable in both arms. Therefore, the favorable risk-benefit profile, also in these heavily pretreated patients, strongly supports the use of this combination as first-line therapy of MBC patients resistant to anthracyclines and taxanes. However, it has been recently reported by Mavroudis *et al*. (2006) that the same combination (with slight differences in schedule) is not superior to single agent capecitabine. A newer option for these patients is treatment with epothilones, a new class of antineoplastic agents with low susceptibility to tumor resistance mechanisms, and demonstrated clinical activity in patients pretreated with anthracyclines, taxanes, and capecitabine. Vahdat *et al*. (2007) demonstrated in a randomized phase 3 trial that the combination of

ixabepilone and capecitabine has superior efficacy to capecitabine alone in terms of progression-free survival (5.8 vs. 4.2 months, respectively, $p = 0.0003$) and response rate (35% vs. 14%, respectively, $p < 0.0001$), with manageable toxicity.

The combination of vinorelbine and capecitabine represents a further option of treatment, due to the favourable toxicity profile of the two drugs, the convenience of oral drug administration (feasible for both the drugs) and the *in vivo* synergistic activity. Welt *et al.* (2005) reported that this combination has significant antitumor activity in women with advanced breast cancer, even after previous treatment with anthracyclines and taxanes, with a response rate of 55% and a time to progression of 8 months.

Finally, the addition of vinorelbine or gemcitabine to platinum salts has been evaluated in phase II studies and was active in previously treated patients, including high-dose/stem-cell failures. However, despite the high response rates observed, these combinations have been characterized by significant hematological toxicity, often requiring dose reduction and treatment discontinuation.

Combinations with liposomal anthracyclines

No definitive data are available regarding the activity of liposomal formulation of anthracyclines in metastatic breast cancer patients pretreated with conventional anthracyclines. Liposomal anthracyclines (pegylated and non-pegylated) have been developed to improve the therapeutic index of conventional anthracyclines: they maintain the efficacy of conventional anthracyclines and show a favorable toxicity profile, reducing many toxicities associated with these drugs. Moreover, a lack of cross-resistance with free anthracyclines has been reported, supporting the use of liposomal antracyclines in patients previously treated with conventional anthracyclines. In a phase 2 study, Al-Batran *et al.* (2006) evaluated the activity of pegylated liposomal doxorubicin (PLD) as single agent in patients with metastatic breast cancer previously treated with conventional anthracyclines. The overall clinical benefit rate was 24% and there was no significant difference between patients who received PLD > 12 months and those who received PLD ≤ 12 months since last anthracycline treatment for metastatic disease (clinical benefit of 25% vs. 24.1%, respectively). In this setting of patients, a high rate of objective responses has been observed with the combination of pegylated liposomal doxorubicin and docetaxel, paclitaxel, gemcitabine, vinorelbine. However, no phase 3 studies to date have been reported testing these combinations over a standard first line treatment.

Batist *et al.* (2006) reported the results of a retrospective analysis on the efficacy of non-pegylated liposomal doxorubicin as first-line treatment of patients with metastatic breast cancer who had received prior adjuvant doxorubicin. In this analysis, data were pooled from two prospective phase 3 randomized clinical trials, comparing non-pegylated liposomal doxorubicin versus conventional doxorubicin in combination with cyclophosphamide and as single agents, respectively. A significant difference for overall response rates (31% vs. 11%, $p = 0.04$) and median time to treatment failure (4.2 versus 2.1 months, $p = 0.001$) in favour of non-pegylated liposomal doxorubicin, was observed. Moreover, treatment with non-pegylated liposomal doxorubicin

was associated with a significantly reduced risk of developing cardiotoxicity as compared with conventional doxorubicin ($p = 0.001$). These studies support the therapeutic option of including a liposomal doxorubicin (pegylated or not) in a two-drug combination regimen for the treatment of MBC patients who have had prior adjuvant anthracycline exposure. The questions regarding the choice between pegylated or non-pegylated formulations of liposomal doxorubicin and the best combination regimen remain unanswered, and should be resolved by well designed, prospective randomized clinical trials.

CURRENT OPTIONS OF TREATMENT FOR HER-2 POSITIVE MBC PATIENTS

What should be the first-line treatment of choice for HER-2-positive MBC patients, pretreated (or not) with anthracyclines-containing adjuvant regimens and who need to be treated with chemotherapy? The combination of trastuzumab and conventional anthracyclines should be avoided in clinical practice, due to the high risk of cardiac toxicity reported with this schedule. Reasonable options of treatment in this setting of patients are trastuzumab in combination with taxanes, combinations without anthracyclines and taxanes or combinations with liposomal anthracyclines (Table 29.2).

Trastuzumab and anthracyclines

In the pivotal trial of Slamon *et al.* (2001), demonstrating the superior efficacy of addition of trastuzumab to chemotherapy, the trastuzumab-anthracycline combination was associated with a cardiac dysfunction of any grade in 27% of patients, 16% of whom developed cardiac heart failure; while cardiac dysfunction and cardiac heart failure were observed in 8% and 3%, respectively, of patients treated with anthracycline-based chemotherapy alone. Despite the high rate of cardiotoxicity, and due to the high rate of activity, the combination of trastuzumab with anthracyclines-based chemotherapy has remained attractive for clinical research, especially for evaluating the role of epirubicin, which is known to be less cardiotoxic than doxorubicin. Untch *et al.* (2004) conducted a phase 1/2 dose escalation, parallel group trial, to investigate the cardiac safety and the activity of the triple combination of trastuzumab, epirubicin, at two rising doses (60 and 90 mg/m^2), and cyclophosphamide, as first line treatment of non anthracycline pretreated MBC patients (the HERCULES study). The treatment was active, reporting a response rate > 60% in both groups. However, a high incidence of asymptomatic cardiac dysfunction was observed (48% of patients in the lower epirubicin dose group and 52% of patients in the higher dose group), making this triple combination with epirubicin quite toxic. The combination of trastuzumab with epirubicin and docetaxel as first line therapy for MBC was evaluated by Venturini *et al.* (2006), in a single arm phase 2 study, having cardiac safety as a primary end point. One third of patients were anthracycline pretreated. This triple combination produced an overall response rate of 66.7%, but the overall cardiac toxicity incidence was 22%; moreover, 11% of patients experienced a severe cardiac event, inducing the authors to conclude that the relative benefits and the risk of cardiotoxicity associated with this treatment did not compare favourably

TABLE 29.2. Randomized clinical trials dedicated to HER-2-positive metastatic breast cancer patients.

Author, year	Study phase	Pts (no)	Treatment line	Treatment arms	OS (mos)	TTP (mos)	RR (%)	Any grade CD (%)	Severe CD (%)
Slamon *et al*., 2001	Phase III	188	First	Paclitaxel + Trastuzumab	22.1	6.9*	41*	13	2
				vs Paclitaxel	18.4	3	17	1	1
		281	First	Doxorubicin (or Epi) + Cyclophosphamide + Trastuzumab	26.8	7.8*	56*	27	16
				vs Doxorubicin (or Epi) + Cyclophosphamide	21.4	6.1	42	8	3
Marty *et al*., 2005	Phase II	186	First	Docetaxel + Trastuzumab	31.2	11.7	71	17	2.2
				vs Docetaxel	22.7	6.1	34	8	0
Gasparini *et al*., 2007	Phase II	123	First	Paclitaxel + Trastuzumab	nr	9.9	75	1.7	0
				vs Paclitaxel	nr	6.6	56.9	1.7	0
Robert *et al*., 2006	Phase III	196	First	Paclitaxel + Trastuzumab + Carboplatin	nr	10.7*	52*	0	0
				vs Paclitaxel + Trastuzumab	nr	7.1	36	2	2

* statistically significant differences; nr: not reported; CD: cardiac dysfunction

with the reported efficacy and tolerability of schedules that combine trastuzumab with single-agent therapies. A strategy to reduce the risk of cardiotoxicity due to concomitant administration of trastuzumab with anthracyclines, is the sequential use of trastuzumab after completion of an anthracycline based chemotherapy. This strategy is based on the hypothesis that the cardiotoxicity associated with this combination is due to an exacerbation of anthracycline damage by a direct mechanism via the HER-2 protein. This hypothesis has been supported by the evidence that rates of cardiotoxicity appear lower and the syndrome less severe when trastuzumab administration is temporally separated from anthracycline exposure, as observed by Seidman *et al*. (2002). In a phase 2 parallel group trial, Bianchi *et al*. (2003) investigated the safety of doxorubicin plus paclitaxel followed by paclitaxel and trastuzumab, administered either concomitantly with both components or only with paclitaxel, as first line therapy of patients not pretreated with anthracyclines. As expected, patients treated with concomitant trastuzumab and doxorubicin experienced a higher incidence of cardiotoxicity than those treated with sequential therapy (50% vs. 6%). It is worth noting that the incidence of cardiac toxicity was very low with the sequential schedule and that no symptomatic heart failure was reported. Although no conclusions can be drawn because of the small sample size, overall response rate (87.5%) and time to progression (20.4 and 12.8 months for the concomitant and the sequential schedules respectively) were higher than in trials in which trastuzumab was combined with doxorubicin or paclitaxel individually.

Trastuzumab and taxanes

The combination of trastuzumab with taxanes should be the preferred therapeutic option, considering the positive results in terms of efficacy and safety of the randomized trials published in recent years. The efficacy and safety of the combination

of trastuzumab and paclitaxel as front-line therapy of HER-2-positive MBC patients, was first demonstrated by Slamon *et al.* (2001). Four-hundred sixty-nine women, who had not received previous treatment for advanced disease, were randomized to receive chemotherapy or chemotherapy plus weekly trastuzumab. Patients who had received anthracyclines in the adjuvant setting or who were not suitable to receive anthracyclines (n = 188), received 3-weekly paclitaxel. All other patients received an anthracycline plus cyclophosphamide. The addition of trastuzumab to chemotherapy significantly improved all clinical outcomes. Median time to progression was 7.4 months in the combination group, whereas in the group treated with chemotherapy alone it was 4.6 months ($p < 0.001$). Compared with chemotherapy alone, combination treatment was associated with a significantly higher response rate (50% vs. 32%, $p < 0.001$), a longer duration of response (median 9.1 vs. 6.1 months, $p < 0.001$), and a longer time to treatment failure (median 6.9 vs. 4.5 months, $p < 0.001$). The improvement was significant in both the chemotherapy (either paclitaxel or anthracycline based) subgroups. Median survival was 25.1 months in the combination group and 20.3 months in the group that received chemotherapy alone ($p = 0.046$). The risk of death was reduced by 18–20% in the subgroups treated with trastuzumab, but the result might be diluted as it included patients given chemotherapy alone who received open-label trastuzumab after the occurrence of disease progression. Trastuzumab given in combination with chemotherapy resulted in an increased incidence of anemia, leukopenia, diarrhoea, and infections, but these events were not severe and in general were quite manageable. In particular, paclitaxel and trastuzumab were well tolerated, also considering cardiac toxicity that was reported in 13% of patients (severe in only 2% of cases). A successive analysis of health-related quality of life by Osoba *et al.* (2002), revealed that a higher proportion of patients receiving trastuzumab and chemotherapy achieved improvement in global quality of life ($p < 0.05$), as compared to patients treated with chemotherapy alone, providing additional support for the benefit of combination treatment.

The growing interest of improving treatment efficacy and tolerability led to the development of new schedules or combinations. A randomized phase 2 clinical trial of Gasparini *et al.* (2007) demonstrated good tolerability and high activity of the combination of weekly paclitaxel and trastuzumab (response rate 75% and median progression-free survival 9.9 months). Cardiotoxicity with the combination treatment was comparable to that seen with trastuzumab monotherapy. The addition of trastuzumab to docetaxel as first line treatment of MBC was evaluated in a randomized phase II study by Marty *et al.* (2005). One-hundred eighty-eight patients were assigned to receive the combination treatment or 3-weekly docetaxel alone. The combination therapy was shown to be superior to chemotherapy alone in terms of response rate (61% vs. 34%), time to progression (median 11.7 vs. 6.1), time to treatment failure (median 9.8 vs. 5.3 months) and overall survival (31.2 vs. 22.7 months), although a formal comparison between the arms should not be made, because the study had a phase 2 trial design. Hematological toxicity was slightly more common with the combination

than with docetaxel alone, as reported in previous trials. Cardiotoxicity incidence was acceptable (any grade cardiac dysfunction 17%), with one patient in the combination arm experiencing symptomatic heart failure (1%). Notably, another patient experienced congestive heart failure 5 months after discontinuation of trastuzumab because of disease progression and following treatment with an investigational anthracycline for 4 months. Based on this trial, the European Medicine Agency (EMEA) approved the combination of trastuzumab and docetaxel as first-line therapy in HER-2-positive metastatic breast cancer.

Trastuzumab combinations without anthracyclines and/or taxanes

The increasing use of taxanes in the adjuvant setting has heightened the demand for alternative trastuzumab-based treatment options for women with MBC who are not candidates for taxane-containing regimen. The combination of trastuzumab and vinorelbine, that demonstrated synergistic activity in preclinical setting, was successfully evaluated as first line therapy in a phase 2 study by Burstein *et al.* (2003), who reported a 68% of objective responses in this setting of patients. Trastuzumab cardiotoxicity appeared not to be worsened by the addition of vinorelbine, with a maximum reported incidence of severe toxicity of 3%. Bartsch *et al.* (2006) investigated a combination with oral vinorelbine, a formulation that may be more convenient for patients, as first or second line therapy, and reported promising results with a response rate of 53% and a median time to progression of 10 months. Moreover, women treated with this combination therapy did not experience any cardiac adverse event, even if the small sample size has to be considered in drawing conclusions. Based on the activity, safety, and pharmacokinetics data of trastuzumab monotherapy administered on a 3-weekly schedule reported by Baselga *et al.* (2005), an alternative 3-weekly schedule of trastuzumab in combination with intravenous vinorelbine was investigated by De Maio *et al.* (2007). Fifty patients were enrolled, and 40 were eligible for response assessment. Overall response rate was 50%; median progression free and overall survival were 9.6 and 22.7 months respectively. Only 6% of patients experienced a cardiac event, but there were no severe events. This schedule was shown to be equally active and no more toxic than weekly schedule, and may be more convenient for patients.

O'Shaughnessy *et al.* (2004) evaluated the activity and safety of the combination of trastuzumab and gemcitabine in patients who were pretreated with anthracyclines and/or taxanes. Overall response rate was ~ 40%, and no symptomatic heart failure was reported, suggesting that this combination is also active and safe in this unfavorable setting. Modest activity was shown by Pegram *et al.* (1998) with the combination of trastuzumab and cisplatin (response rate 24%) in a single trial, which included heavily pretreated patients. There was no evidence of greater cardiotoxicity with this combination than trastuzumab monotherapy, with an incidence of severe cardiac events of 2.6%. A strong preclinical rationale exists for combining trastuzumab and capecitabine, and in a recently published multicenter Japanese trial, Yamamoto *et al.* (2007) confirmed that this combination is active as first or subsequent line of treat-

ment in MBC. Overall response rate was 50% while it was 65% in patients treated with trastuzumab and capecitabine as first-line therapy. No overlapping toxicitites were reported and a grade 1 cardiac adverse event was observed in one patient only.

Trastuzumab and polychemotherapy

The favourable safety profile exhibited by the combinations of trastuzumab plus single agent chemotherapy prompted researchers to evaluate the addition of the antibody to polychemotherapy. Most of the studies investigated combinations with a taxane and a platinum salt. This triple combination showed high response rate, ranging from 65% to 85% in first line therapy and being > 50% also in second line. Pegram *et al*. (2004) conducted two parallel non-randomized phase II studies to evaluate the combination of trastuzumab with docetaxel plus cisplatin or carboplatin in the first or second line treatment of HER-2 overexpressing MBC. Both triple combinations appeared to be highly active and obtained favourable median times to progression (9.9 and 12.7 months, respectively). Toxicity was manageable and consistent with that observed in other clinical settings for the combination of docetaxel plus platinum salts. Finally, a low incidence of clinically significant cardiac dysfunction was reported (2% of severe cardiac events with both combinations) and, as with other trastuzumab-based regimens, the cardiac dysfunction appeared to be reversible with appropriate clinical management. Similarly, Perez *et al*. (2005) evaluated in two parallel studies the addition of 3-weekly trastuzumab to paclitaxel and carboplatin both given weekly or 3-weekly as first line treatment. These schedules were highly active, with a median progression-free survival of 13.8 months for the weekly infusion and 9.9 for the 3-weekly one. Substantial differences emerged between the two schedules with respect to toxicities, with both hematological and non-hematological toxicity profiles favoring the weekly schedule. However, no patient developed symptomatic heart failure in either studies. In a phase 3 trial, Robert *et al*. (2006) evaluated the efficacy of the addition of carboplatin to trastuzumab and paclitaxel, demonstrating an improvement of tumor response rate (36% vs. 52%), the primary objective of the study, and of progression-free survival (10.7 vs. 7.1 months). Only two cases of severe cardiotoxicity were reported in patients treated with trastuzumab plus paclitaxel, confirming the tolerability and the higher activity of a triple combination with taxanes and platinum salts.

The combination of trastuzumab, gemcitabine, and taxanes as first line of treatment of MBC patients was also investigated by Fountzilas *et al*. (2004b) and in other quite small phase II trials, obtaining objective response rate from 45% to 65% and showing a good safety profile, with an incidence of severe cardiac events from 0% to 7%. A single small trial performed by Stemmler *et al*. (2005) evaluated the combination of trastuzumab with gemcitabine plus cisplatin in pretreated MBC patients: response rate was 40%, with a median time to progression of 10.2 months and no cardiotoxicity was observed. Morabito *et al*. (2006a) explored the activity and safety of the addition of trastuzumab to gemcitabine plus vinorelbine for patients with HER-2 overexpressing MBC, pretreated with anthracyclines and/or taxanes and/or trastuzumab. Treatment was well

tolerated, with no cardiac events reported. The response rate was 50%, even in this unfavourable setting, with a median progression-free survival of 7 months. It is worth noting that treatment activity was higher in patients overexpressing HER-2 at 3+ by IHC (response rate 73%), and this result is consistently reported in all the studies that provided a subgroup analysis according to HER-2 status.

Trastuzumab and liposomal anthracyclines

The feasibility of a combination of trastuzumab with pegylated liposomal doxorubicin as first-line therapy of MBC was assessed by Chia *et al*. (2006). In this trial, 43% of patients had received previous adjuvant anthracyclines. The combination was active, with an overall response rate of 52% and a median progression-free survival of 12 months, and exhibited a good safety profile, with an overall incidence of cardiotoxicity in 10% of patients and no congestive heart failure. Phase 2 studies are evaluating the activity and safety of a triple combination of trastuzumab and liposomal pegylated doxorubicin with cyclophosphamide or with docetaxel. The feasibility of trastuzumab and non-pegylated liposomal doxorubicin was demonstrated by Theodoulou *et al*. (2002), in advanced breast cancer patients, in a phase 1–2 study. A very high percentage of objective responses (92.6%) was later reported by Cortes *et al*. (2004), with a triple combination of trastuzumab, weekly paclitaxel and non-pegylated liposomal doxorubicin, in patients with locally advanced or metastatic breast cancer. Moreover, no case of symptomatic cardiac disease was reported with this combination. Two phase 2 studies are currently evaluating the activity of trastuzumab, docetaxel and non-pegylated liposomal doxorubicin in patients with MBC.

CONCLUSIONS AND FUTURE PERSPECTIVES

The shift in the use of anthracyclines and, recently, also of taxanes from the metastatic to the adjuvant treatment of patients affected by breast cancer, is going to progressively increase the number of patients who present with disease recurrence after an adjuvant treatment with these agents. Therefore, treatment of patients with metastatic disease, pretreated with anthracyclines or with anthracyclines and taxanes as adjuvant regimens, represents a significant challenge for oncologists (Figure 29.1). For patients with HER-2-negative MBC considered resistant to anthracyclines and taxanes, no standard chemotherapy has been defined: the combinations of gemcitabine and vinorelbine could be an attractive schedule due to the activity of the two drugs and the favorable toxicity profile. The role of liposomal anthracyclines in this setting needs to be defined, as well as the preferred liposomal formulation (pegylated or non) and the optimal schedule of combination. For patients considered eligible for taxane-containing treatment, a conventional anthracycline should not be included in this combination because it does not improve prognosis. The favorable results of the capecitabine-docetaxel, gemcitabine-paclitaxel, and docetaxel-gemcitabine trials support the use of these combinations as front-line therapy of metastatic breast cancer patients, while docetaxel as single agent or the sequential administra-

tion of docetaxel and capecitabine should be proposed to patients with "less" aggressive disease or at higher risk of toxicity.

For patients with HER-2-positive MBC the combination of trastuzumab and taxanes can be considered a standard option of treatment, while the combination of trastuzumab and vinorelbine can be considered for those patients considered resistant to anthracyclines and taxanes. No definitive data are available regarding the superiority of a 3-drug regimen over a combination of trastuzumab with a single chemotherapeutic agent and regarding the combination of liposomal anthracyclines and trastuzumab.

A large number of molecular-targeted drugs, actually in clinical development in breast cancer, could become part of a standard strategy of first line treatment of patients in the next few years. Bevacizumab, a humanized monoclonal antibody directed against the vascular endothelial growth factor (VEGF)-A ligand, is the most mature target-based agent with anti-angiogenetic activity. After the negative results of the first phase III trial, comparing bevacizumab and capecitabine versus capecitabine alone in pretreated MBC patients, a large, international phase III trial of the Eastern Cooperative Oncology Group (E2100) met the primary end point: in this study Miller *et al*. (2005) showed that the addition of bevacizumab to paclitaxel clearly improved the response rate and the progression-free survival (from 6.1 months to nearly 11 months) in patients with metastatic breast cancer, as compared to paclitaxel monotherapy. Another opportunity of treatment targeting the VEGF pathway is represented by the small molecule inhibitors of VEGF receptors, as reported by a review of Morabito *et al*. (2006b). Sunitinib, a multitargeted receptor tyrosine kinase inhibitor with both direct antiproliferative effects and antiangiogenic properties, targeting the VEGFRs, PDGFR-β, and c-Kit, is being evaluated in metastatic breast cancer patients resistant to anthracyclines and taxanes. Preliminary data show a good safety profile and interesting activity. Several phase III trials are ongoing to evaluate the efficacy of the addition of sunitinib to docetaxel, trastuzumab, capecitabine and to compare sunitinib with chemotherapy or the combination of sunitinib and paclitaxel with paclitaxel and bevacizumab. Finally, although agents directed against the epidermal growth factor receptor (EGFR) have shown promising clinical activity, initial phase 2 studies have suggested that the EGFR tyrosine kinase inhibitors, gefitinib and erlotinib, are not sufficiently active in heavily pretreated metastatic breast cancer patients. Conversely, lapatinib, an oral, small-molecule, dual inhibitor, that blocks downstream signaling pathways of EGFR and HER-2, through inhibition of receptors' autophosphorylation sites, has been shown to significantly improve progression-free survival, when added to capecitabine as compared to capecitabine alone in patients with advanced HER-2-positive breast cancer that had progressed after trastuzumab-based therapy (Geyer *et al*., 2006). However, no significant differences in response rate, time to progression, and overall survival have been demonstrated in HER-2-negative advanced breast cancer patients treated with the combination of paclitaxel and lapatinib as compared with paclitaxel (Di Leo *et al*., 2007). The superiority of the schedule with lapatinib in terms of response rate and TTP has been observed only in a small subgroups of patients whose tumors were HER-2-positive after inclusion in the study, suggesting that the activity

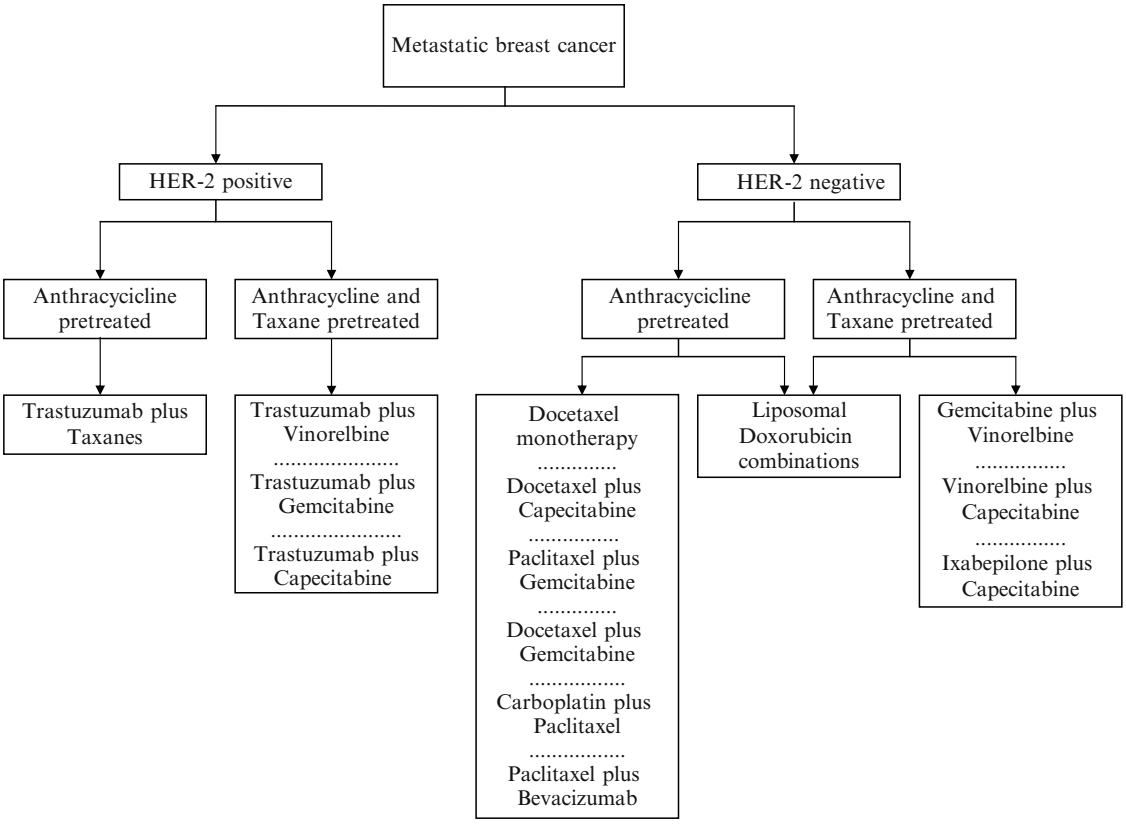

FIGURE 29.1. First-line treatment options for metastatic breast cancer patients pretreated with adjuvant anthracyclines

of this agent is limited to HER-2-positive patients. Several combinations of lapatinib with other agents are currently being tested in clinical trials and should better define the role of lapatinib for breast cancer in metastatic and adjuvant setting.

In conclusion, although current chemotherapy has impacted on survival of MBC patients, there is a common feeling that the research of more or less innovative combinations or sequences of known chemotherapeutic agents are not likely to significantly impact the outcome of metastatic breast cancer patients who have failed most effective drugs in adjuvant. Breast cancer is a heterogeneous disease, characterized by tumor-specific mutations and deregulated cellular pathways. Targeting these pathways with novel agents and combining these agents with chemotherapy may be the best way to fight breast cancer in the future.

REFERENCES

Albain, K.S., Nag, S., Calderillo-Ruiz, G., Jordaan, J.P, Llombart, A., Pluzanska, A., Pawlicki, M., Melemed, A.S., O'Shaughnessy, J., and Reyes J.M. 2004. Global phase III study of gemcitabine plus paclitaxel (GT) vs. paclitaxel (T) as frontline therapy for metastatic breast cancer (MBC): first report of overall survival. *J. Clin. Oncol. 22*: 14 S (Abstr.510).

Al-Batran, S.E., Bischoff, J., von Minckwitz, G., Atmaca, A., Kleeberg, U., Meuthen, I., Morack, G., Lerbs, W., Hecker, D., Sehouli, J.,

Knuth, A., and Jager, E. 2006. The clinical benefit of pegylated liposomal doxorubicin in patients with metastatic breast cancer previously treated with conventional anthracyclines: a multicentre phase II trial. *Br. J. Cancer 94*: 1615–1620.

Bartsch, R., Wenzel, C., Pluschnig, U., Hussian, D., Sevelda, U., Locker, G.J., Mader, R., Zielinski, C.C., and Steger, G.G. 2006. Oral vinorelbine alone or in combination with trastuzumab in advanced breast cancer: results from a pilot trial. *Cancer Chemother. Pharmacol. 57*: 554–558.

Baselga, J., Carbonell, X., Castañeda-Soto, N.J., Clemens, M., Green, M., Harvey, V., Morales, S., Barton, C., and Ghahramaniet, P. 2005. Phase II study of efficacy, safety, and pharmacokinetics of trastuzumab monotherapy administered on a 3-weekly schedule. *J. Clin. Oncol. 23*: 2162–2171.

Batist, G., Harris, L., Azarnia, N., Lee, L.W., and Daza-Ramirez, P. 2006. Improved anti-tumor response rate with decreased cardiotoxicity of non-pegylated liposomal doxorubicin compared with conventional doxorubicin in first-line treatment of metastatic breast cancer in patients who had received prior adjuvant doxorubicin: results of a retrospective analysis. *Anticancer Drugs 17*: 587–595.

Beslija, S., Obralic, N., Basic, H., Tatarevic, A., Naila, M., Banjin, M., Cardzic, A., Sosevic, A., Pasic, A., Ceric, T., and Salkic, B. 2006. Randomized trial of sequence vs. combination of capecitabine (X) and docetaxel (T): XT vs T followed by X after progression as first-line therapy for patients (pts) with metastatic breast cancer (MBC). *J. Clin. Oncol. 24*: 18 S (Abstr.571).

Bianchi, G., Albanell, J., Eiermann, W., Vitali, G., Borquez, D., Vigano', L., Molina, R., Raab, G., Locatelli, A., Vanhauwere, B., Gianni, L., and Baselga, J. 2003. Pilot trial of trastuzumab starting with or after the doxorubicin component of a doxorubicin plus paclitaxel regimen for women with HER2-positive advanced breast cancer. *Clin. Cancer Res. 9*: 5944–5951.

Burstein, H.J., Harris, L.N., Marcom, P.K., Lambert-Falls, R., Havlin, K., Overmoyer, B., Friedlander, Jr., R.J., Gargiulo, J., Strenger, R., Vogel, C.L., Ryan, P.D., Ellis, M.J., Nunes, R.A., Bunnell, C.A., Campos, S.M., Hallor, M., Gelman, R., and Wineret, E.P. 2003. Trastuzumab and vinorelbine as first-line therapy for HER2-overexpressing metastatic breast cancer: multicenter phase II trial with clinical outcomes, analysis of serum tumor markers as predictive factors, and cardiac surveillance algorithm. *J. Clin. Oncol. 21*: 2889–2895.

Chan, S., Romieu, G., Huober, J., Delozier, T., Tubiana–Hulin, M., Lluch, A., Schneeweiss, A., Llombart, A., Carrasco, E., and Fumoleau, P. 2005. Gemcitabine plus docetaxel (GD) versus capecitabine plus docetaxel (CD) for anthracycline-pretreated metastatic breast cancer (MBC) patients (pts): results of a European phase III study. *J. Clin. Oncol. 23*: 16 S (Abstr. 581).

Chia, S., Clemons, M., Martin, L.A., Rodgers, A., Gelmon, K., Pond, G.R., and Panasciet, L. 2006. Pegylated liposomal doxorubicin and trastuzumab in HER-2 overexpressing metastatic breast cancer: a multicenter phase II trial. *J. Clin. Oncol. 24*: 2773–2778.

Cortés, J., Climent, M.A., Lluch, A., Hornedo, J., Gascón, P., Mayordomo, J., Gil, M., Benavides, M., Regueiro, P., Trigo, J., and Baselga, J. on behalf of the Solti Group. 2004. Updated results of a phase II study (M77035) of Myocet® combined with weekly Herceptin® and paclitaxel in patients with HER2-positive locally advanced or metastatic breast cancer (LABC/MBC). 27th Annual San Antonio Breast Cancer Symposium; (Abstr. 3041).

De Maio, E., Pacilio, C., Gravina, A., Morabito, A., Di Rella, F., Labonia, V., Landi, G., Nuzzo, F., Rossi, E., Silvestro, P., Botti, G., Di Bonito, M., Curcio, M.P., Formichelli, F., La Vecchia, F., Staiano, M., Maurea, N., D'Aiuto, G., D'Aiuto, M., Thomas, R., Signoriello, G., Perrone, F., and de Matteis, A. 2007. Vinorelbine plus 3-weekly trastuzumab in metastatic breast cancer. A single-centre phase 2 trial. *BMC Cancer 7*: 50.

Di Leo A, Gomez H, Aziz Z, Zvirbule, Z., Arbushites, M., Oliva, C.R., Koehler, M., Williams, L.S., Dering, J., and Finn, R.S. 2007. Lapatinib (L) with paclitaxel compared to paclitaxel as first-line treatment for patients with metastatic breast cancer: a phase III randomized double-blind study of 580 patients. *J. Clin. Oncol. 25*: 18 S (Abstr.1011).

Fountzilas, G., Kalofonos, H.P., Dafni, V., Papadimitriou, C., Bafaloukos, D., Papakostas, P., Kalogera-Fountzila, A., Gogas, H., Aravantinos, G., Moulopoulos, L.A., Economopoulos, T., Pectasides, D., Maniadakis, N., Siafaka, V.,

Briasoulis, E., Christodoulou, C., Tsavdaridis, D., Makrantonakis, P., Razis, E., Kosmidis, P., Skarlos, D., and Dimopoulos, M.A. 2004a. Paclitaxel and epirubicin versus paclitaxel and carboplatin as first line chemotherapy in patients with advanced breast cancer: a phase III study conducted by the Hellenic Cooperative Oncology Group. *Ann. Oncol. 15*: 1517–1526.

Fountzilas, G., Christodoulou, C., Tsavdaridis, D., Kalogera-Fountzila, A., Aravantinos, G., Razis, E., Kalofonos, H.P., Papakostas, P., Karina, M., Gogas, H., and Skarlos, D. 2004b. Paclitaxel and gemcitabine, as first-line chemotherapy, combined with trastuzumab in patients with advanced breast cancer: a phase II study conducted by the Hellenic Cooperative Oncology Group (HeCOG). *Cancer Invest. 22*: 655–662.

Gasparini, G., Gion, G., Mariani, L., Papaldo, P., Crivellari, D., Filippelli, G., Morabito, A., Silingardi, V., Torino, F., Spada, A., Cancan, M., De Sio, L., Caputo, A., Cognetti, F., Lambiase, A., and Amadori, D. 2007. Randomized Phase II Trial of weekly paclitaxel alone versus trastuzumab plus weekly paclitaxel as first-line therapy of patients with Her-2 positive advanced breast cancer. *Breast Cancer Res. Treat. 101*: 355–365.

Gennari, A., Bruzzi, P., Orlandini, C., Salvatori, B., Donati, S., Landucci, E., Guarneri, V., Rondini, M., Ricci, S., and Conte, P. 2004. Activity of first-line epirubicin and paclitaxel in metastatic breast cancer is independent of type of adjuvant therapy. *Br. J. Cancer 90*: 962–967.

Geyer, C.E., Forster, J., Lindquist, D., Chan, S., Romieu, C.G., Pienkowski, T., Jagiello - Gruszfeld, A., Crown, J., Chan, A., Kaufman, B., Skarlos, D., Campone, M., Davidson, N., Berger, M., Oliva, C., Rubin, S.D., Stein, S., and Cameron, D. 2006. Lapatinib plus capecitabine for HER2-positive advanced breast cancer. *N. Engl. J. Med. 355*: 2733–2743.

Jones, S.E., Erban, J., Overmoyer, B., Budd, G.T., Hutchins, L., Lower, E., Laufman, L., Sundaram, S., Urba, W.J., Pritchard, K.I., Mennel, R., Richards, D., Olsen, S., Meyers, M.L., and Ravdin, P.M. 2005. Randomized phase III trial of docetaxel compared with paclitaxel in metastatic breast cancer. *J. Clin. Oncol. 23*: 5542–5551.

Kardinal, C.G., Perry, M.C., Korzun, A.H., Rice, M.A., Ginsberg, S., and Wood, W.C. 1988. Responses to chemotherapy or chemohormonal therapy in advanced breast cancer patients treated previously with adjuvant chemotherapy. *Cancer 61*: 415–419.

Leonard, R., O'Shaughnessy, J., Vukelija, S., Gorbounova, V., Chan-Navarro, C.A., Maraninchi, D., Barak-Wigler, N., McKendrick, J.J., Harker, W.G., Bexon, A.S., and Twelves, C. 2006. Detailed analysis of a randomized phase III trial: can the tolerability of capecitabine plus docetaxel be improved without compromising its survival advantage? *Ann. Oncol. 17*: 1379–1385.

Martín, M., Ruiz, A., Muñoz, M., Balil, A., García-Mata, J., Calvo, L., Carrasco E., Mahillo, E., Casado, A., Ángel García-Saenz, J., Escudero, M.J., Guillem, V., Jara, C., Ribelles, N., Salas, F., Soto, C., Morales-Vasquez, F., Rodríguez, C.A., Adrover, E., Mel, J.R. 2007. Gemcitabine plus vinorelbine versus vinorelbine monotherapy in patients with metastatic breast cancer previously treated with anthracyclines and taxanes: final results of the phase III Spanish Breast Cancer Research Group (GEICAM) trial. *Lancet Oncol. 8*: 219–225.

Marty, M., Cognetti, F., Maraninchi, D., Snyder, R., Mauriac, L., Tubiana-Hulin, M., Chan, S., Grimes, D., Anto'n, A., Lluch, A., Kennedy, J., O'Byrne, K., Conte, P.F., Green, M., Ward, C., Mayne, K., and Extra, J.M. 2005. Randomized phase II trial of the efficacy and safety of trastuzumab combined with docetaxel in patients with human epidermal growth factor receptor 2–positive metastatic breast cancer administered as first-line treatment: the M77001 Study Group. *J. Clin. Oncol. 23*: 4265–4274.

Mavroudis, D., Ardavanis, A., Boukovinas, I., Varthalitis, I., Syrigos, K., Potamianou, A., Kouroussis, C., and Georgoulias, V. 2006. A multicenter randomized study comparing vinorelbine plus gemcitabine versus capecitabine monotherapy as salvage treatment in patients with advanced breast cancer pretreated with taxane and anthracycline chemotherapy: A preliminary report. *J. Clin. Oncol. 24*: 18 S (Abstr.658).

Miller, K.D., Wang, M., Gralow, J., Dickler, M., Cobleigh, M.A., Perez, E.A., Shenkier, T.N., and Davidson, N.E. 2005. A randomized phase III trial of paclitaxel versus paclitaxel plus bevacizumab as first-line therapy for locally recurrent or metastatic

breast cancer: a trial coordinated by the Eastern Cooperative Oncology Group (E2100). *Breast Cancer Res. Treat. 94* (Suppl 1): S6 Abstr.

Morabito, A., Filippelli, G., Palmeri, S., Cascinu, S., Ferraù, F., Zagonel, V., Gattuso, D., Catalano, V., Capaccetti, B., Franciosi, V., Accurso, V., Scinto, F., and Gasparini, G. 2003. The combination of gemcitabine and vinorelbine is an active regimen as second-line therapy in patients with metastatic breast cancer pretreated with taxanes and/or anthracyclines: a phase I-II study. *Breast Cancer Res. Treat. 78*: 29–36.

Morabito, A., Longo, R., Gattuso, D., Carillio, G., Massaccesi, C., Mariani, L., Bonginelli, P., Amici, S., De Sio, L., Fanelli, M., Torino, F., Monsignori, M., Gasparini, G. 2006a. Trastuzumab in combination with gemcitabine and vinorelbine as second-line therapy for HER-2/neu overexpressing metastatic breast cancer. *Oncol. Rep. 16*: 393–398.

Morabito, A., De Maio, E., Di Maio, M., Normanno, N., and Perrone, F. 2006b. Tyrosine kinase inhibitors of vascular endothelial growth factor receptors in clinical trials: current status and future directions. *Oncologist 11*: 753–764.

Nabholtz, J.M., Senn, H.J., Bezwoda, W.R., Melnychuk, D., Deschênes, L., Douma, J., Vandenberg, T.A., Rapoport, B., Rosso, R., Trillet-Lenoir, V., Drbal, J., Molino, A., Nortier, J.W.R., Richel, D. J., Nagykalnai, T., Siedlecki, P., Wilking, N., Genot, J.Y., Hupperets, P.S.G.J., Pannuti, F., Skarlos, D., Tomiak, E.M., Murawsky, M., Alakl, M., Riva, A., and Aapro, M. The 304 Study Group. 1999. Prospective randomized trial of docetaxel versus mitomycin plus vinblastine in patients with metastatic breast cancer progressing despite previous anthracycline-containing chemotherapy. *J. Clin. Oncol. 17*: 1413–1424.

O'Shaughnessy, J., Miles, D., Vukelja, S., Moiseyenko, V., Ayoub, J.P., Cervantes, G., Fumoleau, P., Jones, S., Lui, W.Y, Mauriac, L., Twelves, C., Van Hazel, G., Verma, S., and Leonard, R. 2002. Superior survival with capecitabine plus docetaxel combination therapy in anthracycline-pretreated patients with advanced breast cancer: phase III trial results. *J. Clin. Oncol. 20*: 2812–2823.

O'Shaughnessy, J.A., Vukelja, S., Marsland, T., Kimmel, G., Ratnam, S., and Pippen, J.E. 2004. Phase II study of trastuzumab plus gemcitabine in chemotherapy-pretreated patients with metastatic breast cancer. *Clin. Breast Cancer 5*: 142–147,

Osoba, D., Slamon, D.J., Burhmore, M., and Murphy, M. 2002. Effects on quality of life of combined trastuzumab and chemotherapy in women with metastatic breast cancer. *J. Clin. Oncol. 20*: 3106–3113.

Pacilio, C., Morabito, A., Nuzzo, F., Gravina, A., Labonia, V., Landi, G., Rossi, E., De Maio, E., Di Maio, M., D'Aiuto, G., Botti, G., Normanno, N., Chiodini, P., Gallo, C., Perrone, F., and de Matteis, A. on behalf of the NCI-Naples Breast Cancer Group. 2006. Is epirubicin effective in first line chemotherapy of metastatic breast cancer (MBC) after an epirubicin-containing adjuvant treatment? A single centre phase III trial. *Br. J. Cancer 94*: 1233–1236.

Pegram, M.D., Lipton, A., Hayes, D.F., Weber, B.L., Baselga, J.M., Tripathy, D., Baly, D., Baughman, S.A., Twaddel, T., Glaspy, J.A., and Slamon, D.J. 1998. Phase II study of receptor-enhanced chemosensitivity using recombinant humanized anti-p185 HER2/neu monoclonal antibody plus cisplatin in patients with HER2/neu-overexpressing metastatic breast cancer refractory to chemotherapy treatment. *J. Clin. Oncol. 16*: 2659–2671.

Pegram, M.D., Pienkowski, T., Northfelt, D.W., Eiermann, W., Patel, R., Fumoleau, P., Quan, E., Crown, J., Toppmeyer, D., Smylie, M., Riva, A., Blitz, S., Press, M.F., Reese, D., Lindsay, M.A., and Slamon, D.J. 2004. Results of two open-label, multicenter phase II studies of docetaxel, platinum salts, and trastuzumab in HER2-positive advanced breast cancer. *J. Natl. Cancer Inst. 96*: 759–769.

Perez, E.A., Suman, V.J., Rowland, K.M., Ingle, J.N., Salim, M.C.L., Flynn, P.J., Mailliard, J.A., Kardinal, C.G. Krook, J.E., Thrower, A.R., Visscher, D.W., and Jenkins, R.B. 2005. Two concurrent phase II trials of paclitaxel/carboplatin/trastuzumab (weekly or every-3-week schedule) as first-line therapy in women with HER2-overexpressing metastatic breast cancer: NCCTG study 983252. *Clin. Breast Cancer 6*: 425–432.

Pierga, J.Y., Asselain, B., Jouve, M., Diéras, V., Carton, M., Laurence, V., Girre, V., Beuzeboc, P., Palangié, T., Dorval, T., and Pouillart, P. 2001.

Effect of adjuvant chemotherapy on outcome in patients with metastatic breast carcinoma treated with first-line doxorubicin-containing chemotherapy. *Cancer 91*: 1079–1089.

Robert, N., Leyland-Jones, B., Asmar, L., Belt, R., Ilegbodu, D., Loesch, D., Raju, R., Valentine, E., Sayre, R., Cobleigh, M., Albain, K., McCullough, C., Fuchs, L., and Slamon, D.J. 2006. Randomized phase III study of trastuzumab, paclitaxel, and carboplatin compared with trastuzumab and paclitaxel in women with HER-2–overexpressing metastatic breast cancer. *J. Clin. Oncol. 24*: 2786–2792.

Seidman, A., Hudis, C., Pierri, M.K., Shak, S., Paton, V., Ashby, M., Murphy, M., Stewart, S.J., and Keefe, D. 2002. Cardiac dysfunction in the trastuzumab clinical trials experience. *J. Clin. Oncol. 20*: 1215–1221.

Sjöström, J., Blomqvist, C., Mourisden, H., Pluzanska, A,. Ottosson-Lönn, S., Bengtsson, N.O., Ostenstad, B., Mjaaland, I., Palm-Sjövall, M., Wist, E., Valvere, V., Anderson, H., and Bergh, J. 1999. Docetaxel compared with sequential methotrexate and 5-fluorouracil in patients with advanced breast cancer after anthracycline failure: A randomised phase III study with crossover on progression by the Scandinavian Breast Group. *Eur. J. Cancer 35*: 1194–1200.

Slamon, D.J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., Fleming, T., Eiermann, W., Wolter, J., Pegram, M., Baselga, J., and Norton, L. 2001. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N. Engl. J. Med. 344*: 783–792.

Soto, C., Torrecillas, S., Reyes, S., Ramirez, M., Perez, L., Cervantes, G., Gonzalez, F., Tellez, E., Cortes, P., and Benitez. H. 2006. Capecitabine (X) and taxanes in patients (pts) with anthracycline-pretreated metastatic breast cancer (MBC): sequential vs.combined therapy results from a MOSG randomized phase III trial. *J. Clin. Oncol. 24*: 18 S (Abstr. 570).

Stemmler, H.J., Kahlert, S., Brudler, O., Beha, M., Muller, S., Stauch, B., Heinemann, V. 2005. High efficacy of gemcitabine and cisplatin plus trastuzumab in patients with HER2-overexpressing metastatic breast cancer: a phase II study. *Clin. Oncol. 17*: 630–635.

Theodoulou, M., Campos, S.M., Batist, G., Winer, E., Norton, L., Hudis, C., and Welles, L. 2002. TLC D99 (D, Myocet) and Herceptin (H) is safe in advanced breast cancer (ABC): final cardiac safety and efficacy analysis. *J. Clin. Oncol. 21*: (Abstr.216).

Untch, M., Himsl, I., Kahlert, S., Lueck, H.J., Eidtmann, H., Du Bois, A., Meerpohl, H.G., Thomssen, C., Harbeck, N., Jackisch, C., Kreienberg, R., Emons, G., Wallwiener, D., Wiese, W., Schaller, G., Kuhn, W., Muscholl, M., Pauschinger, M., and Langer, B. 2004. Anthracycline and trastuzumab in breast cancer treatment. *Oncology 18*: 59–64.

Vahdat, L.T., Thomas, E., Li R., Jassem, J., Gomez, H., Chung, H., Peck, R., Mukhopadhyay, P., Klimovsky, J., and Roché, H. 2007. Phase III trial of ixabepilone plus capecitabine compared to capecitabine alone in patients with metastatic breast cancer (MBC) previously treated or resistant to an anthracycline and resistant to taxanes. *J. Clin. Oncol. 25*: 18 S (Abstr. 1006).

Venturini, M., Bruzzi, P., Del Mastro, L., Garrone, O., Bertelli, G., Guelfi, M., Pastorino, S., Rosso, R. and Sertoli, M.R. 1996. Effect of adjuvant chemotherapy with or without anthracyclines on the activity and efficacy of first-line cyclophosphamide, epidoxorubicin, and fluorouracil in patients with metastatic breast cancer. *J. Clin. Oncol. 14*: 764–773.

Venturini, M., Bighin, C., Monfardini, S., Cappuzzo, F., Olmeo, N., Durando, A., Puglisi, F., Nicoletto, O., Lambiase, A., and Del Mastro, L. 2006. Multicenter phase II study of trastuzumab in combination with epirubicin and docetaxel as first-line treatment for HER2-overexpressing metastatic breast cancer. *Breast Cancer Res. Treat. 95*: 45–53.

Welt, A., von Minckwitz, G., Oberhoff, C., Borquez, D., Schleucher, R., Loibl, S., Harstrick, A., Kaufmann, M., Seeber, S., and Vanhoefer, U. 2005. Phase I/II study of capecitabine and vinorelbine in pretreated patients with metastatic breast cancer. *Ann. Oncol. 16*: 64–69.

Yamamoto, D., Iwase, S., Kitamura, K., Odagiri, H.. Yamamoto, C., and Nagumo, Y.A. 2008. Phase II study of trastuzumab and capecitabine for patients with HER2-overexpressing metastatic breast cancer: Japan Breast Cancer Research Network (JBCRN) 00 Trial. *Cancer Chemother. Pharmacol. 61*: 509–514.

30
Estrogen Receptor-Negative and HER-2/neu-Positive Locally Advanced Breast Carcinoma: Therapy with Paclitaxel and Granulocyte-Colony Stimulating Factor

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HORMONE RECEPTOR STATUS AND RESPONSE TO CHEMOTHERAPY

Most investigators have identified negative hormone receptor status as the strongest predictive factor of response to neoadjuvant chemotherapy. Subset analyses taking hormone receptor status into account were performed in several trials evaluating the role of neoadjuvant chemotherapy in either large operable or locally advanced breast cancer. There is a general agreement that the benefit of aggressive anthracycline/taxane-based chemotherapy is much more evident in estrogen receptor (ER)-negative patients, whereas a very modest therapeutic gain can be obtained with this approach in ER-positive patients. In a European cooperative trial, (Gianni *et al*., 2005) doxorubicin/cyclophosphamide followed by paclitaxel chemotherapy induced a 23% pathological Complete Response (pCR) rate in the breast (20% in breast plus axilla). The pCR rate was 10% in ER-positive and 45% in ER-negative patients. In the multivariate analysis, only ER status was significantly associated with pathologic complete response (odds ratio for ER negative, 5.77; 95% confidence interval, 3.49–9.52; $P < 0.0001$).

Untch *et al*. (2002) compared a dose-dense (q2wk) sequence of three cycles of epirubicin (150 mg/m2) followed by three cycles of paclitaxel (250 mg/m2) with four every 3 weeks cycles of a combination of both drugs (Epi 90 mg/m^2 and Paclitaxel 175 mg/m^2). Treatment had the same duration of 12 weeks, but the total dose for both drugs was higher in the sequential dose-dense treatment (EPI 450 vs. 270 mg/m^2; Paclitaxel 750 vs. 525 mg/m^2). The pCR rate (absence of invasive tumor in the breast and axilla) was significantly

higher in the sequential dose-dense arm (18% vs. 10%). Also, in this study ER-negative patients showed a much higher probability of achieving a pCR (25% vs. 8%; $p < 0.0001$).

To evaluate the prognostic impact of the addition of paclitaxel to doxorubicin-based neoadjuvant chemotherapy, Mazouni *et al.* (2007) performed a pooled analysis of results from seven consecutive neoadjuvant chemotherapy trials conducted at M.D. Anderson Cancer Center from 1974 to 2001, and including 1,079 patients. Four hundred and twenty-six (39.5%) patients received taxane-based neoadjuvant therapy. pCR rates and survival times were analyzed as a function of chemotherapy regimen and ER status. Multivariate logistic and Cox regression analysis were carried out to identify variables associated with pCR and survival. Patients with ER-negative cancer had higher overall pCR rate than patients with ER-positive tumors (20.1% vs. 4.9%, $P < 0.001$). In ER-negative patients, the pCR rates were 29% and 15% with or without a taxane ($P < 0.001$). In ER-positive patients, the pCR rates were 8.8% and 2.0% with or without a taxane ($P < 0.001$). In multivariate analysis, clinical tumor size ($P < 0.001$), ER-negative status ($P < 0.001$) and inclusion of a taxane ($P = 0.01$) were independently associated with pCR. For patients with pCR, survival was similar regardless of ER status or the type of regimen that induced pCR.

Similar data had been reported by Cristofanilli *et al.* (2004) in a retrospective analysis of 240 inflammatory breast cancer patients treated at the same Institution in six consecutive trials between 1973 and 2000. Patients were treated with Fluorouracil, Adriamycin, Cyclophosphamide (FAC) in the first four trials (1973–1993), and with FAC followed by paclitaxel in the last two trials (1994–2000). The median overall survival and progression-free survival (PFS) were better in the patients treated with paclitaxel, and these differences reached statistical significance in the patients with ER-negative disease (median overall survival: 32 vs. 54 months; $P = 0.03$).

Colleoni *et al.* (2004) reviewed the pretreatment biopsies and histologic specimens at final surgery of 399 patients with large or locally advanced breast cancer (cT2-T4, N0-2, M0), who were treated with preoperative chemotherapy. The chance of obtaining pathological complete remission for patients with endocrine nonresponsive tumors compared with those having some estrogen receptor or progesterone receptor expression was 4.22 (95% confidence interval, 2.20–8.09, 33.3% vs. 7.5%).

Ring *et al.* (2004) retrospectively evaluated the correlation between estrogen receptor status and pathological complete response (pCR) to neoadjuvant chemotherapy in 435 patients with operable breast cancer. Patients whose tumors were ER-negative were more likely to achieve a pCR than patients who were ER positive (21.6 vs. 8.1%, $P < 0.001$).

The role of ER status in predicting outcome after adjuvant chemotherapy has also been evaluated. A very accurate retrospective analysis of three CALGB adjuvant trials focusing on the role of ER status has been recently published (Berry *et al.*, 2006). Of particular interest is the analysis of the last two studies (9344 and 9741). In the CALGB 9344 trial, the addition of paclitaxel to doxorubicin-cyclophosphamide was evaluated. The absolute difference in 5-year disease-free survival was 8.2% in ER-negative patients, and 2.1% in ER-positive, the reduction of

risk being 25% and 12%, respectively. The observed difference was highly significant in ER-negative and not significant in ER-positive patients. In the CALGB 9741 trial, paclitaxel was administered in all treatment arms. The 2 × 2 factorial design aimed at comparing the sequential to the combined treatment, and the standard to the dose-dense schedule. In the overall population a significantly better DFS was observed with the dose-dense approach (2 vs. 3 weeks). However, the improvement in outcome was statistically significant only in ER-negative patients (risk reduction 24% and 5-year DFS gain 9.1%). In ER-positive patients the risk reduction was 8% only, with a 5-year gain of 2.8%.

HER2/neu status and response to chemotherapy

Human epidermal growth factor receptor 2 (HER-2) is overexpressed in 25–30% of breast cancer, suggesting a role for overexpression in tumorigenesis. This overexpression is most commonly the result of gene amplification. Several lines of evidence support the role of HER-2 overexpression in the pathogenesis and poor outcome of human tumors. A number of these studies have shown that breast cancers that overexpress HER-2 have a more aggressive course and higher relapse and mortality rates. In retrospective analyses of adjuvant studies HER2 overexpression was also associated with better response to more aggressive chemotherapy.

Pritchard *et al.* (2006) retrospectively analyzed the data of the trial, which had shown the superiority of Cyclophosphamide, Epirubicin, Fluorouracil (CEF) on Cyclophosphamide, Methotrexate, Fluorouracil (CMF) in the adjuvant treatment of node-positive breast cancer patients. Amplification of HER-2 was associated with a poor prognosis regardless of the type of treatment. In patients whose tumors showed amplification of HER-2, CEF was superior to CMF when assessed on the basis of relapse-free survival (hazard ratio, 0.52; 95% confidence interval, 0.34–0.80; P = 0.003) and overall survival (hazard ratio, 0.65; 95% confidence interval, 0.42–1.02; P = 0.06). For women whose tumors lacked amplification of HER-2, CEF did not improve relapse-free survival (hazard ratio for relapse, 0.91; 95% confidence interval, 0.71–1.18; P = 0.49) or overall survival (hazard ratio for death, 1.06; 95% confidence interval, 0.83–1.44; P = 0.68). The adjusted hazard ratio for the interaction between treatment and HER-2 amplification was 1.96 for relapse-free survival (95% confidence interval, 1.15–3.36; P = 0.01) and 2.04 for overall survival (95% confidence interval, 1.14–3.65; P = 0.02).

Similar results had been reported by Moliterni *et al.* (2003). Patients had been randomly allocated to receive either 12 courses of intravenous CMF or eight courses of the same regimen followed by four cycles of ADM. Relapse Free Survival (RFS) and Overall Survival (OS) were analyzed by a Cox model taking into account treatment, HER-2 status, and the interaction between treatment and HER-2 status, adjusting for the effect of other known clinical and biopathologic factors. Improved RFS and OS were observed in the HER-2 positive subgroup after treatment with CMF plus ADM versus CMF alone. With a median follow-up of 15 years, the hazard ratio (HR) for RFS was 0.83 in HER-2 positive tumors and 1.22

in HER-2 negative tumors. The effect of treatment was more evident on OS in HER-2 positive patients (Hazard Ratio = 0.61; CI 0.32–1.16) than in HER-2 negative patients (HR = 1.26).

The hypothesis that HER-2 positive patients could substantially benefit from a more aggressive treatment seems confirmed by the data reported by Del Mastro *et al*. (2005). The authors retrospectively evaluated the role of HER-2 status in patients enrolled in a phase III study comparing standard FEC21 (5-fluorouracil, epirubicin, and cyclophosphamide, administered every 3 weeks) vs. dose-dense FEC14 (the same regimen repeated every 2 weeks). Disease-free and overall survival were compared within each HER-2 subgroup and within each treatment arm. Among FEC21-treated patients, both Disease free survival (Hazard Ratio = 2.07; 95% CI 1.27–3.38) and overall survival (Hazard Ratio = 2.47; 95% CI 1.34–4.57) were significantly worse in HER-2 positive patients than in HER-2 negative patients. Among FEC14-treated patients, differences in either Disease free survival (Hazard Ratio = 1.21; 95% CI 0.65–2.24) or overall survival (Hazard Ratio = 1.85; 95% CI 0.88–3.89) between HER-2 positive and HER-2 negative patients were not statistically significant. Interaction analysis suggested that the use of dose-dense FEC14 might remove the negative prognostic effect of HER-2 overexpression on Disease free survival and overall survival.

The role of paclitaxel in improving response in HER-2 positive patients with metastatic disease was addressed by Konecny *et al*. (2004). The authors retrospectively investigated the response of patients with known HER-2 status to treatment with epirubicin-paclitaxel chemotherapy compared with treatment with epirubicin-cyclophosphamide chemotherapy. Patients with HER-2 positive tumors had a statistically significantly greater objective response rate than patients with HER-2 negative tumors to treatment with epirubicin-paclitaxel (76% vs. 50%, respectively; P = 0.005) but not to treatment with epirubicin-cyclophosphamide (46% vs. 33%; P = 0.130). The objective response rate associated with epirubicin-paclitaxel was greater than that associated with epirubicin-cyclophosphamide for both HER-2 positive tumors (76% vs. 46%; P = 0 .004) and HER-2 negative tumors (50% vs. 33%; P = 0.002). However, the improvement in the objective response rate associated with epirubicin-paclitaxel, compared with that associated with epirubicin-cyclophosphamide, was greater for patients with HER-2 positive tumors (adjusted odds ratio = 3.64, 95% CI = 1.48–8.92; P = 0.005) than for patients with HER-2 negative tumors (adjusted odds ratio = 1.92, 95% Confidence Interval = 1.01–3.64; P = 0.046). Among patients with HER-2 positive tumors, those who received epirubicin-paclitaxel had better progression-free survival and overall survival than those who received epirubicin-cyclophosphamide. Conversely, among patients with HER-2 negative tumors, those who received epirubicin-paclitaxel and those who received epirubicin-cyclophosphamide had similar progression-free survival and overall survival.

The role of HER-2 overexpression in affecting the efficacy of neoadjuvant chemotherapy has been evaluated in several studies. Penault-Llorca *et al*. (2007) found a threefold more frequent pCR rate for HER-2-positive patients (23.5%) than for HER-2-negative patients (7%).

Andre *et al*. (2007) examined the correlation between HER-2 expression and pathologic complete response to paclitaxel followed by Fluorouracil, Adriamycin, Cyclophosphamide preoperative chemotherapy in breast cancer. Retrospective analysis of data including 534 treated patients was performed. Of the 534 patients, 105 (20%) had HER-2 overexpressing breast cancer. The pCR rates were 33% and 15% for patients with HER-2 positive and HER-2 negative tumors ($P < 0.001$). The 5-year relapse-free survival rates were 94% and 70% in HER2+ tumors with or without pCR ($P = 0.009$). HER-2 overexpression (odds ratio 2.3, 95% CI 1.3–3.9, $P = 0.004$), estrogen receptor status, grade and weekly schedule of paclitaxel were each significantly and independently associated with pCR in multivariate analysis. When patients were stratified by ER status, the pCR rates were 50% for HER-2 positive/ER negative, 30% for HER-2 negative/ER negative, 19% for HER-2 positive/ER positive, and 6% for HER-2 negative/ER positive tumors. A strong positive correlation between HER-2 overexpression and pCR achievement was also found ($P = 0.017$) in the GEPARTRIO trial (Rody *et al*., 2007).

At the MDACC, Rouzier *et al*. (2005) tried to determine whether different molecular subtypes of breast cancer also respond differently to preoperative chemotherapy. Gene expression profiling was done with Affymetrix U133A microarrays. The basal-like and HER-2 positive subgroups were associated with the highest rates of pathologic complete response, 45% (95% Confidence Interval, 24–68) and 45% (95% Confidence Interval, 23–68), respectively; whereas luminal tumors had a pathologic complete response rate of 6% (95% Confidence Interval, 1–21). No pathologic complete response was observed among the normal-like cancers (95% Confidence Interval, 0–31). Molecular class was not independent of conventional cliniocopathologic predictors of response such as estrogen receptor status and nuclear grade. None of the 61 genes associated with pathologic complete response in the basal-like group were associated with pathologic complete response in the HER-2 positive group, suggesting that the molecular mechanisms of chemotherapy sensitivity may vary between these two estrogen receptor-negative subtypes.

PACLITAXEL-BASED CHEMOTHERAPY AS PRIMARY TREATMENT OF LARGE-OPERABLE OR LOCALLY-ADVANCED BREAST CANCER

Taxanes (paclitaxel and docetaxel) are generally considered among the most active molecules in the treatment of breast cancer. It has been hypothesized that the weekly administration of paclitaxel gives a substantial therapeutic advantage, because the shorter interval assures a more prolonged cumulative exposure to the drug, and less opportunity for the emergence and regrowth of drug resistant cell clones (Seidman *et al*., 1998).

Weekly paclitaxel was tested alone in metastatic breast cancer, resulting in a high dose intensity, negligible hematologic toxicity, and high therapeutic activity. A clear pharmacokinetic advantage was also demonstrated, because a weekly paclitaxel dose of 100 mg/m^2 given over 1-h gave a peak concentration and AUC similar to those obtained with higher dose given in 3-h every 3 weeks.

There is evidence in favor of a better therapeutic index for the weekly paclitaxel

administration, also in patients with less advanced disease. Green *et al.* (2005) reported the results of a randomized trial comparing the regimen paclitaxel q3wk ×4 followed by FAC ×4 with the regimen of weekly paclitaxel ×12 followed by Fluorouracil, Adriamycin Cyclophosphamide (FAC) ×4. A 28.8% pathological complete response rate was obtained with weekly paclitaxel, as compared to a 13.6% pathological complete response rate with the tri-weekly paclitaxel followed by FAC. In the last few years, increasing interest has arisen about the role of platinum compounds in the treatment of breast cancer patients. Several cisplatin-based regimens have been tested in the neoadjuvant setting, showing high antitumor activity (Ezzat *et al.*, 2004; Orlando *et al.*, 2001).

In the mid-1990s Frasci *et al.* (1999) started the assessment of a weekly triplet regimen including cisplatin, epirubicin, and paclitaxel (PET regimen) in breast cancer patients. In a preliminary phase I study, nine different dose levels were tested, keeping the dose of cisplatin fixed to 30 mg/m^2. The doses of epirubicin and paclitaxel were safely increased up to 40 and 85 mg/m^2, respectively, without G-CSF support. However, due to frequent treatment delay or dose reduction, the actually delivered dose intensity was 64% of that planned. Therefore, the dose escalation was continued with the addition of G-CSF from day 3 to 5 each week. Doses of epirubicin and paclitaxel of 50 and 120 mg/m^2 were administered without observing relevant hematologic toxicity. However, due to the occurrence of severe nonhematologic toxicity (mucositis, and diarrhoea) in some patients it was decided to stop the escalation and to consider this dose level as the one advisable for phase II. This weekly approach was evaluated in phase II and III trials including women with either LABC or large operable disease (Frasci *et al.*, 2000, 2005, 2006). The results of this 10-year cumulative experience are reported here.

WEEKLY CISPLATIN-EPIRUBICIN-PACLITAXEL WITH G-CSF SUPPORT IN LOCALLY ADVANCED BREAST CANCER

METHODS

Between December 1997 and May 2007 a total of 216 patients with T4 a-d and/or N2 breast cancer, aged < 75, received weekly cisplatin-epirubicin-paclitaxel with G-CSF support as primary treatment. Table 30.1 outlines the main patient characteristics. Overall, 138 patients had T4a-b-c disease, while 51 women had inflammatory carcinoma, and 27 N2 disease. Estrogen or progesterone receptors were positive in 133 and 118 patients, respectively. Forty-eight patients were HER-2 positive. Putting ER and HER-2 status together, patients were: 122 ER positive/HER-2 negative; 11 ER positive/HER-2 positive; 46 ER negative/ HER-2 negative; 37 ER negative/ HER-2 positive.

Treatment schema: Epirubicin 50 mg/m^2 was administered as an i.v., bolus, followed by paclitaxel 120 mg/m^2 as a 1-h infusion, and cisplatin 30 mg/m^2 as a 30 min infusion, weekly for a maximum of 12 cycles. Recombinant human G-CSF 300 µg/day was also given subcutaneously on days 3–5 of each week. Short-term forced hyperhydration (1 L of saline over 2 h) and prophylaxis

TABLE 30.1. LABC: demographics.

Characteristic	No. of patients (total 216)
Age	
Median (range)	53 (27–73)
< 65/≥65	152/64
T4 N0	50
T4 N1	139
Any T N2	27
Breast tumor	
T4a–c/d	138/51
Histology	
Ductal	131
Lobular	69
Mixed	8
Mucinous	5
Other	3
Grading	
I	39
II	73
III	95
Unknown	9
Menopausal status	
Pre-/post-menopausal	97/119
Hormone receptors status	
ER: yes/no	133/83
PgR: yes/no	118/98
HER/neu status	
pos/neg	48/168

for nausea/vomiting (HT3 receptor antagonists) were also performed. Prophylaxis for hypersensitivity reactions consisted of dexamethasone 8 mg i.v. and promethazine 50 mg i.m. plus ranitidine 50 mg i.v. 30 min before paclitaxel administration. Within 4 weeks from the end of chemotherapy patients underwent surgery. Breast sparing surgery was performed whenever feasible. It consisted of quadrantectomy together with standard level I and II axillary lymph-node dissection.

Four cycles of CMF were delivered after surgery in patients showing at pathologic assessment < 4 axillary lymph nodes involved (including pCR).

Additional four FEC (epirubicin 60 mg/m^2 instead of methotrexate) cycles were administered in those women showing four or more axillary nodes involved. On completion of post-operative chemotherapy, radiotherapy was performed in all patients who underwent conservative surgery, as well as in those submitted to mastectomy but with more than three axillary nodes involved, or muscle, skin and/or nipple involvement, or had G3 tumor at diagnosis.

Hormone treatment was also given on completion of postoperative chemotherapy in ER and/or PgR positive patients. LH-RH analogue for 2 years together with tamoxifen for 5 years was administered in premenopausal women. Among the 89 ER positive postmenopausal women, 50 received tamoxifen and 39 an aromatase inhibitor for at least 5 years. Eight out of 48 HER-2 positive patients received 1-year Trastuzumab treatment (8 mg/kg loading dose, followed by 6 mg/kg q3wk), starting within 1-month from the completion of adjuvant chemotherapy.

Dose adjustments according to toxicity

Chemotherapy was given at full doses if neutrophil count was ≥1.5 10^9/L, and platelet count was ≥100 × 10^9/L. Doses were reduced by 50% if neutrophil counts were < 1.5/10^9/L and ≥1.0/10^9/L, or platelet counts were < 100 × 10^9/L and ≥75/10^9/L. In the case of grade ≥ 3 neutropenia, grade ≥ 2 thrombocytopenia, or grade > 1 non-hematological toxicity the treatment was always omitted. In the presence of grade 4 neutropenia, febrile neutropenia, grade 4 thrombocytopenia, grade 4 anaemia, grade 3–4 non-hematological toxicity (except for alopecia) doses were reduced by 25% in the subsequent administrations.

Response evaluation criteria

Clinical tumor response was assessed within 2 weeks from the end of chemotherapy. For pathologic assessment of response, the

amount of residual epithelial neoplastic cells in the tumor mass and the location of malignant component (invasive *vs*. intraductal) were taken into account. Response in the breast was scored as follows: Class I (absence of residual malignant epithelial cells), class II (persistence of only *in situ* residual malignant component), class III (only focal invasive tumor residuals), class IV (no substantial modifications in the tumor mass). Patients showing a class I or II response in the breast, together with absence of axillary involvement were considered as pCR.

RESULTS

Response

All the 216 enrolled patients were included into the response analysis on an "intent to treat basis". Overall, 192 women completed the 12 planned weekly cycles. Twelve out of 24 patients who did not complete chemotherapy showed progression of disease, while in the remaining 12 patients severe toxicity (emesis 3, mucositis 3, neuropathy 2, fatigue 4) was the cause of the early discontinuation. At the clinical restaging, 51 complete and 126 partial responses were recorded, giving an 82% overall response rate. Additional 27 patients showed a minor regression or stabilization of the tumor.

Overall, 196 women underwent surgery. Breast sparing surgery was performed in a total of 93 patients. Thirty-two patients showed absence of residual malignant epithelial cells, either invasive or intraductal, in the breast specimen. Only *in situ* residual tumor cells were found in additional 24 women. Therefore, a total of 56 patients achieved a pCR rate in the breast. A pPR in the breast (i.e., only focal invasive tumor residuals in the removed breast tissue) was recorded in 78 patients. Overall, 89 women showed negative axilla. Among the 107 patients with persistence of tumor in the axilla, 52 had 1–3 lymph nodes involved, and 55 four or more nodes. Forty-one patients showed an absence of invasive tumor in both breast and axilla; giving a pCR rate of 19% (Table 30.2). Pathological complete responders had the following immunohistochemical baseline characteristics: 9/122 ER positive/HER-2 negative, 3/11 ER positive/HER-2 positive, 15/46 ER negative/HER-2 negative, 14/37 ER negative/HER-2 positive, the pCR rate being 7.3%, 27%, 32.6%, and 37.8%, in the 4 groups, respectively.

After a median follow-up of 50 (range, 5–116) months, 109 patients had progressed or relapsed, and 47 had died, 5-year progression/relapse-free survival and overall survival being 47% and 69%, respectively (Figure 30.1). Thirty-four progressions/relapses were registered in the 51 women with inflammatory breast cancer, as compared to 75 of these events in patients with noninflammatory disease, 5-year progression/relapse-free survival being 43% and 52%, respectively. At a median follow-up of 53 (range, 4–109)

TABLE 30.2. LABC: pathological assessment.

	No. of patients (%) (total assessed 196)
Breast	
Class I	32 (16.3)
Class II	24 (12.2)
Class III	78 (40)
Cass IV	62 (31.5)
Axilla	
N0	89 (45.4)
N1–3	52 (26.5)
N > 3	55 (28.1)
pCR (class I + II and N0)	41 (19)[a]

[a] Calculated on the whole population (216 patients).

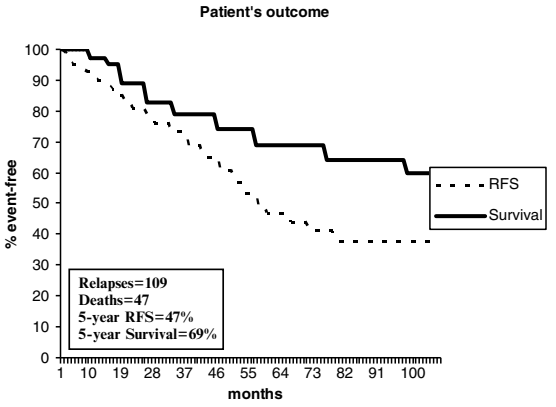

FIGURE 30.1. Relapse-free and overall survival in the 216 LABC patients

months, 13 relapses (11 distant) and 6 deaths were registered among the 41 patients who had achieved a pCR, giving a 5-year disease-free survival and overall survival of 69% and 81%, respectively. Overall, 61 out of 133 patients with ER positive tumor had progression or relapse at the time of the present analysis, as compared to 48 out of 83 patients with ER negative tumor. In the latter group, failures were 23/46 in HER-2 negative and 25/37 in HER-2 positive patients. Five-year progression/relapse-free survival was 59%, 43% and 31% in ER positive, ER negative/HER-2 negative and ER negative/HER-2 positive patients, respectively.

Toxicity

Hematological toxicity was not a major problem, except for anemia. Overall 115 patients experienced a grade ≥ 3 neutropenia. Grade 4 occurred in 37 cases, but neutropenic fever was observed in only 13 patients, with no deaths. Severe thrombocytopenia was very uncommon, grade 3–4 occurring in only 14 patients. Severe anaemia occurred in 26 (12%) patients. Overall, nonhematological toxicity was much more frequent and troublesome. Severe emesis, loss of appetite, diarrhoea, and fatigue occurred in 28 (13%), 34 (18%), 24 (11%) and 41 (19%) of patients, respectively, causing early chemotherapy discontinuation in a total of eight patients. Neurotoxicity and mucositis were also common side effects in both arms. Overall, 79 (36%) patients complained of sensory neuropathy, but it was severe in only seven patients. In two patients it occurred very early, so causing definitive treatment discontinuation. In additional five patients neuropathy impaired after completion of chemotherapy. Severe mucositis was recorded in 28 (13%) patients, and in three cases it caused the definitive treatment discontinuation. Renal, liver, and cardiac toxicity were very uncommon. There were two cases of grade 3 liver toxicity, while severe renal toxicity never occurred. Overall, nine patients had a left ventricular ejection fraction decrease below 50%, but no patients showed clinical signs of congestive heart failure.

WEEKLY CISPLATIN -EPIRUBICIN-PACLITAXEL WITH G-CSF SUPPORT IN LARGE OPERABLE DISEASE

METHODS

Between May 1998 and February 2007 113 patients with large operable breast cancer (T2-3, T size > 3 cm and N0-1), aged < 70 years, with no prior chemotherapy or hormone treatment, received weekly cisplatin-epirubicin-paclitaxel with G-CSF support as primary treatment. Table 30.3 outlines the main patient characteristics. Overall, 48 patients had T2 and 65 T3 disease, clinical N0 and N1 were 39 and 74, respectively. Estrogen or

TABLE 30.3. Large operable: demographics.

Characteristic (total 113)	No. of patients (%)
Age	
Median (range)	52 (29–70)
< 65/≥65	72/41
Breast	
T2	48 (42)
T3	65 (58)
Axilla	
N0	39 (35)
N1	74 (65)
Histology	
Ductal	63
Lobular	31
Mixed	6
Mucinous	5
Other	8
Grading	
I	23
II	37
III	47
Unknown	6
Menopausal status	
Pre-/post-menopausal	52/61
Hormone receptors status	
ER: yes/no	66/47
PgR: yes/no	59/54
HER/neu status	
pos/neg	29/84

progesterone receptors were positive in 66 and 59 patients, respectively. Twenty-nine patients were HER-2 positive. Putting ER and HER-2 status together, patients were: 57 ER positive/ HER-2 negative; 9 ER positive/ HER-2 positive; 27 ER negative/ HER-2 negative; 20 ER negative/ HER-2 positive.

Treatment schema: Epirubicin 50 mg/m^2 was administered as an i.v. bolus, followed by paclitaxel 120 mg/m^2 as a 1-h infusion, and cisplatin 30 mg/m^2 as a 30 min infusion, weekly for a maximum of eight cycles. Recombinant human G-CSF 300 µg/day was also given subcutaneously on days 3–5 of each week. Short-term forced hyperhydration (1 L of saline over 2 h) and prophylaxis for nausea/vomiting (HT3 receptor antagonists) were also given. Prophylaxis for hypersensitivity reactions consisted of dexamethasone 8 mg i.v. and promethazine 50 mg i.m. plus ranitidine 50 mg i.v. 30 min before paclitaxel administration.

Patients underwent surgery within 4 weeks from the end of chemotherapy. Breast sparing surgery was performed whenever feasible. It consisted of quadrantectomy together with standard level I and II axillary lymph-node dissection.

Four cycle of CMF were delivered after surgery in patients showing at pathologic assessment < 4 axillary lymph nodes involved (including pCR). Additional four FEC (epirubicin 60 mg/ m^2 instead of methotrexate) cycles were administered in those women showing four or more axillary nodes involved. On completion of post-operative chemotherapy, radiotherapy was performed in all patients who underwent conservative surgery, as well as in those submitted to mastectomy but with > 3 axillary nodes involved, muscle, skin and/or nipple involvement, or had G3 tumor at diagnosis. Six out of 29 HER-2 positive patients received 1-year Trastuzumab treatment (8 mg/kg loading dose, followed by 6 mg/kg q3wk), starting within 1-month from the completion of adjuvant chemotherapy. Hormone treatment was also given on completion of postoperative chemotherapy in ER and/or PgR positive patients. LH-RH analogue for 2 years together with tamoxifen for 5 years was administered in premenopausal. Among the 42 ER positive postmenopausal women, 25 received tamoxifen and 17 an aromatase inhibitor for at least 5 years.

RESULTS

Compliance

All but one women received the planned 8 weekly treatment cycles. An at least

1-week delay due to hematological toxicity (neutrophil count below 1,000, or platelets < 75,000, or hemoglobin < 8 g/L), or persistence of grade > 1 nonhematological toxicity on day of recycling, occurred in 24 patients. A dose reduction was performed in 37 patients for overall 95 cycles. Eighty-four out of 113 (74%) patients actually received ≥ 80% of the planned dose-intensity.

RESPONSE

All the 113 enrolled patients underwent surgery. Thus, they were evaluated for both clinical and pathological responses. Forty-two women (37%) showed at clinical restaging absence of residual tumor in the breast and axilla. An additional 61 women (54%) obtained a partial regression of the tumor, giving a 91% overall response rate. The remaining 10 patients achieved only a minor regression or stabilization of the tumor, but nevertheless underwent surgery. Overall, 69 out of 113 (61%) patients underwent breast-sparing surgery. A modified radical mastectomy had been considered in 41 of them at diagnosis.

Table 30.4 summarizes pathological response data. Thirty-nine patients (34%) showed absence of residual malignant epithelial cells, either invasive or intraductal in the breast specimen (class I). An additional 15 (13%) women had only *in situ* residual tumor cells (class II). Therefore, 54/113(47%) women obtained a pathological complete regression of the tumor in the breast. A pathological partial response in the breast (class III = only focal invasive tumor residuals in the removed breast tissue) was recorded in 32 patients. In the remaining 27 women no substantial modifications in the tumor mass were observed (class IV). Overall, 64 women (56%) showed negative axilla. Among the 49 patients with persistence of tumor in the axilla, 26 had 1–3 lymph-nodes involved, and 23 four or more.

TABLE 30.4. Large operable disease: pathological assessment.

	No. of patients (%) (total assessed 113)
Breast	
Class I	39 (34)
Class II	15 (13)
Class III	32 (28.3)
Cass IV	27 (24.7)
Axilla	
N0	64 (56)
N1-3	26 (23)
N > 3	23 (21)
pCR (class I + II and N0)	39 (34.5)[a]

[a] Calculated on the whole population (113 patients).

Thirty-nine patients (34.5%) had, at pathologic assessment, absence of invasive tumor in both breast and axilla. Pathological complete responders had the following immunohistochemical baseline characteristics: 9/57 (15.8%) ER positive/HER-2 negative, 2/9 (22%) ER positive/HER-2 positive, 15/27 (56%) ER negative/HER-2 negative, 13/20 (65%) ER negative/HER-2 positive.

At a 53-month median follow-up (range; 3–101), 23 relapses, and 12 deaths had occurred, 5-year relapse-free survival and overall survival being 69% and 85%, respectively (Figure 30.2). Seven out of 39 pathological complete responders had a distant relapse, 5-year Distant- relapse free survival being 86%. Four out of seven pCRs who relapsed had ER negative/HER-2 positive tumor. Taking the baseline immunohistochemical features into consideration relapses were distributed as follows: 10/57 (17.5%) ER positive/HER-2 negative, 2/9 (22%) ER positive/HER-2 positive, 6/27 (22.5%) ER negative/HER-2 negative, and 5/20 (25%) ER negative/

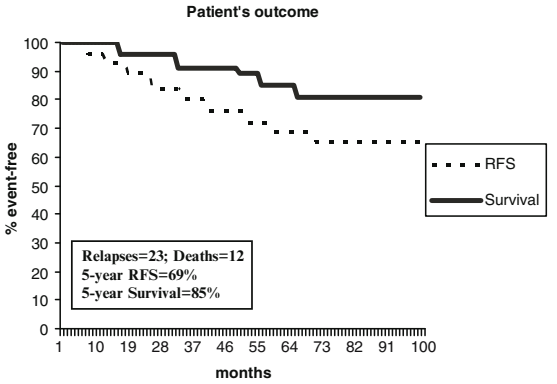

FIGURE 30.2. Relapse-free and overall survival in the 113 patients with large operable disease

HER-2 positive. Five-year relapse-free survival was 71%, 73%, and 59% in ER positive, ER negative/HER-2 negative, and ER negative/HER-2 positive patients, respectively.

TOXICITY

No treatment-related deaths occurred. Thirty-one (27.4%) patients showed grade 3–4 neutropenia, but only in five of them neutrophils fell below 500/μL, with three episodes of febrile neutropenia. Severe thrombocytopenia occurred in six (5.3%) patients, and only four (3.5%) patients required red blood cell transfusions due to hemoglobin fall below 8 g/L. Alopecia was almost universal. Among the other nonhematological side effects, emesis, fatigue, loss of appetite and mucositis were the most common, although they were well controlled with steroids administration. In particular, ~ 60% of patients complained of nausea/vomiting, but only in eight (7%) cases was it severe. Severe fatigue was registered in 11 (10%) women. Diarrhoea was also quite frequent, and in six (5.3%) patients it was severe. A 25% dose reduction was performed in all cases, and chemotherapy could be completed without occurrence of other episodes of severe diarrhoea. Other nonhematological toxicities such as mucositis, skin toxicity, and neuropathy, caused rarely patients' complaints in the present study. Only six (5.3%) patients suffered from severe stomatitis, and three had palmar plantar erithrodystesia. Overall 32 (28.3%) patients complained of mild or moderate paresthesias in the hands and feet, but in only one case was it severe. A complete recovery from neurotoxicity occurred within 3 months in all but one women. Musculoskeletal symptoms like transient arthralgias and myalgias occurred in overall 39 patients, but they were severe in only four cases, and generally responded well to anti-inflammatory drugs. In the majority of cases these symptoms were related to G-CSF administration. Cardiac toxicity was almost absent. Only three patient developed a decline in left ventricular ejection fraction by more than 15%, but congestive heart failure never occurred. Finally, four episodes (none of them severe) of transient increase of the AST/ALT serum levels were observed without any clinical sign of liver dysfunction.

DISCUSSION

This discussion attempts to provide answers to the following questions: (1) Is there a clear indication for the use of paclitaxel in aggressive carcinomas of the breast identified by the ER negative and/or HER-2 positive status?; and (2) Are different paclitaxel schedules, sometimes requiring G-CSF support more effective in these patients?

The MDACC experience focusing on both LABC (Cristofanilli *et al.*, 2004) and large operable disease (Mazouni *et al.*, 2007) would seem to confirm, beyond any

doubt that the addition of paclitaxel substantially improves the chance of pCR achievement and long-term survival in ER positive breast cancer patients. In another MDACC retrospective analysis (Andre *et al*., 2007), both ER negative and HER-2 positive were independently associated with higher pCR rate in patients receiving neoadjuvant paclitaxel followed by Fluorouracil, Adriamycin, Cyclophosphamide. Indeed, pCR rate was 50% in HER-2 positive/ER negative, 30% in HER-2 negative/ER negative, 19% in HER-2 positive/ER positive, and 6% in HER-2 negative/ER positive patients, respectively. Paclitaxel had been delivered with two different schedules in these patients (triweekly or weekly). The weekly schedule was also independently associated with better pCR rate at multivariate analysis.

Paclitaxel, either weekly or triweekly, was administered alone in the MDACC trials. Therefore, routinely G-CSF administration was not performed. Indeed, especially when the weekly schedule is employed, the administration of paclitaxel as a single agent does not require G-CSF support. The administration of 80–175 mg/m^2 of paclitaxel each week produces tumoricidal, but not myelotoxic serum concentration. In view of that, we are able to deliver a cumulative dose of paclitaxel exceeding 400 mg/m^2 over 3 weeks, with less myelotoxicity than that associated to 200–250 mg/m^2 given as a 1- to 24-h infusion every 3 weeks.

A quite different philosophy underlay the SICOG trials. Weekly paclitaxel was administered in combination with relevant doses of epirubicin and cisplatin in order to deliver a high cumulative dose of these three drugs in a shorter time. Thus, the routine administration of G-CSF support was necessary in these patients. In the SICOG LABC series, 12 weekly cycles of cisplatin-epirubicin-paclitaxel (PET) plus G-CSF produced 29 pCRs in the 83 ER negative patients (35%) treated. If the HER-2 status is also considered, pCR rate was 32.6% and 37.8% in ER negative/HER-2 negative and ER negative/HER-2 positive patients, respectively. This figure seems very interesting, especially if we consider that the duration of the treatment was very short. Gianni *et al*. (2005) reported seven pCRs in 31 ER-negative LABC patients (23%) who had preoperatively received three cycles of doxorubicin 60 mg/m^2 + paclitaxel 200 mg/m^2 every 3 weeks, followed by 12 weekly cycles of paclitaxel 80 mg/m^2, so the treatment lasting more than 21 weeks. Guarneri *et al*. (2006) reported the findings of a retrospective analysis including 1,731 patients with stage I–III noninflammatory breast cancer treated between 1988 and 2005 with primary chemotherapy at the MDACC. Ninety-one percent of patients received anthracycline-based chemotherapy, and 66% received additional taxane therapy. Treatment lasted usually 4–6 months. Three hundred and forty-two patients had stage IIIB-IIIC disease. One hundred and twenty-five of them were ER-negative. Seventeen (14%) pCRs were observed in this group.

On the basis of these data the weekly PET regimen appears to be a very effective neoadjuvant treatment for women with ER negative and/or HER-2 positive LABC. It is interesting to remark that these results were obtained at the price of a moderate myelotoxicity. Indeed, the risk of life-threatening sepsis was much lower in the SICOG experience than that generally reported with other preoperative regimens. Nonhematological toxicity, mainly mucositis, fatigue, and neurotoxicity may be sometimes troublesome with

this regimen, and are in most cases cumulative. That is the main reason why this treatment cannot be prolonged beyond 12 cycles. It would be interesting to evaluate this regimen as a part of a sequential approach. Perhaps, patients could receive after 12 weekly PET cycles an additional 12-week treatment with drugs having non overlapping toxicity, such as gemcitabine, vinorelbine, or capecitabine.

The combination of targeted agents with PET could be another effective way to increase the rate of pCRs. The addition of weekly trastuzumab to PET could reasonably increase the rate of pCRs in HER-2 positive patients. The addition of Bevacizumab might also result into a better efficacy in these patients.

The very wide range of the follow-up time (5–116 months) in this SICOG series, makes it difficult to draw firm conclusions on the survival outcome of these patients. The 47% 5-year relapse-free survival observed in this study appears quite promising, although this figure cannot be considered remarkably different from that reported by other investigators with more standard approaches. Two reasons can explain why it is difficult to substantially improve the rate of cure in locally advanced breast cancer. First, whatever regimen is given, the rate of pCR still remains very low in the general population, so the very high proportion of no pCR patients strongly affect the outcome of the whole population. Secondly, the risk of distant metastasis still remains substantial in LABC patients achieving pCR. In the SICOG series 13 relapses (11 distant) and 6 deaths were registered among the 41 patients who had achieved a pCR, giving a 5-year relapse-free and overall survival of 69% and 81%, respectively. In a very well conducted retrospective analysis, Gonzales-Angulo *et al.* (2005) showed that the risk of distant metastasis was 7.5% in pathological complete responders who had operable disease before treatment, as compared to 25% in those pathological complete responders who had IIIB, IIIC, or inflammatory breast cancer at diagnosis.

If we perform a split analysis of long-term outcome, according to ER and HER-2 status, we can observe that estimated 5-year relapse-free survival was better in ER positive/HER-2 negative patients (59%) than in ER negative/HER-2 negative (43%) and ER negative/HER-2 positive (31%) patients, despite the much lower pCR rate achieved in the first group. The strong protective role of adjuvant hormone treatment (perhaps, emphasized by the use of aromatase inhibitors in a relevant proportion of patients) can explain this finding.

Some considerations deserve to be made on the SICOG large operable series. Although the relatively small sample size does not permit to draw definitive conclusions, it is worth-noting that 15/27 (56%) ER negative/HER-2 negative, and 13/20 (65%) ER negative/HER-2 positive patients achieved a pathological complete regression of the tumor in both breast and axilla, with a preoperative treatment of only 8 weeks. This figure appears certainly better than that reported by Gianni *et al.* (2005) and Untch *et al.* (2002), especially if we consider that all patients of the SICOG series had a true large tumor (T2–T3).

In the trial conducted by Gianni *et al.* (2005), 6 months of preoperative chemotherapy produced a 45% pCR in the breast in ER negative patients. In the trial conducted by Untch *et al.* (2002), which included also LABC patients, a 25% pCR rate was observed in ER negative

patients. In the retrospective analysis conducted by MDACC investigators (Andre *et al*., 2007), a 30% and 50% pCR rate was registered in ER negative/HER-2 negative and ER negative /HER-2 positive patients, respectively, with a treatment much longer than that administered in the SICOG series.

With a very high proportion of ER negative patients achieving a pCR with the PET regimen, we can expect a relevant impact of chemotherapy on the long-term outcome of these patients. Indeed, 5-year relapse-free survival was 73% in ER negative/HER-2 negative patients, similar to that observed in ER positiv patients. The risk of relapse was higher in ER negative/HER-2 positive patients, the estimated 5-year survival being 59% in this group. Because the number of patients ER negative/HER-2 positive was quite low, it is difficult to define whether the higher risk of relapse is attributable to more frequent relapse in pCRs, in no-pCR, or in both. It must be remarked, however, that 4/13 ER negative/HER-2 positive pCRs had relapsed as compared to 3/26 pCRs with different immunehistochemical feature at diagnosis. The administration of Trastuzumab in combination with either preoperative or postoperative chemotherapy could result in an overall substantial reduction of the risk of relapse. This statement is strongly supported by the MDACC data (Buzdar *et al*., 2007), and the results of the randomized trials in the adjuvant setting show a 55% reduction of the risk with the addition of Trastuzumab to chemotherapy.

The activity data obtained with just 8 weekly PET cycles in women with T2–T3 tumor are of interest also because they were associated with mild toxicity. Sepsis and severe thrombocytopenia were almost anecdotical in this series, which included more than 100 patients. Severe hematological toxicity is common in patients receiving standard anthracycline/taxane combinations, and sometimes it may cause hospitalization and even death. Seven deaths occurred in NSABP B27 trial (Bear *et al.,* 2003), with 21.2% of patients experiencing febrile neutropenia. Nonhematological toxicity was also acceptable in this series of patients receiving 8 rather 12 PET cycles. The very favorable toxicologic profile makes it reasonable to hypothesize a prolongation of treatment by administering a noncross-resistant regimen, in those patients who are expected not to obtain pCR after eight PET cycles. The assessment of response by means of dynamic MRI and FDG-PET may allow the prediction of pathological findings with an accuracy of 90% or more (Rousseau et al., 2006; Padhani et al., 2006). In patients showing residual disease at this assessment, a second-line chemotherapy combined with targeted agents (i.e., Bevacizumab in HER-2 negative and Lapatinib in HER-2 positive, if they had already received Herceptin) should be administered before submitting the woman to surgery.

In conclusion, the weekly administration of the cisplatin-epirubicin-paclitaxel combination together with G-CSF support is a very effective preoperative approach in women with aggressive (ER negative and/or HER-2 positive) breast cancer, either large operable or locally-advanced. Given the occurrence of cumulative nonhematological toxicity, this treatment cannot be prolonged for more than 12 cycles. In order to increase the chance of pCR achievement, a sequential approach consisting of eight PET cycles followed by a second-line regimen (plus

a targeted agent) in patients who are not predicted to get a pCR, should be highly recommended.

REFERENCES

Andre, F., Mazouni, C., Liedtke, C., Kau, S.W., Frye, D., Green, M., Gonzalez-Angulo, A.M., Symmans, W.F., Hortobagyi, G.N., and Pusztai, L. 2008. HER-2 expression and efficacy of preoperative paclitaxel/FAC chemotherapy in breast cancer. *Breast Cancer Res. Treat. 108*: 183–190.

Bear, HD., Anderson, S., Brown, A., Smith, R., Mamounas, EP., Fisher, B., Margolese, R., Theoret, H., Soran, A., Wickerham, D.L., and Wolmark, N.; National Surgical Adjuvant Breast and Bowel Project Protocol B-27. 2003. The effect on tumor response of adding sequential preoperative docetaxel to preoperative doxorubicin and cyclophosphamide: preliminary results from National Surgical Adjuvant Breast and Bowel Project Protocol B-27. *J. Clin. Oncol. 21*: 4165–4174.

Berry, D.A., Cirrincione, C., Henderson, I.C., Citron, M.L., Budman, D.R., Goldstein, L.J., Martino, S., Perez, E.A., Muss, H.B., Norton, L., Hudis, C., and Winer, E.P. 2006. Estrogen-receptor status and outcomes of modern chemotherapy for patients with node-positive breast cancer. *JAMA. 295*: 1658–1667.

Buzdar, A.U., Valero, V., Ibrahim, N.K., Francis, D., Broglio, K.R., Theriault, R.L., Pusztai, L., Green, M.C., Singletary, S.E., Hunt, K.K., Sahin, A.A., Esteva, F., Symmans, W.F., Ewer, M.S., Buchholz, T.A., and Hortobagyi, G.N. 2007. Neoadjuvant therapy with paclitaxel followed by 5-fluorouracil, epirubicin, and cyclophosphamide chemotherapy and concurrent trastuzumab in human epidermal growth factor receptor 2-positive operable breast cancer: an update of the initial randomized study population and data of additional patients treated with the same regimen. *Clin. Cancer Res. 13*: 228–233.

Colleoni, M., Viale, G., Zahrieh, D., Pruneri, G., Gentilini, O., Veronesi, P., Gelber, R.D., Curigliano, G., Torrisi, R., Luini, A., Intra, M., Galimberti, V., Renne, G., Nole, F., Peruzzotti, G., and Goldhirsch, A. 2004. Chemotherapy is more effective in patients with breast cancer not expressing steroid hormone receptors: a study of preoperative treatment. *Clin. Cancer Res. 10*: 6622–6628.

Cristofanilli, M., Gonzalez-Angulo, A.M., Buzdar, A.U., Kau, S.W., Frye, D.K., and Hortobagyi, G.N. 2004. Paclitaxel improves the prognosis in estrogen receptor negative inflammatory breast cancer: the M. D. Anderson Cancer Center experience. *Clin. Breast Cancer 4*: 415–419.

Del Mastro, L., Bruzzi, P., Nicolo, G., Cavazzini, G., Contu, A., D'Amico, M., Lavarello, A., Testore, F., Castagneto, B., Aitini, E., Perdelli, L., Bighin, C., Rosso, R., Venturini, M. 2005. HER-2 expression and efficacy of dose-dense anthracycline-containing adjuvant chemotherapy in breast cancer patients. *Br. J. Cancer 93*: 7–14.

Ezzat, A.A., Ibrahim, E.M., Ajarim, D.S., Rahal, M.M., Raja, M.A., Tulbah, A.M., Al-Malik, O.A., Al-Shabanah, M., and Sorbris, R. 2004. Phase II study of neoadjuvant paclitaxel and cisplatin for operable and locally advanced breast cancer: analysis of 126 patients. *Br. J. Cancer 90*: 968–974.

Frasci, G., D'Aiuto, G., Comella, P., Apicella, A., Thomas, R., Capasso, I., Di Bonito, M., Cartenì, G., Biglietto, M., De Lucia, L., Maiorino, L., Piccolo, S., Bianchi, U., D'Aniello, R., Lapenta, L., and Comella, G. 1999. Cisplatin-epirubicin-paclitaxel weekly administration in advanced breast cancer: a phase I study of the Southern Italy Cooperative Oncology Group. *Breast Cancer Res. Treat. 56*(3): 239–252.

Frasci, G., D'Aiuto, G., Comella, P., Thomas, R., Capasso, I., Di Bonito, M., Rivellini, F., Cartenì, G., De Lucia, L., Maiorino, L., D'Aniello, R., Frezza, P., Lapenta, L., and Comella, G. 2000. Cisplatin-epirubicin-paclitaxel weekly administration with G-CSF support in advanced breast cancer. A Southern Italy Cooperative Oncology Group (SICOG) phase II study. *Breast Cancer Res. Treat. 62*(2): 87–97.

Frasci, G., D'Aiuto, G., Comella, P., Thomas, R., Botti, G., Di Bonito, M., D'Aiuto, M., Romano, G., Rubulotta, M.R., and Comella G. 2005. A 2-month cisplatin-epirubicin-paclitaxel (PET) weekly combination as primary systemic therapy for large operable breast cancer: a phase II study. *Ann. Oncol. 16*: 1268–1275.

Frasci, G., D'Aiuto, G., Comella, P., Thomas, R., Botti, G., Di Bonito, M., De Rosa, V., Iodice, G.,

Rubulotta, M.R., and Comella G; Southern Italy Cooperative Oncology Group (SICOG). 2006. Weekly cisplatin, epirubicin, and paclitaxel with granulocyte colony-stimulating factor support vs triweekly epirubicin and paclitaxel in locally advanced breast cancer: final analysis of a sicog phase III study. *Br. J. Cancer 95*: 1005–1012.

Gianni, L., Baselga, J., Eiermann, W., Guillem Porta, V., Semiglazov, V., Lluch, A., Zambetti, M., Sabadell, D., Raab, G., Llombart Cussac, A., Bozhok, A., Martinez-Agullo, A., Greco, M., Byakhov, M., Lopez, J.J., Mansutti, M., Valagussa, P., and Bonadonna G; European Cooperative Trial in Operable Breast Cancer Study Group. 2005. Feasibility and tolerability of sequential doxorubicin/paclitaxel followed by cyclophosphamide, methotrexate, and fluorouracil and its effects on tumor response as preoperative therapy. *Clin. Cancer Res. 11*(24 Pt 1): 8715–8721.

Gonzalez-Angulo, A.M., McGuire, S.E., Buchholz, T.A., Tucker, S.L., Kuerer, H.M., Rouzier, R., Kau, S.W., Huang, E.H., Morandi, P., Ocana, A., Cristofanilli, M., Valero, V., Buzdar, A.U., and Hortobagyi, G.N. 2005. Factors predictive of distant metastases in patients with breast cancer who have a pathologic complete response after neoadjuvant chemotherapy. *J. Clin. Oncol. 23*: 7098–7104.

Green, M.C., Buzdar, A.U., Smith, T., Ibrahim, N.K., Valero, V., Rosales, M.F., Cristofanilli, M., Booser, D.J., Pusztai, L., Rivera, E., Theriault, R.L., Carter, C., Frye, D., Hunt, K.K., Symmans, W.F., Strom, E.A., Sahin, A.A., Sikov, W., and Hortobagyi GN. 2005. Weekly paclitaxel improves pathologic complete remission in operable breast cancer when compared with paclitaxel once every 3 weeks. *J. Clin. Oncol. 23*: 5983–5992.

Guarneri, V., Broglio, K., Kau, S.W., Cristofanilli, M., Buzdar, A.U., Valero, V., Buchholz, T., and Meric. 2006. Prognostic value of pathologic complete response after primary chemotherapy in relation to hormone receptor status and other factors. *J. Clin. Oncol. 24*(7): 1037–1044.

Konecny, G.E., Thomssen, C., Luck, H.J., Untch, M., Wang, H.J., Kuhn, W., Eidtmann, H., du Bois, A., Olbricht, S., Steinfeld, D., Mobus, V., von Minckwitz, G., Dandekar, S., Ramos, L., Pauletti, G., Pegram, M.D., Janicke, F., and Slamon, D.J. 2004. Her-2/neu gene amplification and response to paclitaxel in patients with metastatic breast cancer. *J. Natl. Cancer Inst. 96*: 1141–1151.

Mazouni, C., Kau, S.W., Frye, D., Andre, F., Kuerer, H., Buchholz, T., Symmans, W., Anderson, K., Hess, K., Gonzalez-Angulo, A., Hortobagyi, G.N., Buzdar, A., and Pusztai, L. 2007. Inclusion of taxanes, particularly weekly paclitaxel, in preoperative chemotherapy improves pathologic complete response rate in estrogen receptor-positive breast cancers. *Ann. Oncol. 18*: 874–880.

Moliterni, A., Menard, S., Valagussa, P., Biganzoli, E., Boracchi, P., Balsari, A., Casalini, P., Tomasic, G., Marubini, E., Pilotti, S., and Bonadonna, G. 2003. HER2 overexpression and doxorubicin in adjuvant chemotherapy for resectable breast cancer. *J. Clin. Oncol. 21*: 458–462.

Orlando, L., Colleoni, M., Curigliano, G., Nolè, F., Ferretti, G., Masci, G., Peruzzotti, G., Minchella, I., Intra, M., Veronesi, P., Viale, G., and Goldhirsch, A. 2001. Chemotherapy with vinorelbine, cisplatin and continuous infusion of 5-fluorouracil in locally advanced breast cancer: a promising low-toxic regimen. *Anticancer Res. 21*(6A): 4135–4139.

Padhani, A.R., Hayes, C., Assersohn, L., Powles, T., Makris, A., Suckling, J., Leach, M.O., and Husband, J.E. 2006. Prediction of clinicopathologic response of breast cancer to primary chemotherapy at contrast-enhanced MR imaging: initial clinical results. *Radiology 239*: 361–374.

Penault-Llorca, F., Abrial, C., Mouret-Reynier, M.A., Raoelfils, I., Durando, X., Leheurteur, M., Gimbergues, P., Tortochaux, J., Cure, H., and Chollet, P. 2007. Achieving higher pathological complete response rates in HER-2-positive patients with induction chemotherapy without trastuzumab in operable breast cancer. *Oncologist 12* : 390–396.

Pritchard, K.I., Shepherd, L.E., O'Malley, F.P., Andrulis, I.L., Tu, D., Bramwell, V.H., and Levine, M.N.; National Cancer Institute of Canada Clinical Trials Group. 2006. HER-2 and responsiveness of breast cancer to adjuvant chemotherapy. *N. Engl. J. Med. 354*: 2103–2111.

Ring, A.E., Smith, I.E., Ashley, S., Fulford, L.G., and Lakhani, S.R. 2004. Oestrogen receptor status, pathological complete response and prognosis in patients receiving neoadjuvant chemotherapy for early breast cancer. *Br. J. Cancer 91*: 2012–2017.

Rody, A., Karn, T., Gatje, R., Ahr, A., Solbach, C., Kourtis, K., Munnes, L, Loibl, S., Kissler, S., Ruckhaberle, E., Holtrich, U., von Minckwitz, G., and Kaufmann, M. 2007. Gene expression profiling of breast cancer patients treated with docetaxel, doxorubicin, and cyclophosphamide within the GEPARTRIO trial: HER-2, but not topoisomerase II alpha and microtubule-associated protein tau, is highly predictive of tumor response. *Breast 16*: 86–93.

Rousseau, C., Devillers, A., Sagan, C., Ferrer, L., Bridji, B., Campion, L., Ricaud, M., Bourbouloux, E., Doutriaux, I., Clouet, M., Berton-Rigaud, D., Bouriel, C., Delecroix, V., Garin, E., Rouquette, S., Resche, I., Kerbrat, P., and Chatal, J.F., and Campone, M. 2006. Monitoring of early response to neoadjuvant chemotherapy in stage II and III breast cancer by [18F]fluorodeoxyglucose positron emission tomography. *J. Clin. Oncol. 24*: 5366–5372.

Rouzier, R., Perou, C.M., Symmans, W.F., Ibrahim, N., Cristofanilli, M., Anderson, K., Hess, K.R., Stec, J., Ayers, M., Wagner, P., Morandi, P., Fan, C., Rabiul, I., Ross, J.S., Hortobagyi, G.N., and Pusztai, L. 2005. Breast cancer molecular subtypes respond differently to preoperative chemotherapy. *Clin. Cancer Res. 11*: 5678–5685.

Seidman, A.D., Hudis, C.A., Albanell, J., Tong, W., Tepler, I., Currie, V., Moynahan, M.E., Theodoulou, M., Gollub, M., Baselga, J., and Norton, L. 1998. Dose-dense therapy with weekly 1-hour paclitaxel infusions in the treatment of metastatic breast cancer. *J. Clin. Oncol. 16*(10): 3353–3361.

Untch, M., Konekny, G., and Ditsch, N. 2002. Dose-dense sequential epirubicin-paclitaxel as preoperative treatment of breast cancer: results of a randomised AGO study. *Proc. Am. Soc. Clin. Oncol. 21*: 34a (abstr. 133).

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Breast Cancer: Side Effects of Tamoxifen and Anastrozole

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INTRODUCTION

Breast cancer remains the commonest malignancy amongst women with a lifetime risk of between 10% to 12%. The incidence continues to increase with almost half a million women dying of the disease worldwide. There are now 45,000 and 15,000 deaths per annum in the United States and United Kingdom, respectively, and the inexorable rise in numbers of women suffering from breast disease is particularly notable in those countries that previously had a relatively low incidence of breast cancer but have now adopted Western lifestyles with changes in reproductive behaviour and greater usage of the oral contraceptive pill. Despite the continued rise in incidence of breast cancer, mortality rates have fallen over the past 2 decades, which is attributable to a combination of screening, heightened public awareness of the disease and the introduction of adjuvant systemic therapies. These epidemiological observations emphasize the hormone dependency of breast cancer and the importance of endocrine factors for tumor initiation and promotion. Breast cancer is a predominantly post-menopausal disease in which more than three-quarters of tumors are hormone responsive. This hormone dependency of breast cancer interacts with environmental and genetic factors to determine incidence and progression of the disease. However, it is the clinical response of these tumors to hormonal manipulation, which provides a unique therapeutic opportunity; tamoxifen has served as a prototype for the development of targeted therapies at the laboratory-clinical interface. Molecular technologies have permitted elucidation of mechanisms for tissue specific action and led to newer selective estrogen receptor modulators (SERM's) with potentially greater anti-tumor efficacy and attenuated uterotrophic profile. Nonetheless, publications over the past 4–5 years have emphasized the risks of thromboembolism and endometrial carcinoma associated with tamoxifen use, which has accelerated application of other hormonal agents for treatment and chemoprevention

of breast cancer. Indeed, there has been a resurgence of interest in hormonal therapies with the advent of third-generation aromatase inhibitors (AI) that represent the most significant advance in endocrine management of breast cancer since the introduction of tamoxifen more than 30 years ago. Recent data showing a survival advantage for aromatase inhibitors versus tamoxifen alone in both the advanced and adjuvant settings has heralded a major shift in standard first-line endocrine therapies for both advanced and early stage disease and perhaps ultimately chemoprevention of breast cancer.

Like many novel agents for treatment of breast cancer, AI's were initially used for advanced disease and Buzdar *et al*. (1996) showed that they offered advantages over tamoxifen and progestins as first- and second-line therapies, respectively. Aromatase inhibitors are widely used in the neoadjuvant setting for hormone sensitive tumors, and Smith *et al*. (2005) demonstrated that these agents can permit subsequent breast conservation surgery when mastectomy would otherwise have been indicated. However, it is in the adjuvant setting that AI's have stimulated much interest and generated an element of uncertainty in the optimum form of adjuvant hormonal therapy for post-menopausal women with estrogen receptor positive tumors. It seems likely that any blanket policy is no longer appropriate and a selective strategy with tailoring of therapy based on risk of relapse is the preferred option. Those patients at greatest risk of relapse may benefit most from an upfront AI, whilst those with lower hazard rates for relapse may be best treated with an 'early switch' regimen involving tamoxifen for 2–3years followed by an AI for a total duration of 5 years. Benefits in terms of disease-free and overall survival must be balanced against longer-term adverse effects on bone health and cognitive function as well as cost. Some patients at very low risk of relapse may derive minimal additional benefit from incorporation of an AI into their treatment schedule and should receive tamoxifen only.

The three oral AI's, anastrozole, letrozole, and exemestane are of comparable efficacy and are potentially interchangeable. Longitudinal studies must be undertaken with gathering of longer term data on side-effect profiles before any definitive pronouncements on clinical utility. There are particular concerns about severe oestrogen depletion amongst women receiving an AI for chemoprevention and ongoing evaluation of treatment related morbidity is essential.

This chapter will focus on the side-effects of tamoxifen and the AI anastrozole within the context of their clinical application. The biological rationale for hormone manipulation will be outlined and an overview of the principle trials will be provided, evaluating anastrozole versus tamoxifen in the advanced, adjuvant, and chemopreventive settings.

BIOLOGICAL MODELS

The concept of hormonal manipulation as a therapeutic goal is based on biological principles and an understanding of mammary tumorigenesis derived from preclinical models and *in vitro* studies of breast cancer cell lines. The first evidence for a direct role of estrogens in the development of breast cancer came from the use of

ablative endocrine therapies for the treatment of metastatic breast cancer. Schinzinger (1889) suggested that breast cancer was hormonally responsive and might regress following removal of the ovaries. Beatson (1896) was the first surgeon to perform such a procedure for breast cancer, reporting a favourable response to oophorectomy in a small group of pre-menopausal women with disseminated disease. Surgical methods of hormonal ablation included not only oophorectomy, but also more intrusive procedures such as adrenalectomy and hypohysectomy. They were associated with a higher morbidity and usually reserved as 'second-line' endocrine therapies for relapse following primary ovarian suppression.

Beatson's clinical observations were made more than a century ago, and the physiological basis for regression of breast cancer was unknown at that time. Estrogens were isolated in crystalline form in the 1930s and were soon implicated in the initiation and promotion of mammary tumors in rodents by Lacassagne (1932) amongst others. The selective accumulation of radiolabelled synthetic oestrogens in target organs which responded to these hormones supported a direct role for oestrogens in normal breast development and physiology. Furthermore, uptake of radiolabelled systemic oestradiol by 7,12 dimethylbenzanthracene (DBMA) – induced rat mammary tumors suggested that oestrogens may also be involved in promoting tumor growth by acting directly upon breast cancer cells via putative oestrogen receptors. Curiously, mean plasma levels of oestrogen did not and have never been found to correlate with breast cancer risk. Moreover, administration of high dose oestrogen could lead paradoxically to regression of advanced breast cancer.

The discovery of the estrogen receptor by Toft and Gorski in the mid-1960s consolidated understanding of oestrogen stimulated growth. Oestrogen binding in uterine tissue of immature rats led Jensen (1968) to formulate an early model for oestrogen mediated events in which oestrogen interacted directly with target cells via cytoplasmic receptors, oestrogen receptors (ER). Subsequent translocation of the ligand/receptor to the nucleus and its interaction with DNA resulted in modulation of gene transcription (oestrogen responsive genes). The uptake of tritiated oestradiol by breast tumor samples was essentially 'all-or-none' and this dichotomous response led to designation of tumor as ER positive or ER negative. This heralded the modern era of endocrine therapy in which the clinical response of advanced breast cancer could be predicted from the ER content of metastatic lesions and later of primary tumors. Approximately, half of all advanced breast cancers are ER positive, 60% of which will respond to hormonal manipulation compared with only 5–10% of ER negative tumors. Modern methods of immunoassay classify tumors as ER rich or ER poor. The system attributed to Allred (1998) has been developed to more accurately define ER expression to predict response to endocrine treatments. Tumors are assigned an intensity score (1–3) and a frequency score (0–5) and ER levels are based on an aggregate score from 0 (no expression) to 8 (strong expression). Differential responses between aromatase inhibitors and tamoxifen have been observed for lower levels of expression (0–3).

ANTI-HORMONAL THERAPIES

The growth stimulatory effects of estrogens upon hormonally sensitive tissues can be opposed by either interfering with the ligand-receptor interaction at the cellular level or by suppressing oestrogen production.

Anti-Estrogens. Characterisation of the ER and its mechanism of action provided an alternative approach to hormonal manipulation. Instead of inducing a state of oestrogen deprivation by surgical intervention, the action of estrogen could be blocked at the level of the receptor by competitive antagonism. Initial interest in sex hormone antagonists lay in their potential as oral contraceptive agents and it was the failure of tamoxifen in this capacity, which ultimately changed its clinical destiny. This agent was found by Jordan and Koerner (1975) to block binding of estrogen to the human ER. Consistent with this ER blocking activity, tamoxifen was reported by Jordan (1974) to suppress DMBA-induced carcinogenesis in rodent mammary tumors and inhibited growth of ER positive cancer cell lines *in vitro*.

Tamoxifen was first used in the treatment of advanced breast cancer with response rates of up to 70% (Ravdin *et al*., 1992) in those patients expressing both ER and progesterone receptor (PR) and 30% of unselected patients. However, it is in the adjuvant setting that tamoxifen has found widespread application in recent years as standard hormonal therapy for large numbers of both pre- and post-menopausal women with breast cancer. It is a relatively nontoxic therapy that permits effective targeting of micrometastatic tumor foci at distant sites which are preexistent in many patients presenting with 'early breast cancer'. Much of the clinical success of tamoxifen is attributable not only to its effectiveness, but also its favourable side-effect profile which makes it a potential candidate for chemoprevention.

Tamoxifen is classified as an anti-estrogen whose primary action is to competitively antagonize estrogen at the level of cellular receptors. However, it also possesses estrogen agonist properties on non-breast tissues such as the endometrium and bone. On the one hand, these may confer incidental benefits in terms of enhanced bone mineral density; Saarto *et al*. (1996) confirmed that tamoxifen can improve lipid profiles. On the other, Jordan (2001) points out that this agonist activity increases the incidence of endometrial cancer and may compromise the fundamental anti-tumor activity of tamoxifen.

The term selective estrogen receptor modulator or SERM encompasses this duality of action. SERM's are non-steroidal compounds which interact with the ER, resulting in degrees of dimerisation of the two structurally related forms of the receptor (alpha and beta) whose distribution permits variable and tissue specific activation of estrogen response genes. Tamoxifen is the prototype of this group of SERM's and possesses a triphenylbutene core and basic side chain. The tissue specificity results in anti-estrogenic activity on breast tissue but partial estrogen agonist activity has been demonstrated for endometrial tissue by Assikis *et al*. (1992) and for bone by Love *et al*. (1992). Tamoxifen modulates serum lipid profiles in an estrogenic manner with a reduction in LDL fraction and maintenance or increase in HDL levels reported by Love *et al*. (1991). Despite a favourable modulation in the ratio of HDL/LDL cholesterol,

this has not been translated into a reduced incidence of ischaemic heart disease or cardiac mortality. Paech *et al*. (1997) have elegantly shown how the configuration of the SERM-oestrogen receptor complex determines the recruitment of coactivators and corepressors that bind to the external surface of the complex and activate estrogen response elements. The pattern of transcriptional complexes is programmed by this SERM-estrogen receptor complex which in turn is governed by the precise chemical formula and structure of the ligand. Newer SERM's have been developed with weaker estrogen agonist properties and attenuated uterotrophic effects. Individual SERM's have a clinical signature with a range of structure-activity profiles that are site specific and confer differential and noncorrelative mixed agonist/antagonist activity between species and tissues.

Aromatase Inhibitors. Newer SERM's with attenuated uterotrophic activity together with selective estrogen receptor downregulators (SERD's) are under development and evaluation. Nonetheless, approaches to endocrine manipulation have shifted away from directly targeting the ER to inducing states of estrogen deprivation in post-menopausal women, using highly selective oral aromatase inhibitors that do not affect adrenal synthesis of glucocorticoids and mineralocorticoids.

Following cessation of ovarian function at the menopause, the principle source of estrogens are from peripheral synthesis in adipose tissue and the adrenal glands. The enzyme estrogen synthetase or aromatase is present within these tissues together with skeletal muscle and two-thirds of breast tumors. This cytochrome p450 enzyme converts the androgens testosterone and androstenedione to estradiol and estrone, respectively. This process of aromatization in peripheral tissues releases relatively small amounts of estrogen into the general circulation compared with ovarian sources in premenopausal women. However, van Landeghem *et al*. (1985) have found that local concentrations of estrogen within the post-menopausal breast can reach levels comparable to the premenopausal state due to local production by breast adipose tissue supplemented by intratumoral aromatase activity.

Aromatase inhibitors block peripheral aromatization of androgens and reduce both serum and intra-mammary tissue levels of estrogen, and have therapeutic efficacy in post-menopausal women only. When ovarian function remains intact, aromatase inhibitors can produce a reflex increase in levels of GnRH (luteinising hormone/follicle stimulating hormone), which in turn will stimulate the ovaries and can lead to supra-physiological levels of estrogen in premenopausal women. Stein *et al*. (1990) have investigated the use of GnRH antagonists in conjunction with aromatase inhibitors as a possible strategy for hormonal manipulation in younger premenopausal women who are strongly ER positive.

All of the 'third-generation' aromatase inhibitors share the convenience of being once daily oral preparations with low toxicity and are classified as TYPE 1 or TYPE 2 depending on their mechanism of action. The three main agents in current clinical usage are exemestane, anastrozole, and letrozole. The former is a steroidal analogue of androstenedione that binds *irreversibly* to the steroid binding site of the enzyme with inactivation and destruction of the enzyme. By contrast, the latter

two agents are non-steroidal compounds (triazoles) which bind *reversibly* to the p450 component of the haem site.

Despite differing mechanisms of action, both type I and II aromatase inhibitors have been shown (Lonning *et al*., 2003) to reduce enzyme activity and circulating levels of estrogen by ≥ 98%. *In vitro* studies with a variety of cell systems (including breast cancer cell lines) suggest that letrozole is ≥ 10-fold potent than anastrozole at inhibiting aromatization. Moreover, Geisler *et al*. (2002) demonstrated that letrozole induces a more profound decrease in whole body aromatization and levels of plasma estrogen (estrone [E1], estrone sulphate [E1S]) in postmenopausal breast cancer patients than anastrozole. These differences in the pharmacodynamic profiles of these two agents has not translated into any gains in time to progression (TTP) for letrozole in the context of metastatic breast cancer and their clinical relevance remains uncertain.

The following sections provide an overview of the clinical applications of tamoxifen and the aromatase inhibitor anastrozole in the context of advanced and early stage breast cancer together with their potential as chemopreventive agents. This will include a critical discussion of the side effect profiles for each agent, and how these should be assimilated into any overall assessment of clinical utility based on cost:risk:benefit analysis.

ADVANCED BREAST CANCER

Tamoxifen was first used in the management of advanced breast cancer in postmenopausal women for whom 30% of unselected and 60% of estrogen receptor-positive patients respond. By contrast, fewer than 10% of patients with a paucity of receptors respond to tamoxifen. A high proportion of patients who initially respond to tamoxifen will relapse and all patients eventually acquire resistance. Aromatase inhibitors have shown superior efficacy and better tolerability compared with conventional second line hormonal therapies such as progestins and aminoglutethimide, with response rates ranging from 8% to 24%.

Two seminal randomized trials have compared anastrozole with tamoxifen in postmenopausal women with ER positive and/or PgR positive or receptor unknown advanced breast cancer. In the North American trial, undertaken by Nabholtz *et al*. (2000), 353 patients were randomly allocated either tamoxifen (20 mg/day) or anastrozole (1 mg/day) as first line treatment, whilst almost twice this number of patients were randomized to receive similar treatment schedules in the European trial (Bonneterre *et al*., 2000). Though the North American trial showed a statistically significant ($p = 0.005$) improvement in TTP with an aromatase inhibitor (11.1 months versus 5.6 months), a combined analysis suggested comparable efficacy and anastrozole was recommended as an alternative to tamoxifen as first line treatment of advanced breast cancer in postmenopausal women. Though the North American trial showed an improvement in TTP, Nabholtz *et al*. (2001) have subsequently shown that there was no difference in objective response rates nor overall survival. By contrast, the larger European study showed no difference in any of these endpoints with an average TTP of 8 months for the two treatment groups. It is noteworthy that in the North American trial, nearly all patients had documented hormonal status

compared with only half those in the European trial. It therefore seems likely that the receptor unknown group contained a greater number of hormone receptor negative tumors. Furthermore, 39% and 22% of patients had received prior tamoxifen in the North American and European trials evaluating anastrozole, respectively. Aromatase inhibitors may be of value as first line treatment of advanced disease in patients who have previously received adjuvant tamoxifen, and are therefore less likely to respond to this agent in the metastatic setting.

A pooled analysis of eight prospective randomized studies involving 1,615 patients treated with an aromatase inhibitor (letrozole, anastrozole, fadrozle, formestane or exemestane) and 1,623 with tamoxifen for metastatic breast cancer has recently been presented by Carlini *et al.* (2005). Despite considerable heterogeneity amongst these trials, the hazard rate for risk of TTP for aromatase inhibitors compared with tamoxifen was 0.83 ($p < 0.001$). When confined to non-steroidal aromatase inhibitors, the HR was 0.79 ($p < 0.001$). Overall response rates were higher for aromatase inhibitors (relative risk 1.18; $p < 0.01$), but no difference in overall survival emerged from this pooled analysis. However, failure to demonstrate an overall survival advantage may partially be attributable to the confounding effects of chemotherapy which many ER positive postmenopausal women have received. The safety profile of aromatase inhibitors in terms of thromboembolic events was superior, and this echoes the findings from the North American study in respect of anastrozole. This coupled with a lower incidence of vaginal bleeding (relative risk 0.36; $p < 0.01$) is an important factor in assessing the risk:benefit ratio for aromatase inhibitors particularly in the adjuvant and chemopreventive settings.

NEOADJUVANT HORMONAL THERAPY

By analogy with neoadjuvant chemotherapy, hormonal treatment can be used pre-operatively with the intention of downstaging tumors. This may render inoperable tumors operable and permit breast conservation in patients who might otherwise require mastectomy. Hormonal therapies are less toxic and potential side-effects of chemotherapy can be avoided in elderly patients and those with a poor performance status. Furthermore, Buzdar *et al.* (2003) have reported that patients with hormone receptor positive disease are less likely to achieve a complete pathological tumor response from preoperative chemotherapy schedules. Urruticoechea (2005) and others have confirmed that aromatase inhibitors consistently outperform tamoxifen in the neoadjuvant setting, when endpoints include not only response rates, but also breast conservation rates.

The IMPACT study (Immediate Preoperative Arimidex, Tamoxifen or Combined with Tamoxifen) led by Smith *et al.* (2005) randomized 330 patients with operable hormone responsive breast cancer to 3 months of anastrozole alone, tamoxifen alone or a combination of the two. The objective clinical response rates in the three groups were 37.2%, 36.1%, and 39.4%, respectively. Though these response rates were not significantly different, anastrozole was more effective at downstaging tumors to permit breast conservation surgery (BCT) according to surgeon

assessment (46% considered suitable for BCT after anastrozole versus 22% for tamoxifen; p = 0.03). However, there was no significant difference in breast conservation rates when analysed on the basis of actual surgery performed at 3 months (44% versus 31%, respectively; p = 0.23). Despite evidence for higher response rates for the other non-steroidal aromatase inhibitor letrozole compared with anastrozole in the neoadjuvant setting (55% versus 37.2%), Urriticoechea (2005) found that treatment of primary operable ER positive tumors with letrozole or anastrozole for 2 weeks results in similar changes in the proliferation marker Ki-67. There is some evidence for a preferential decrease in mean Ki-67 levels in ER positive/HER2 negative patients receiving 3–4 months of preoperative letrozole. Ellis *et al.* (2005) found a significant fall in mean Ki-67 levels in HER2 negative patients, but levels remained unaltered in HER2 positive patients ($p < 0.0001$). These results suggest that HER2 positive patients may more readily develop resistance to aromatase inhibitor therapy. Inhibition of HER2 overexpression in tumor xenografts with a combination of gefitinib, trastuzumab and pertuzumab (GTP) can restore or increase ER expression thus overcoming endocrine resistance and allowing further hormonal manipulation.

ADJUVANT HORMONAL THERAPY

Tamoxifen has been the mainstay of adjuvant systemic hormonal treatment of breast cancer for the past 30 years. Though there are extensive data confirming the clinical efficacy of tamoxifen in prolonging both disease-free and overall survival, its dominance is being challenged by the emergence of the oral aromatase inhibitors that may combine greater efficacy with a more favourable side-effect profile (particularly in terms of thromboembolism and endometrial cancer). Use of aromatase inhibitors in the adjuvant setting is being investigated with two principle approaches – head to head and sequencing trials with an early or late switch.

ADJUVANT TRIALS OF TAMOXIFEN

The series of overviews by the Early Breast Cancer Trialists Collaborative Group (EBCTCG) (1998) undertaken at 5 yearly intervals have confirmed that tamoxifen reduces rates of local relapse and improves overall survival. The most recent analysis involving 37,000 patients reveals benefits for all age groups irrespective of menopausal and nodal status. At least 5 years of tamoxifen therapy confers a proportional reduction in mortality of 26% and up to 47% reduction in local recurrence at 10 year follow up. These benefits are confined to patients with ER- positive tumors, though the number of patients with ER-negative tumors was small with fewer events. When ER- negative tumors were excluded from the analysis, absolute risk reductions for local recurrence were 14.9% for node negative patients and 15.2% for node positive patients ($p < 0.001$). Absolute mortality reductions for node negative and node positive groups were 5.6% and 10.9%, respectively ($p < 0.0001$).

The NSABP B-14 trial reported by Fisher *et al.* (2001) rerandomised node negative patients who remained disease-free after 5 years of adjuvant tamoxifen to a further 5 years of the same or placebo.

This revealed no further reduction in disease recurrence nor improvement in longer term survival. Tamoxifen is a cytostatic agent and there is a theoretical rationale for extended adjuvant endocrine treatment. However, the survival benefits of tamoxifen continue beyond 5 years, although much of the effect on recurrence occurs before cessation of tamoxifen treatment. This carry-over effect is clinically opportune and of particular importance in the context of the B-14 trial results that fail to reveal any benefit from more prolonged tamoxifen therapy. Furthermore, this study showed no statistically significant reduction in contralateral breast cancers following the second randomization. Continued therapeutic intervention may not impart any overall survival gain with respect to either the primary tumor or contralateral breast cancer. In the case of 10 years of tamoxifen, a net detrimental effect may ensue from adverse side-effects such as increased thromboembolism and gynaecological problems. Indeed, the most recent analysis of the B-14 trial shows a significantly shorter disease-free and overall survival for those patients treated for 10 years.

ADJUVANT TRIALS OF AROMATASE INHIBITORS

Adjuvant trials of AI's have investigated anastrozole and other third-generation oral agents using a variety of schedules including head to head comparisons of tamoxifen versus an AI, aromatase inhibitors in combination with tamoxifen and early sequencing with a switch to an AI after 2–3 years of tamoxifen.

ATAC trial. The ATAC (Arimidex, Tamoxifen, alone or in combination) study is the largest of these adjuvant trials, and Baum *et al*. (2002) were the first to publish results. More than 9,000 patients were enrolled in the original three way randomization. Following primary loco-regional treatment post-menopausal women were randomized to receive tamoxifen alone, anastrozole alone, or a combination of the two drugs for a total of 5 years. The rationale for the combination arm was based on differing mechanisms of action for tamoxifen and anastrozole, which were thought to be potentially synergistic. However, the combination arm was subsequently abandoned due to lack of any additional efficacy compared to tamoxifen alone with further evaluation confined to the monotherapy arms where 6,186 patients received one or other agent. Early results of this trial at a median follow up of 33 months revealed a statistically significant benefit for anastrozole over tamoxifen in terms of local recurrence (disease-free survival), time to recurrence and incidence of contralateral breast cancer. This initial analysis also demonstrated significant advantages for anastrozole in respect of treatment related side-effects, which included serious adverse events such as endometrial cancer and thromboembolism.

The most recent update of this trial by the ATAC trialists group (2005) provided data at 68 months follow up. This showed a continuing divergence of the curves for disease-free survival with evidence of a carry-over effect and a reduction in time to distant recurrence in favour of anastrozole. In hormone receptor positive patients, anastrozole increased disease-free survival by 17% (HR 0.83 95% CI 0.73–0.94; $p = 0.01$) representing an absolute risk reduction of 2.5% at 5 years. Though there was a reduction in distant disease-free survival (HR 0.93 95% CI 0.80–1.07), this was not significant and no benefit was

apparent for overall survival (HR 0.97 95% CI 0.85–1.12; p = 0.70). However, only 60% of all deaths within this study were attributable to breast cancer. There was a relative reduction of 53% in the incidence of contralateral cancers in hormone receptor positive patients (26 in anastrozole group versus 53 in tamoxifen group). There is evidence from a limited subgroup analysis of the ATAC trial that patients with ER positive but PgR-negative tumors and those with overexpression of HER2-neu may derive proportionately greater benefit from anastrozole (HR 0.48).

Italian (ITA) and Austrian trials (ABCSG 8 and ARNO 95). Each of these three individual trials have investigated an early switch (2–3 years) from adjuvant tamoxifen to anastrozole in post-menopausal women with hormone responsive breast cancer (Table 31.1). Results of the Italian trial were reported by Boccardo *et al.* (2001) and the Austrian trials by Jakesz *et al.* (2005a). The design of these trials was broadly similar and addressed the same question. This formed the basis and justification for a meta-analysis performed by Jonat and presented at the San Antonio Breast Cancer Symposium in 2005 and subsequently published in The Lancet Oncology (Jonat *et al.,* 2007). There was some lack of heterogeneity and certain key differences between the trials which are summarized in Table 31.1. Some of the trials randomized patients at the start of adjuvant systemic hormonal treatment (i.e., immediately after surgery), whilst others randomized after 2–3 years of tamoxifen (i.e., at the time of switching). There was also variation in the stage distribution of patients and in particular the proportion of node positive patients, and hence the absolute risk of relapse. Despite these limitations, combined group analysis was employed using raw data rather than processed results of individual trials. A previous combined analysis of the Austrian trials by Jakesz *et al.* (2005b) showed that switching to anastrozole after 2 years of tamoxifen therapy significantly improved event-free survival at a median follow up of 28 months (HR 0.60 95% CI 0.44–0.80; p = 0.0009).

TABLE 31.1. Summary of patient characteristics in early switch trials of tamoxifen and anastrozole used for meta-analysis.

ITALIAN (ITA) TRIAL (448 pts)
– 99% patients node positive
– Grade III tumors included
– > 50% patients underwent mastectomy
ABCSG 8 TRIAL (2,262 pts)
– Randomised immediately after surgery (not at point of switching)
– Grade I and II tumors
– 25% node positive
– 80% breast conservation
ARNO 95 (962 pts)
– Similar to ABCSG 8 but patients randomised at time of switching to anastrozole

The meta-analysis of the two Austrian trials combined with the Italian trial involved a total of 4,006 patients of whom 2009 switched from tamoxifen to anastrozole and 1997 remained on tamoxifen for 5 years. The median follow up across all trials was 30 months and incorporated 10,000 person years. The principle aim of the meta-analysis was to determine whether improvements in event-free survival translate into benefits in long term outcomes (overall survival). For each therapy arm, the distribution of event-free and overall survival was estimated using the Kaplan-Meier technique. The results showed a statistically significant disease-free survival advantage for switching from tamoxifen to anastrozole (HR 0.59; p = 0.001).

In particular, there was a significant improvement in overall survival with a

29% reduction in risk of death from breast cancer (HR 0.71; p = 0.038). A Forest plot of individual trials revealed that improvement in survival only reached significance in the ARNO 95 trial. Analysis of the ABCSG 8 trial alone at a median follow up of 30 months showed that switching to anastrozole after 2 years of tamoxifen significantly reduced the incidence of local events. The magnitude of the risk reduction was greater when analysed from the point of switching (40%) compared with the time of randomization, which included the first 2 years of tamoxifen (24%). Furthermore, Jakesz *et al*. (2005a) showed that the disease-free survival advantage for switching from tamoxifen to anastrozole was lost when the analysis was done from the time of randomization (p = 0.07) rather than at therapy switch (p = 0.01). However, there was no overall survival advantage (HR 0.93; p = 0.726). The authors concluded from this meta-analysis that switching from tamoxifen to anastrozole after 2–3 years results in: (1) fewer recurrences, (2) benefits in terms of overall survival, and (3) favourable safety profiles. It was recommended that post-menopausal patients with hormone responsive breast cancer should be switched to anastrozole upon completion of 2–3 years of tamoxifen treatment. The methodology employed for this meta-analysis has been questioned and the lack of homogeneity is of some concern. Moreover, the ARNO 95 and ITA trials are relatively small compared with the ABCSG 8 trial and have correspondingly fewer events.

An element of uncertainty now prevails over the optimum form of adjuvant endocrine therapy for post-menopausal women with hormone receptor positive tumors. This has been generated by emerging data from the above trials and clarification is now demanded on how best to advise and manage patients who are newly diagnosed with breast cancer or have already received some years of tamoxifen. The American Society of Clinical Oncology Technology Assessment Winer *et al.* (2005) recommends that adjuvant hormonal therapy for this group of patients should include an aromatase inhibitor prescribed either as initial therapy or sequenced after tamoxifen for 2–3 years (early switch) or 5 years duration. It is implicit from this consensus that 5 years of tamoxifen alone is no longer considered adequate therapy for any group of postmenopausal estrogen receptor positive patients. This statement represented a major shift in management strategy for breast cancer patients and was taken one step further in the article published on behalf of the ATAC Trialists Group (2005). This advocated that not only should 5 years of an aromatase inhibitor upfront be the preferred hormonal option for these women, but that anastrozole should be the agent of choice. Benefits in terms of disease-free and overall survival must be balanced against longer term risks (bone health; cognitive function) and costs.

There is no clear evidence that initial treatment with tamoxifen for 2–3 years prevents subsequent bone loss, but those patients who are not osteopaenic at the start of endocrine treatment are less likely to develop osteoporosis secondary to estrogen deprivation. An upfront aromatase inhibitor might be indicated in those patients at higher risk of relapse for whom the amplitude of the hazard peak for recurrence is proportionately greater in magnitude and could be suppressed or 'smoothed out' (Epanechinikov kernel) by an aromatase inhibitor more

effectively than tamoxifen. Baum (2004) explains that as this peak occurs in the first 2–3 years after primary treatment, such an effect would only be possible with an upfront aromatase inhibitor and not an early switch. For those patients with lower hazards for relapse within the first 2–3 years, sequential therapy with tamoxifen (2–3 years) followed by an aromatase inhibitor may be more appropriate and cheaper. Finally, it should be noted that a group of older patients may exist with a very low risk of relapse for whom 5 years of tamoxifen remains the most cost-effective adjuvant endocrine option. However, issues of cost are complex and should take into account subsequent adverse events prevented. Locker (2004) has emphasized that any health economic analysis is dependent on the particular healthcare system within which patients are treated.

The evidence available at the present time favours an early switch policy for the majority of patients with hormone receptor positive post-menopausal breast cancer. The absolute benefits of an aromatase inhibitor for the average patient are very small in the first 36 months, and some argue that the additional benefit of an aromatase inhibitor during the first 2–3 years is difficult to justify. Only 3.7% of patients have an early relapse in the first 2–3 years. Punglia *et al*. (2005) used a Markov analysis to develop models which simulated 10 year disease-free survival amongst post-menopausal ER positive women with early breast cancer. According to this analysis, switching from tamoxifen to an aromatase inhibitor after 2–3 years leads to a modest gain in disease-free survival compared with monotherapy with an upfront aromatase inhibitor for 5 years (relative risk reduction 6%). Furthermore, this early switch regimen appeared superior to a late switch from tamoxifen to an aromatase inhibitor after 5 years which did not result in further improvements in 10 year survival. However, Cuzik *et al*. (2006) have criticized this analysis on the basis of heterogeneity of endpoints. Some of the trials included used disease-free survival (ATAC, IES, Coombes *et al*. (2004), BIG 1–98, (Thurlimann, 2005), ITA (Boccardo *et al*., 2001)), whilst others time to recurrence (MA.17, ABCSG 8, ARNO 95). They maintain that there is a 'dilutionary effect' of deaths without recurrence when disease-free survival is used which augments the relative benefits of a switch strategy. A variation of the Markov method was used to demonstrate that when time to recurrence was taken as the primary endpoint, upfront aromatase inhibitors were the favoured option. This so-called 'deep model' attempts to explain observed events in terms of underlying biological mechanisms and offers a potential explanation for the greater efficacy of aromatase inhibitors in ER positive/PgR negative tumors compared with ER positive/PgR positive ones.

CHEMOPREVENTION

TAMOXIFEN

Recent trials have established the principle of chemoprevention and confirmed that tamoxifen can reduce the incidence of breast cancer, although this advantage has yet to demonstrate a reduction in disease specific mortality. These trials have concomitantly highlighted the dilemma of administering a chemopreventive agent to healthy women which not only has significant side-effects but may also lead to an increase in all-cause mortality.

Analysis of adjuvant trials by the EBCTCG (1998) have confirmed that at least 5 years of tamoxifen therapy can

reduce the incidence of contralateral breast cancer by 47%. Initial observations on the reduction in contralateral tumors together with laboratory data on suppression of dimethylbenzanthracene-induced mammary carcinoma in rodents with continuous tamoxifen administration led to three major trials of tamoxifen as a chemopreventive agent. The largest of these trials (NSABP P-1) was spearheaded by Fisher *et al.* (1998) and revealed a reduction in the cumulative incidence of invasive and non-invasive breast cancer of 49% ($p < 0.0001$) and 50% ($p < 0.002$), respectively. Initial analyses of the smaller European studies reported by Veronesi *et al.* (1998) and Powles *et al.* (1998) revealed that 5 years of tamoxifen conferred no chemoprotective effect. A follow up analysis of the Italian study by Veronesi *et al.* (1998) reinforces earlier conclusions that tamoxifen does not significantly reduce the incidence of breast cancer in hysterectomized women at usual or slightly reduced risk of disease. These differences in trial outcome have been attributed to several factors, including population size and intrinsic levels of risk among trial participants. There was a high rate of attrition within the Italian study with more than one-quarter of patients withdrawing and compliance estimated at no more than 70%. Though a significant preventive effect has not emerged for nonusers of HRT, those patients who ever used HRT have an incidence of breast cancer comparable to non-users. This has led some to conclude that tamoxifen may compensate for the proliferative effects of HRT upon breast tissue and partially negate the increased risk of breast cancer associated with HRT use. These results suggest that combined HRT and tamoxifen may keep to a minimum any increased breast cancer risk, whilst harnessing the benefits of HRT on cardiovascular osteoporotic events and avoiding any harmful effects on morality in longer term breast cancer survivors.

The IBIS-1 trial (2002) involved the double blind randomization of 7,000 healthy women at increased risk of breast cancer to either 5 years of tamoxifen therapy or placebo. Tamoxifen reduced the incidence of breast cancer by one-third and combined analyses of all chemoprevention trials have shown an overall reduction of 38% in the incidence of invasive and noninvasive malignancy. The risk:benefit ratio for tamoxifen is shifted in the chemopreventive setting, where otherwise healthy women receive a pharmacological intervention for which the benefits are less tangible. Day *et al.* (1999) remind us that the absolute benefit for an individual woman must be balanced against risks of endometrial cancer and thromboembolic phenomena, together with other side-effects such as hot flushes, sexual dysfunctioning, and cataracts. Healthy women may be affected with life-threatening conditions when they may never have developed breast cancer. It was this strong desire to minimize side-effects that sporned the STAR trial, a head to head comparison of tamoxifen with raloxifene as chemopreventive agents. Like tamoxifen, raloxifene is a triphenylethylene and classified as a SERM. The latter had been found by Cummings *et al.* (1999) to be associated with an incidental risk reduction of > 77% for breast cancer in the MORE trial for osteoporosis. This trial recruited patients with osteoporosis and no family history of breast cancer. Initial results of the STAR trial have been reported by Wickerham *et al.* (2006). This trial specifically aimed to evaluate the risk:benefit

ratio for these two agents, but some have commented that the trial is comparing "apples and pears". Initial results have shown that raloxifene is similar to tamoxifen in reducing the incidence of invasive breast cancer by 50% (statistically equivalent; 167 versus 163 cancers respectively). However, it appears to have little effect on noninvasive forms of the disease, suggesting that raloxifene may interfere with the progression of *in situ* to invasive disease, but have no impact on premalignant to *in situ* transition.

In terms of side-effects, raloxifene is at best moderately superior to tamoxifen and at worse of marginal statistical significance. Though there were fewer cases of uterine cancer in the raloxifene group (23/4,712) compared with the tamoxifen arm (36/4,732), it should be noted that the trial recruited only post-menopausal women more than half of whom had undergone hysterectomy and were also at higher risk for development of breast cancer as determined by the Gail model. There was a slight decrease in the number of women suffering from DVT or PE in the raloxifene group, but no difference was found in incidence of stroke. Though these results of the STAR trial were heralded by some as a breakthrough, they do not show an overwhelming benefit for raloxifene over tamoxifen in terms of efficacy, side-effects and cost.

AROMATASE INHIBITORS

In theory, the most effective agent for preventing breast cancer should be one that creates an hormonal environment depleted of estrogens. Aromatase inhibitors are associated with a greater reduction of contralateral breast cancer in adjuvant trials than tamoxifen and are currently under investigation as chemopreventive agents. The IBIS II is a multicentre trial which randomizes healthy women at increased risk of breast cancer to either anastrozole or placebo. Other aromatase inhibitors are likely to be candidates for chemoprevention trials in the future. Though these agents can only be used in post-menopausal women, they could be combined with GnRH antagonists as a chemopreventive strategy in premenopausal women. However, there are concerns about side-effects of profound estrogen deprivation (increased fractures, musculoskeletal pain), and it remains unclear for how long women should be treated with a chemopreventive agent. It is unknown whether a single 'pulse' of treatment (e.g., 5 years) is sufficient and whether any carry-over effect occurs. It would not be feasible to target young teenage girls with a combination of an aromatase inhibitor/GnRH agonist for a prolonged period of time. Delivery of a short pulse of treatment at a critical stage in a woman's life may induce changes in the breast tissue which confer longer term protection and avoids the adverse effects of more prolonged therapy.

Apart from concerns regarding longer term effects of estrogen deprivation, there are significant cost issues relating to use of aromatase inhibitors as chemopreventive agents in a managed healthcare system. It has been calculated by Jordan (2006) that the cost of preventing 300 breast cancers with an aromatase inhibitor is between £40 and £50 million pounds compared with £2 million for tamoxifen and £23 million for raloxifene. SERM's can prevent osteoporosis and decrease numbers of fractures which potentially incur future additional costs to the health service. The development of a SERM that combines enhanced

risk reduction for breast cancer with incidental benefits in other tissues may be a more promising approach to chemoprevention than aromatase inhibitors.

SIDE-EFFECTS OF HORMONAL THERAPIES

TAMOXIFEN

Much data have now accrued testifying to the clinical efficacy of tamoxifen in treatment and prevention of breast cancer. Clinical benefits relate not only to antitumor effects but also to sequelae of oestrogen agonist activity. There are concerns, however, over adverse effects of tamoxifen which have focused recently on heightened risks of thromboembolism and endometrial carcinoma in postmenopausal women. Adjuvant trials of tamoxifen have generally revealed a two- to threefold increased risk for each of these events. In the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14 study in which patients received either 5 years of tamoxifen or placebo in the principal randomization, thromboembolic events were higher in the tamoxifen group (1.3% versus 0.4%), with two patients dying from pulmonary emboli in this group of 1,404 patients. Other studies have shown comparable levels of enhanced relative risk (e.g., the NSABP P-1 study; relative risk 3.01 (pulmonary embolism) and 1.6 (deep venous thrombosis)). In the context of breast cancer treatment the risk:benefit ratio is overwhelmingly in favour of tamoxifen therapy, but the situation is different for those patients receiving tamoxifen as a prophylactic agent. Under these circumstances, tamoxifen is being administered to healthy women for whom the benefits are less tangible than for patients with breast cancer for whom improvements in local recurrence and overall survival rates are well documented. The recently published results of the International Breast Cancer Prevention Study (IBIS) confirm an increased risk of thromboembolic events, which is consistent with, and of the same order of magnitude, as previous adjuvant and prophylactic trials of tamoxifen. This latest analysis of the IBIS study not only reaffirmed the increased risk of thromboembolic events (odds ratio 2.5, 95% CI 1.5–4.4; $p = 0.001$) but also highlighted that thromboembolism was a relative contributor to nonbreast cancer deaths, with two possible deaths from pulmonary embolism in the tamoxifen arm of the IBIS study. An overview of the three main tamoxifen prevention trials revealed an overall increased relative risk for thromboembolic events of 1.9 (95% CI 1.4–2.6; $p < 0.0001$) which was similar for women under and above 50 years of age. Most of the thromboembolic events occurred within 3 months of major surgery or following a period of immobility, suggesting that appropriate preventative measures should be taken to reduce the risk of deep venous thrombosis and pulmonary embolism associated with tamoxifen therapy (including temporary cessation of therapy at the time of major elective surgery).

The IBIS-1 trial showed no statistically significant differences in cerebrovascular or cardiac events. These included: (i) all cerebrovascular events ($p = 0.86$), (ii) stroke or cerebrovascular accident ($p = 0.84$), (iii) transient ischaemic attack ($p = 0.34$), (iv) all cardiac events ($p = 0.44$) (v) myocardial infarction ($p = 1.00$), (vi) coronary revascularisation ($p = 1.00$), and (vii) angina ($p = 0.64$). Similarly, adjuvant trials of tamoxifen have shown no difference in

nonbreast cancer mortality between tamoxifen and control in respect of all cardiac (myocardial infarction) and vascular deaths (CVA, stroke, TIA only) ($2p > 0.1$). However, the relatively small number of vascular deaths may have precluded statistical demonstration of any reduction in these parameters (of the order 10–15%) attributable to tamoxifen.

The potential risk of hepatic neoplasia with prolonged tamoxifen therapy is often cited. However, Jordan (1999) points out that the incidence of such tumors is extremely low in women on longer-term tamoxifen, and the predicted risk based on animal data of DNA adducts and liver carcinogenesis in rats has not translated into clinical reality. Indeed, within adjuvant tamoxifen trials, there were fewer deaths linked to liver disease (hepatoma or primary liver cancer) within the tamoxifen group compared with the control group (12 cases versus 9 cases).

The estrogen agonist activity of tamoxifen includes uterotrophic effects encompassing endometrial thickening, polyps, and carcinoma. Jordan (1999) concludes that tamoxifen appears to stimulate a preexisting hypertrophic state rather than initiating carcinogenesis, with endometrial carcinoma occurring after relatively short durations of therapy. Moreover, there is no increase in DNA adduct formation in human uterine tissue and thickening of the endometrium occurs secondary to stromal expansion rather than endometrial hyperplasia. Tamoxifen induces a two- to threefold increased incidence of endometrial carcinoma, but absolute rates remain modest and are estimated by Jordan (1999) at 2/1,000 women years annual relative risk. In the NSAPB P-1 chemoprevention study, rates of endometrial cancer increased from 0.91 per 1,000 in the placebo group to 2.30 per 1,000 in the tamoxifen group, yielding a relative risk of 2.53 (95% confidence interval, 1.35–4.97). This increased risk affects women over 50 years of age with no stage migration and all tumors in the tamoxifen group being FIGO (International Federation of Gynaecology and Obstetrics) stage I and amenable to treatment. In a case control study, however, it was found that at least 5 years of adjuvant tamoxifen was associated with up to sevenfold increased risk of endometrial cancer and tumors of higher stage (FIGO III and IV) and more aggressive histology. Nonetheless, even allowing for a significantly worse endometrial cancer specific survival amongst tamoxifen users (0.2 per 1,000 excess of deaths from endometrial cancer) for all prevention trials, the overall benefits of tamoxifen in the adjuvant setting far outweigh any adverse gynaecological effects, and women should continue to receive tamoxifen as adjuvant treatment of breast cancer for which mortality from recurrent disease far exceeds that of endometrial malignancy. In the adjuvant trials involving 5 years of tamoxifen the absolute increase in incidence of endometrial cancer was only approximately half of the decrease in contralateral breast cancer. Furthermore, Love *et al.* (1999) have found no clear evidence that early detection with transvaginal ultrasound or endometrial biopsies (blind or hysteroscopically targeted) improves outcome in asymptomatic women on tamoxifen. Any preexisting lesion should be excluded on baseline gynaecological examination, but the most appropriate surveillance schedule remains uncertain; Juneja *et al.* (2002) have suggested

annual clinical examination for all women on tamoxifen, whilst Vosse *et al*. (2002) recommend an examination after 3 years of tamoxifen therapy. Abnormal bleeding requires complete gynaecological evaluation with concomitant increased anxiety and healthcare costs. Newer agents such as aromatase inhibitors and SERM's possess an attenuated uterotrophic profile that may offer cost-benefit advantages in respect of monitoring.

AROMATASE INHIBITORS

The aromatase inhibitors have emerged as a new class of hormonal therapies which are at least as effective and possibly superior to tamoxifen for treatment of metastatic breast cancer and are challenging the primacy of tamoxifen in the adjuvant setting. Furthermore, AI's appear to have a greater impact on the incidence of contralateral breast cancer than tamoxifen, and are being evaluated in the chemopreventive setting (IBIS-II). Though these agents are associated with fewer thromboembolic events there are potential adverse effects from longer term estrogen deprivation, including bone loss and impaired cognition.

Buzdar *et al*. (2006) have recently published a comprehensive side-effect profile of anastrozole and tamoxifen within the context of the ATAC trial. With a median follow up of 68 months, fewer than 10% of patients remained on trial treatment at the time of analysis and all of these had < 12 months scheduled treatment remaining. Data were analysed with stringent statistical criteria (99% CI) and a p-value of < 0.01 for significance level (non-predefined events). Prespecified checklists were not used in order to minimize the chance of reporting bias. Though this might have influenced absolute event rates, any effect would be comparable between the tamoxifen and anastrozole groups.

There was no significant difference for all adverse events recorded during treatment or within 14 days of discontinuation between tamoxifen and anastrozole (RR 0.99; 95% CI 0.98–1.00; p = 0.2). However, differences did emerge for the following categories in favour of anastozole:

1. Treatment related adverse events (RR 0.89; 95% CI 0.86–0.92; p < 0.0001)
2. Adverse events leading to withdrawal (RR 0.78; 95% CI 0.68–0.89; p = 0.0002)
3. Serious adverse events (RR 0.93; 95% CI 0.86–0.99; p = 0.03)
4. Treatment related serious adverse events (RR 0.53; 95% CI 0.43–0.64; p = 0.0001)

Results of this analysis reinforced previous findings that anastrozole is associated with fewer serious adverse events including thromboembolism (2.8% versus 4.5%; p = 0.0004), uterine malignancy (0.22% versus 0.76%; p = 0.02), and cerebrovascular events (2.0% versus 2.8%; p = 0.03). Rates of cardiovascular morbidity were similar between the two groups (angina 2.3% versus 1.6%; p = 0.07, myocardial infarction 1.2% versus 1.1%; p = 0.7). Though the annual fracture rate was higher in the anastrozole group (22.6 versus 15.6 fractures per 1,000 women years; HR 1.43; 95% CI 1.21–1.68; p < 0.0001), this has not increased over time and the excess fractures appear confined to the axial skeleton and upper limbs. Of note, the incidence of hip fractures was similar (and relatively low) for both groups, which is a reassuring finding bearing in mind the clinical and economic impact of fractures at this site within the general population.

Morever, fracture rates were not modulated by use of biphosphonates, which was low in both the tamoxifen and anastrozole groups (10% versus 7%; odds ratio 1.51; 95% CI 1.24–1.84; $p < 0.0001$). Symptoms of arthralgia were more common in the anastrozole group but this was not of a severity which prompted widespread withdrawal from treatment (13 versus 6). Thus, with more prolonged follow up further adverse events have not occurred in the anastrozole group and statistical significance has been maintained for the more favourable differences in predefined side-effect categories.

An exploratory analysis of nonpredefined events within the ATAC trial revealed advantages for anastrozole compared with tamoxifen in terms of adverse gynaecological events, hysterectomy rates, vaginal moniliasis, and urinary tract infections (see Table 31.1). However, reduced libido and dysparuenia were more commonly reported amongst patients taking anastrozole. Interestingly, the fourfold higher hysterectomy rate in the tamoxifen group may reflect a lower threshold by gynaecologists for performing this procedure amongst women with benign tamoxifen induced uterine changes (polyps, endometrial thickening).

Consistent with the overall increased fracture rate (11% versus 8%; $p = 0.0001$), patients within the anastrozole group were more likely to spontaneously report osteopenia or osteoporosis (11% versus 7%. odds ratio 1.49; 99% CI 1.18–1.88; $p < 0.0001$). The incidence of the latter rapidly increases with age in post-menopausal women and monitoring of bone health amongst patients receiving aromatase inhibitors is mandatory. Stimulation of osteoclastic activity is further supported by evidence of higher alkaline phosphatase levels and hypercalcaemia in women prescribed anastrozole compared with tamoxifen. Biphosphonates may be indicated for states of osteopenia in addition to established osteoporosis. Though muscle cramps were reported more frequently in the tamoxifen group (8% versus 4%; odds ratio 0.54; 99% CI 0.42–0.72), other musculoskeletal symptoms such as carpal tunnel syndrome and upper limb parasthesiae were more common in the anastrozole group (3% versus 1%; $p < 0.001$ and 7% versus 5%; $p < 0.0001$, respectively). This accords with a general increase in musculoskeletal complaints amongst users of aromatase inhibitors, which has been documented by Coombes *et al*. (2004, 2007) for exemestane within the Intergroup Exemestane study.

Measurement of lipid profiles were performed sporadically rather than systematically within the ATAC trial. The limited data available demonstrate relative hypercholesterolaemia within the anastrozole group (9% versus 3%; odds ratio 2.73; 99% CI 2.02–3.69; $p < 0.0001$). However, this apparent difference may be due to loss of the lipid-reducing properties of tamoxifen rather than any direct effect from anastrozole *per se*. Indeed, data from other studies suggest that this particular AI is clinically 'lipid-neutral'. It is noteworthy that this latest follow up analysis from the ATAC trial has not shown any difference in the number of cardiovascular deaths between the two groups (tamoxifen 1% versus anastrozole 2%). Trials involving other AI's have alluded to potential increased cardiovascular mortality and hence a differential side-effect profile or pharmacokinetics between individual AI's rather than a class effect.

The risk of death from breast cancer was statistically similar for anastrozole compared with tamoxifen (HR 0.88; 95% CI 0.75–1.05; p = 0.02), but there were more deaths overall in the tamoxifen group and more non-breast cancer related deaths in the anastrozole group.

It was concluded that any differences in these additional non-predefined events are unlikely to influence the choice of adjuvant hormonal therapy generally. However, Buzdar *et al*. (2006) have expressed lingering concerns about the longer term impact of AI's and estrogen deprivation on lipid profiles and bone health, together with other consequences of disruption of estrogen-based signaling. The ATAC trialists group has assimilated recurrence and side-effect data into global risk:benefit indices. For one of these indices (GI-WHI), the ATAC adverse events have been 'mapped' into corresponding fields used by the Women's Health Initiative (WHI). The other index is the GI-DFS-SAE which is standard methodology used in the Women's Health Initiative Study. The results of this synthesis have been presented as Kaplan-Meier curves of cumulative rates over time and the smoothed annual hazard rates show a continuous benefit for anastrozole throughout the 5 year treatment period, but this is greatest in the first 1–2 years. This suggests that patients at higher risk of local relapse may derive proportionately more benefit from an AI used upfront. It is perhaps premature to conclude from the data presented that anastrozole should be employed as first line adjuvant hormonal treatment for all post-menopausal estrogen receptor-positive patients. This processing of the data into global indices is methodologically sound, but is open to interpretation. Moreover, in terms of health economics, such an index does not incorporate costs which ultimately will influence any final decision on treatment options in a managed healthcare system.

PATIENT INFORMATION

It is important to be able to communicate the information on side effects of tamoxifen and anastrozole to patients. Jenkins *et al*. (2001) have outlined the reasons for this. Firstly it empowers patients, enabling them to help make an informed decision. It aids management of side effects as patients are aware of them before they present and so know what to expect and when to seek medical attention. Thirdly, patients like to be informed despite others preferring to leave the decision making to the professionals whose care they are under. Understanding the side effects and benefits may increase compliance. Finally, the National Institute of Clinical Excellence (NICE) guidelines on Cancer Services state that patients should have access to high quality information to aid decision making. Information is provided by a combination of nurses, oncologists, surgeons, and patient information leaflets. Nurses spend the most time with patients and surgeons the least. Information leaflets allow patients to read about information they may not have been able to fully absorb during a consultation.

It is important that information on the side effects of treatments is conveyed to patients in a consistent manner and a recent study by McGurk *et al.* (2000) addressed this. Information providers in breast care (nurses, surgeons, and oncologists)

were interviewed in 16 UK hospitals as to which side effects they consistently reported during the initial clinic consultation when hormonal therapy was commenced. The study examined the information patients were provided with on the side effects of tamoxifen and anastrozole. The main findings are discussed below:

Within all teams the more serious side effects were successfully addressed. Vasomotor symptoms were the most frequently mentioned side effects for both treatments (hot flushes and sweats) and patients were always told about these side effects by a team member, as well as about vaginal symptoms (dryness, itching, discharge, and bleeding) following tamoxifen. Almost every team discussed the possibility of thrombo-embolic events, endometrial cancer and weight gain with tamoxifen. Only about half the team discussed gastrointestinal side effects and eye problems with tamoxifen use. With anastrozole every team mentioned bone density loss and all but one team addressed arthralgia. Half the teams mentioned vaginal and gastrointestinal symptoms. Other side effects were mentioned less frequently. Although most of the serious side effects were mentioned by every team, it is of concern that 1 team failed to consistently mention endometrial cancer by any member of the team.

In the UK, breast care is provided by multidisciplinary teams and it was noted in the study that it was the breast care nurses and oncologists but not the surgeons who spent most of the time discussing side effects with the patients. This may reflect delegation of adjuvant discussions by surgeons to other members of the team. Nurses were found to be less likely to report bone density loss and arthralgia to patients as possible side effects. If surgeons are to delegate these discussions, then this highlights the importance of nurses who are well informed about respective side effects so ensuring their discussion with patients. The study further showed that side effects were mentioned with frequencies not necessarily corresponding with the literature's interpretation of their frequencies or clinical relevance. It is important not to mislead patients when giving them the side effect profiles, especially if they are to be involved in their treatment decisions.

The study also found problems with patient information leaflets. There were some serious omissions: The potential effect on bone density was not discussed in the majority of leaflets and some failed to mention vaginal bleeding and thrombo-embolic events. Out of date leaflets were being used and many contained irrelevant information. Perhaps there is a need for a national standardised leaflet on side effects that every hospital uses which is evidence based, has the most relevant information and is regularly updated. In conclusion, the consistency and quality of information could be improved. It is reasonable for different members of the team to discuss different aspects of treatment as long as all the key side effects are elaborated. Teams need to be aware of the litertature; information leaflets require improvement and there needs to be a uniform national consensus of which side effects are key and merit discussion.

CONCLUSIONS

Aromatase inhibitors represent the most significant advance in the endocrine man-

agement of breast cancer since the introduction of tamoxifen more than 30 years ago. They now have an established role as first- and second-line treatment for metastatic breast cancer where they offer advantages over tamoxifen and progestins in terms of several clinical parameters including response rates, TTP, and side-effect profile.

The optimal strategy for incorporation of aromatase inhibitors into standard adjuvant endocrine schedules remains unclear. Benefits in terms of disease-free and overall survival must be balanced against longer term risks (bone health; cognitive function) and costs. There is no clear evidence that initial treatment with tamoxifen for 2–3 years prevents subsequent bone loss, but patients who are not osteopaenic at the start of endocrine treatment are less likely to develop osteoporosis secondary to estrogen deprivation. An upfront aromatase inhibitor might be indicated in those patients at higher risk of relapse for whom the amplitude of the hazard peak for recurrence is proportionately greater in magnitude and could be suppressed or 'smoothed out' (Epanechinikov kernel) by an aromatase inhibitor more effectively than tamoxifen. As this peak occurs in the first 2–3 years after primary treatment, such an effect would only be possible with an upfront aromatase inhibitor and not an early switch. For those patients with lower hazards for relapse within the first 2–3 years, sequential therapy with tamoxifen (2–3 years) followed by an aromatase inhibitor may be more appropriate and cheaper. Finally, it should be noted that a group of older patients may exist (with a very low risk of relapse) for whom 5 years of tamoxifen remains the most cost-effective adjuvant endocrine option. However, issues of cost are complex and should take into account of subsequent adverse events prevented. Any health economic analysis is dependent on the particular healthcare system within which patients are treated. Furthermore, patients themselves must be involved in treatment decisions and be informed of side effect profiles for adjuvant hormonal agents. These should include both acute problems such as vasomotor symptoms and arthralgias, as well as longer term issues relating to bone loss and induction of second malignancies.

There are concerns regarding the impact of severe estrogen depletion in women receiving aromatase inhibitors for chemoprevention. The use of 'add-on' agents to minimize the complications of aromatase inhibitors (e.g., biphosphonates) detracts from a preventative strategy in the context of healthy women. Ongoing evaluation of treatment related morbidity will provide important data for accurate analysis of the relative risks, benefits, and costs of these agents. This will in turn inform management decisions and permit a more tailored approach to endocrine treatment for individual patient subgroups.

REFERENCES

Allred, D.C., Harvey, J.M., Berardo, M., and Clark, G.M. 1998. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod. Path. 11*: 155–168.

The Arimidex, Tamoxifen, Alone or in Combination Trialists' Group, Buzdar, A., Howell, A., Cuzick, J., Wale, C., Distler, W., Hoctin-Boes, G., Houghton, J., Locker, G.Y., and Nabholtz, J.M. 2006. Comprehensive side-effect profile of anastrozole and tamoxifen as adjuvant treatment for early-stage breast cancer: long term safety analysis of the ATAC trial. *Lancet Oncol. 7*: 633–643.

Assikis, V.J., and Jordan, V.C. 1992. Gynaecologic effects of tamoxifen and the association with endometrial carcinoma. *Int. J. Gynaecol. Obstet. 49*: 241–257.

ATAC Trialists Group. 2005. Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years adjuvant treatment for breast cancer. *Lancet 365*: 60–62.

Baum, M. 2004. ATAC update. Are there now sufficient data to replace tamoxifen with anastrozole as first line therapy for hormone receptor positive post-menopausal breast cancer. 27th San Antonio Breast Cancer Symposium, San Antonio, TX December 8–11.

Baum, M., Buzdar, A., Cuzik, J., Forbes, J., Houghton, J.H., Klijn, J.G., and Sahmoud, T. 2002. Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early breast cancer: first results of the ATAC randomized trial. *Lancet 359*: 2131–2139.

Beatson, G. 1896. On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment with illustrative cases. *Lancet 2*: 104–107.

Boccardo, F., Rubagotti, A., Amoroso, D., Mesiti, M., Romeo, D., Caroti, C., Farris, A., Cruciani, G., Villa, E., Schieppati, G., and Mustacchi, G. 2001. Sequential tamoxifen and anastrozole versus tamoxifen alone in the adjuvant treatment of postmenopausal breast cancer patients: results of an Italian cooperative study. *J. Clin. Oncol. 19*: 4209–4215.

Bonneterre, J., Thurlimann, B., Robertson, J.F.R., Krzakowski, M., Mauriac, L., Koralewski, P., Vergote, I., Webster, A., Steinberg, M., and von Euler, M. 2000. Anastrozole versus tamoxifen as first-line therapy for advanced breast cancer in 668 postmenopausal women: results of the Tamoxifen or Arimidex Randomised Group Efficacy and Tolerability study. *J. Clin. Oncol. 18*: 3748–3757.

Buzdar, A., Jonat, W., Howell, A., Jones, S.E., Blomqvist, C., Vogel, C.L., Eiermann, W., Wolter, J.M., and Azab, M. 1996. Anastrozole, a potent and selective aromatase inhibitor, versus megestrol acetate in postmenopausal women with advanced breast cancer; results of overview analysis of two phase III trials. *J. Clin. Oncol. 14*: 2000–2011.

Buzdar, A., Valero, V., and Theriault, R.L. 2003. Pathological complete response to chemotherapy is related to hormone receptor status. *Breast Cancer Res. Treat. 82*: abstract 302.

Carlini, P., Bria, E., Ferretti, G., Felici, A., Papaldo, P., Fabi, A., Ruggeri, E., Cognetti, F., Terzoli, E., and Giannarelli, D. 2005. New aromatase inhibitors (AI's) as 1st line endocrine therapy (ET) in metastatic breast cancer (MBC): a pooled analysis of 3238 women from 8 phase III trials. ASCO Annual Meeting, Orlando, Florida. Abstract 602.

Coombes, R.C., Hall, E., Gibson, L.J., Phil, M., Paridaens, R., Jassem, J., Delozier, T., Jones, S.E., Alvares, I., Bertelli, G., Ortmann, O., Coates, A.S., Bajetta, E., Dodwell, D., Coleman, R.E., Fallowfield, L.J., Mickiewicz, E., Anderson, J., Lonning, P.E., Cocconi, G., tewart, A., Stuart, N., Snowdon, C.F., Carpentieri, M., Massimini, G., and Bliss, J.M. 2004. A randomized trial of exemestane after two to three years of tamoxifen therapy in postmenopausal women with primary breast cancer. *N. Engl. J. Med. 350*: 1081–1092.

Coombes, R.C., Kilburn, L.S., Snowdon, C.F., Paridaens, R., Coleman, R.E., Jones, S.E., Jassem, J., Van de Velde, C.J.H., Delozier, T., Alvarez, I., Del Mastro, L., Ortmann, O., Diedrich, K., Coates, A.S., Bajetta, E., Holmberg, S.B., Dodwell, D., Mickiewicz, E., Andersen, J., Lønning, P.E., Cocconi, G., Forbes, J., Castiglione, M., Stuart, N., Stewart, A., Fallowfield, L.J., Bertelli, G., Hall, E., Bogle, R.G., Carpentieri, M., Colajori, E., Subar, M., Ireland, E., and Bliss, J.M., on behalf of the Intergroup Exemestane Study. 2007. Survival and safety of exemestane versus tamoxifen after 2–3 years' tamoxifen treatment (Intergroup Exemestane Study): a randomized controlled trial. *Lancet 369*: 559–570.

Cummings, S.R., Eckert; S., Krueger; K.A., Grady; D., Powles; T.J., Cauley; J.A., Norton; L., Nickelsen; T., Bjarnason, N.H., Morrow, M., Lippman, M.E., Black, D., Glusman, J.E., Costa, A., and Craig Jordan, V.C. 1999. The effect of raloxifene on risk of breast cancer in postmenopausal women: results of the more randomized trial. *JAMA. 281*: 2189–2197.

Cuzik, J., Sasieni, P., and Howell, A. 2006. Should aromatase inhibitors be used as initial treatment

or sequenced after tamoxifen. *Br. J. Cancer 94*: 460–464.

Day, R., Ganz, P.A., Costantino, J.P., Cronin, W.M., Wickerham, D.L., and Fisher, B. 1999. Health-related quality of life and tamoxifen in breast cancer prevention: a report from the National Surgical Adjuvant Breast and Bowel Project P-1. *J. Clin. Oncol. 17*: 2659–2669.

Early Breast Cancer Trialists Collaborative Group 1998. Tamoxifen for early breast cancer: an overview of the randomized trials. *Lancet 351*: 1451–1461.

Ellis, M.J., Tao, Y., Young, O., White, S., Proia, A.D., Murray, J., Renshaw, L., Faratian, D., Thomas, J., Dowsett, M., Krause, A., Evans, D.B., Miller, W.R., and Dixon, J.M. 2005. Estrogen-independent cell proliferation occurs in the majority of estrogen receptor positive HER2 gene-amplified primary breast cancers: evidence from a combined analysis of 2 independent neoadjuvant letrozole studies. ASCO Annual Meeting., Florida (abstract 9538).

First results from the International Breast Cancer Intervention Study (IBIS-1): a randomized prevention trial. 2002. *Lancet 360*: 817–824.

Fisher, B., Costantino, J.P., Wickerham, D.L., Redmond, C.K., Kavanah, M., Cronin, W.M., Vogel, V., Robidoux, A., Dimitrov, N., Atkins, J., Daly, M., Wieand, S., Tan-Chiu, E., Ford, L., Wolmark, N., and other National Surgical Adjuvant Breast and Bowel Project Investigators. 1998. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 study. *J. Natl. Cancer Inst. 90*: 1371–1388.

Fisher, B., Dignam, J., Bryant, J., and Wolmark, N. 2001. Five versus more than 5 years of tamoxifen for lymph node negative breast cancer: updated findings of the National Surgical Adjuvant Breast and Bowel Project B-14 randomised trial. *J. Natl. Cancer Inst. 93*: 684–690.

Geisler, J., Haynes, B., Anker, G., Dowsett, M., and Lønning, P.E. 2002. Influence of letrozole and anastrozole on total body aromatization and plasma estrogen levels in postmenopausal breast cancer patients evaluated in a randomized, cross-over study. *J. Clin. Oncol. 20*: 751–757.

Jakesz, R., Gnant, M., Greil, R., Tausch, C., Samonigg, H., Kwasny, W., Kubista, E., Stierer, M., Luschin, G., and Mittlboeck, M. 2005a. The benefits of sequencing adjuvant tamoxifen and anastrozole in postmenopausal women with hormone-responsive early breast cancer: 5 year analysis of ABCSG Trial 8. *Breast Cancer Res. Treat. 94*: S10 (abstract 13).

Jakesz, R., Jonat, W., Gnant, M., Mittlboeck, M., Greil, R., Tausch, C., Hilfrich, J., Kwasny, W., Menzel, C., Samonigg, H., Seifert, M., Gademann, G., Kaufmann, M., Wolfgang, J., ABCSG, and the GABG. 2005b. Switching of post-menopausal women with endocrine responsive early breast cancer to anastrozole after 2 years adjuvant tamoxifen: combined results of ABCSG trial 8 and ARNO 95 trial. *Lancet 366*: 455–462.

Jenkins, V., Fallowfield, L., and Saul, J. 2001. Information needs of patients with cancer: results from a large study in UK cancer centres. *Br. J. Cancer 84*: 48–51.

Jensen, E.V., Suzuki, T., Kawashima, T., Stumpf, W.E., Jungblut, P.W., and DeSombre, E.R. 1968. A two-step mechanism for the interaction of oestradiol with rat uterus. *Proc. Nat. Acad. Sci. USA 59*: 632–638.

Jonat, W., Gnant, M., Boccardo, F., Kaufmann, M., Rubagotti, A., Zuna, I., Greenwood, M., and Jakesz, R. 2007. Effectiveness of switching from adjuvant tamoxifen to anastrozole in postmenopausal women with hormone-sensitive early-stage breast cancer: a meta-analysis. *Lancet Oncol. 7*: 991–996.

Jordan, V.C. 1974. Anti-tumour effect of the anti-oestrogen ICI 46,474 (tamoxifen) in the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model. *J. Steroid Biochem. 5*: 354–359.

Jordan, V.C. 1975. Controversies in Breast Cancer. Prevention of Breast Cancer – A debate on SERM's versus aromatase inhibitors. Edinburgh. September. 4–6.

Jordan, V.C. 1999. Tamoxifen for treatment and prevention of breast cancer. Melville, New York: PRR Inc. pp 117–126.

Jordan, V.C. 2001. Selective estrogen receptor modulation: a personal perspective. *Cancer Res. 61*: 5683–5687.

Jordan, V.C., and Koerner, S. 1975. Tamoxifen (ICI 46, 474) and the human carcinoma 8 S oestrogen receptor. *Eur. J. Cancer 11*: 205–206.

Juneja, M., Jose, R., and Kekre, A.N. 2002. Tamoxifen-induced endometrial changes in postmenopausal women with breast carcinoma. *Int. J. Gynaecol. Obstet. 76*: 279–284.

Lacassagne, A. 1932. Apparition de cancers de la mamelle chez le souris male, soumise a des injections de folliculine. *Renal Acad. 195*: 630–634.

Locker, G.Y. (on behalf of the ATAC Trialists' Group) 2004. Cost-utility analysis of anastrozole versus tamoxifen as primary adjuvant therapy in postmenopausal women with early breast cancer from a US healthcare perspective: the 5 year completed treatment analysis of the ATAC trial. *Breast Cancer Res. Treat. 88*: (suppl 1) S105.

Lonning, P., Pfister, C., Martoni, A., and Zamagni, C. 2003. Pharmacokinetics of third-generation aromatase inhibitors. *Semin. Oncol. 30*: 23–32.

Love, C.D.B., Muir, B.B., Scrimgeour, J.B., Leonard, R.C.F., Dillon, P., and Dixon, J.M. 1999. Investigation of endometrial abnormalities in asymptomatic women treated with tamoxifen and an evaluation of the role of endometrial screening. *J. Clin. Oncol. 17*: 2050–2054.

Love, R.R., Wiebe, D.A., Newcomb, P.A., Cameron, L., Leventhal, H., Jordan, V.C., Feyzi, J., and DeMets, D.L. 1991. Effects of tamoxifen on cardiovascular risk factors in post-menopausal women. *Ann. Intern. Med. 115*: 860–864.

Love, R.R., Mazess, R.B., Barden, H.S., Epstein, S., Newcomb, P.A., Jordan, V.C., Carbone, P.P., and DeMets, D.L. 1992. Effects of tamoxifen on bone mineral density in post-menopausal women with breast cancer. *N. Engl. J. Med. 326*: 852–856.

McGurk, R., Fallowfield L., and Winters Z.E. 2000. Information provision for patients by breast cancer teams about the side effects of hormone treatments. *Eur. J. Cancer 42*: 1760–1767.

Nabholtz, A., Buzdar, A., and Pollak, M. 2000. Anastrozole is superior to tamoxifen as first line therapy for advanced breast cancer therapy in post-menopausal women: results of a North American multicentre randomized trial. *J. Clin. Oncol. 18*: 3758–3767.

Nabholtz, J.M., Bonneterre, J., Buzdar, A., Robertson, J.F., and Thurlimann, B. 2001. Anastrozole is superior to tamoxifen as first-line therapy in hormone receptor positive advanced breast carcinoma. Results of two randomized trials designed for combined analysis. Arimidex Writing Committee; Investigators Committee members. *Cancer 92*: 2247–2258.

Paech, K., Webb, P., Kuiper, G.G.J.M., Nilsson, S., Gustafsson, J., Kushner, P.J., and Scanlan, T.S. 1997. Differential ligand activation of estrogen receptors ER alpha and ER beta at AP-1 sites. *Science 277*: 1508–1510.

Powles, T., Eeles, R., Ashley, S., Easton, D., Chang, J., Dowsett, M., Tidy, A., Viggers, J., and Davey, J. 1998. Interim analysis of the incidence of breast cancer in the Royal Marsden Hospital tamoxifen randomized chemoprevention trial. *Lancet 362*: 98–101.

Punglia, R.S., Kuntz, K.M., Winer, E., Weeks, J.C., and Burstein, H.J. 2005. Optimising adjuvant endocrine therapy in post-menopausal women with early stage breast cancers. *J. Clin. Oncol. 23*: 5178–5187.

Ravdin, P.M., Green, S., Dorr, T.M., McGuire, W.L., Fabian, C., Pugh, R.P., Carter, R.D., Rivikin, S.E., Borst, J.R., Belt, R.J. 1992. Prognostic significance of progesterone receptor in estrogen receptor positive patients with metastatic breast cancer treated with tamoxifen: results of a prospective Southwest Oncology Group Study. *J Clin Oncol. 10*: 1284–1291.

Saarto, T., Blomquist, C., and Enholm, C. 1996. Antiatherogenic effects of adjuvant antiestrogens: a randomized trial comparing the effects of tamoxifen and toremifene on plasma lipid levels in post-menopausal women with node positive breast cancer. *J. Clin. Oncol. 14*: 429–433.

Schinzinger, A. 1889. The artificial menopause and cancer of the breast. *J. Am. Med. Assoc. 131*: 810–816.

Smith, I.E., Dowsett, M., Ebbs, S.R., Dixon, J.M., Skene, A., Blohmer, J.U., Ashley, S.E., Francis, S., Boeddinghaus, I., and Walsh, G., IMPACT Trialists Group. 2005. Neoadjuvant treatment of post-menopausal breast cancer with anastrozole, tamoxifen or both in combination: the Immediate Preoperative Anastrozole, Tamoxifen or Combined with Tamoxifen (IMPACT) Multicenter Double-Blind Randomized trial. *J. Clin. Oncol. 23*: 5108–5116.

Stein, R.C., Dowsett, M., Hedley, A., Gazet, J.C., Ford, H.T., and Coombes, R.C. 1990. The clini-

cal and endocrine effects of 4-hydroxyandrostenedione alone and in combination with goserilin in premenopausal women with advanced breast cancer. *Br. J. Cancer 62*: 679–683.

Thurlimann, B. (on behalf of the BIG 1–98 Colloborative Group and the International Breast Cancer Study Group). 2005. A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. *N. Engl. J. Med. 353*: 2747–2756.

Urruticoechea, A. 2005. Molecular characterization of ER positive breast cancer before and during treatment with the aromatase inhibitors letrozole and anastrozole. ASCO Annual Meeting., Florida (abstract 9554).

van Landeghem, A.A.J., Poortman, J., Nabuurs, M., and Thijssen, J.J.H. 1985. Endogenous concentration and subcellular distribution of estrogens in normal and malignant breast human breast tissue. *Cancer Res. 45*: 2900–2906.

Veronesi, U., Maisonneuve, P., Costa, A., Sacchini, V., Maltoni, C., Robertson, C., Rotmensz, N., and Boyle, P., on behalf of the Italian Tamoxifen Prevention Study. 1998. Prevention of breast cancer with tamoxifen: preliminary findings from the Italian randomized trial among hysterectomised women. *Lancet 352*: 93–97.

Vosse, M., Renard, F., Coibion, M., Neven, P., Nogaret, J.M., and Hertens, D. 2002. Endometrial disorders in 406 breast cancer patients on tamoxifen: the case for less intensive monitoring. *Eur. J. Obstet. Gynaecol. 101*: 58–63.

Wickerham, D.L., Constantino, J.P., Vogel, V., Cronin, Q., Cecchini, R., Atkins, J., Bevers, T., Fehrenbacher, L., Maskill-Stevens, W., and Wolmark, N. 2006. The study of tamoxifen and raloxifene (STAR): Initial findings from the NSABP P-2 breast cancer prevention study. 2006 ASCO Annual Meeting Proceedings Part 1. *J. Clin. Oncol. 24*: No. 18 S LBA5.

Winer, E.P., Hudis, C., Burstein, H.J., Wolff, A.C., Pritchard, K.I., Ingle, J.N., Chlebowski, R.T., Gelber, R., Edge, S.B., Gralow, J., Cobleigh, M.A., Mamounas, E.P., Goldstein, L.J., Whelan, T.J., Powles, T.J., Bryant, J., Perkins, C. Perotti, J., Braun, S., Langer, A.S., Browman, G.P., Mark, R., and Somerfield, M.R. 2005. American Society of Clinical Oncology Technology Assessment on the use of aromatase inhibitors as adjuvant therapy for postmenopausal women with hormone receptor positive breast cancer: Status report 2004. *J. Clin. Oncol. 23*: 619–630.

32
Breast Cancer: Expression of HER-2 and Epidermal Growth Factor Receptor as Clinical Markers for Response to Targeted Therapy

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INTRODUCTION

In this chapter we consider the relationships that involve both HER-2 and epidermal growth factor receptor (EGFR), with a particular focus on the potential use of these two transmembrane growth factor receptors as biomarkers for predicting response to targeted therapeutic agents directed at either or both of them. Effective clinical use of these receptors as biomarkers depends both on an understanding of their complex biological interactions, and an awareness of the shortcomings of current methods of measuring these two cellular constituents in the clinical setting. Here we will review selected aspects of these two topics, and attempt to develop working hypotheses and organizing principles that might be useful in translating basic knowledge into clinical practice.

CELL AND MOLECULAR BIOLOGY OF HER-2/EPIDERMAL GROWTH FACTOR RECEPTOR INTERACTIONS

Because HER-2 has no known ligands, in order for it to initiate signal transduction it must form heterodimers with other members of its membrane-bound tyrosine kinase receptor family. The major heterodimeric partners for HER-2 are EGFR or HER-3. The remaining member of this membrane-bound receptor family, HER-4, does not appear to play a significant role in tumor cell signaling. Individual HER family members can also homodimerize. Activated EGFR homodimers are rapidly internalized and degraded, whereas HER-2/EGFR heterodimers are internalized more

slowly, and are more likely to be recycled to the cell membrane.

The progression of HER-2 overexpressing tumors is thought to be accompanied by the development of chromosomal aneuploidy and by progressive HER-2 amplification. HER-2 amplification can occur early, prior to the development of aneuploidy or later in the course of tumor progression. In tetraploid and aneuploid cells, the number of HER-2 gene copies per cell increases in tandem with the number of chromosome 17 copies per cell, while pure gene amplification increases both the number of HER-2 gene copies and the ratio of HER-2 gene copy number to chromosome 17 copy number per cell. This increased ratio forms the basis for one of the commonly performed clinical fluorescence *in situ* hybridization assays for HER-2.

EGFR can bind various ligands, including EGF itself and TGF alpha. The various combinations of dimers that include EGFR and/or HER-2 can signal through multiple downstream signaling pathways, including the ras/ERK1/2 and PI3 kinase/Akt pathways. However, there are several distinctive known signaling themes. One is a prominent relationship between HER-2/EGFR heterodimerization and ras/ERK signaling. Another is the activation of HER-2/HER-3 heterodimers, which drive PI3 kinase signaling. A third is the involvement of EGFR in the activation of src/STAT signaling. These and other signal transducing pathways that emanate from the EGFR family of membrane tyrosine kinases, in turn, can interact with other growth factor/growth factor receptor systems (e.g., the IGF receptor system (Jones *et al*., 2006)). There is also crosstalk through convergent interactions with promiscuous signaling kinases such as src, and convergence of different signaling pathways on common downstream nodes, such as cyclin D1.

It is apparent from this brief sketch that there are multiple intricate relationships that affect HER-2 and EGFR signaling in breast cancer cells. These relationships become even more complex as they evolve during the course of tumor development and tumor progression. Before pursuing the dynamic behavior of some of these relationships in greater depth, it is useful to establish several quantitative benchmarks.

QUANTITATIVE CONSIDERATIONS

Average levels of HER-2 protein molecules per cell can be obtained by saturation binding studies and Scatchard analysis by ELISA or by cell-based techniques (e.g., radioimmunohistochemistry (Robertson *et al*., 1996), or flow cytometry (Shackney *et al*., 1998)). We have used a flow cytometry-based immunofluorescence method (Shackney *et al*., 1998) where the fluorescence measurement of each cell in the sample is compared with the mean fluorescence of a concomitantly run reference sample, and where the mean number HER-2 molecules per cell in the reference sample had been determined previously by ELISA. Two advantages of this approach are that one also determines the distribution and effective range of HER-2 values per cell in each sample, and that one can obtain correlated measurements of other cell constituents, such as EGFR and ploidy on a cell by cell basis (Shackney *et al*., 2006).

Normal average levels of HER-2 protein are generally in the range of 1×10^4 to $< 1 \times 10^5$ molecules per cell (Robertson *et al*., 1996). The high average levels of HER-2 protein per cell found in HER-2+ cancers generally exceed 1×10^5 molecules per cell and can approach or exceed 1×10^6 molecules per cell. HER-2 gene copy number per cell and RNA expression levels per cell are thought to parallel the levels of protein expression. Robertson *et al*. (1996) have demonstrated a bimodal distribution of HER-2 expression levels in human breast cancers, where the higher peak is associated exclusively with HER-2 gene amplification.

Levels of EGFR in human breast cancers are also mostly in the range of 1×10^4 to $\sim 1 \times 10^5$ molecules per cell (Robertson *et al*., 1996). Interestingly, many of these values are lower than those found in normal breast cells obtained from mammoplasty specimens (Robertson *et al*., 1996). They have claimed an inverse relationship between EGFR levels and HER-2 levels in tumors, which was statistically significant only when high HER-2-expressing tumors with HER-2 amplification were excluded (Robertson *et al*., 1996). Our own quantitative flow cytometry studies suggested a more complex set of relationships (Shackney *et al*., 1998). We observed two major tumor subtypes and one minor tumor subtype based on cellular patterns of HER-2 or EGFR overexpression, as shown in Figure 32.1. The most common pattern was one in which the tumor was aneuploid, HER-2 was overexpressed (mean, $> 2 \times 10^5$ molecules per cell), and the ratio of HER-2/EGFR level per cell exceeded 1. Unfailingly, the highest levels of HER-2 protein expression were observed in the aneuploid cell component of each tumor (Figure 32.1B), and within the aneuploid component, the highest cellular levels of HER-2 were seen in the cells with the highest ploidy levels (not shown). The overexpression of ras was limited almost exclusively to aneuploid, HER-2 overexpressing cells, suggesting the possibility of high levels of ras/ERK signaling in advanced disease (data not shown).

Another major group consisted of tumors that were diploid, tetraploid or aneuploid, that overexpressed EGFR, and exhibited a HER-2/EGFR ratio that was <1 (Figure 32.1); i.e., these tumors exhibited higher levels per cell of EGFR than HER-2. There was a third small group of aneuploid tumors that overexpressed EGFR, but little if any HER-2 (Figure 32.1C). In retrospect, that latter group may have represented basal like breast cancers; such tumors overexpress EGFR but not HER-2, and are frequently aneuploid.

In any case, it seems that there are several different evolutionary pathways in human breast cancer that exhibit distinctive and quantitatively divergent patterns of expression of HER-2 and EGFR. It is also clear that within most individual tumors with HER-2 overexpression, a high ratio between HER-2 expression and EGFR expression is maintained over a broad range of HER-2 and EGFR levels within individual cells in each tumor. The pervasiveness of this relationship would suggest that it plays an important and continuing role in the development and progression of a substantial proportion of HER-2 overexpressing tumors.

Immunohistochemistry, one of the most common clinical methods for determining the presence of HER-2 protein overexpression, is neither quantitative in an absolute sense nor is it particularly sensitive. It has

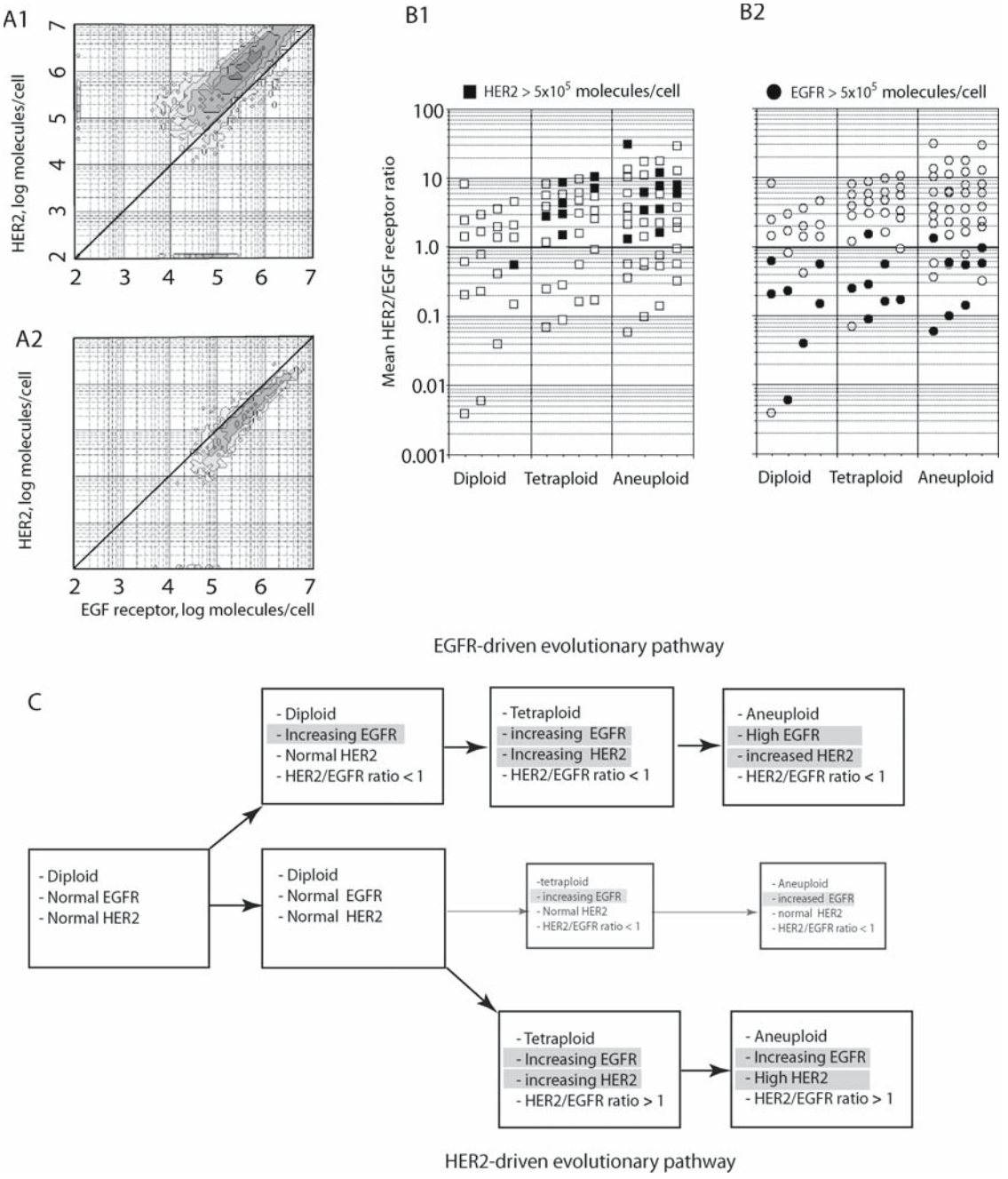

FIGURE 32.1. (A) examples of primary human breast cancers studied by quantitative multiparameter flow cytometry, in which, (A1) the ratio of HER-2/EGFR expression per cell exceeds one, and (A2) the ratio of HER-2/EGFR expression per cell is less than one. Frequency data on individual cells (Z axis) are presented as contour maps. (B) Mean HER-2/EGFR ratios for different tumors, in relation to ploidy. (B1) Samples with absolute mean HER-2 level > 500,000 molecules per cell are shown as black rectangles. (B2) Samples with absolute meanEGFR level < 500,000 molecules per cell are shown as black circles. (C) Proposed evolutionary sequences of breast cancers overexpressing HER-2 and/orEGFR

been estimated that the lower threshold for immunohistochemical detection of HER-2 may be in the range of 200,000–500,000 molecules per cell (Shackney and Shankey, 1997). This would suggest that imunohistochemical methods might be well suited for identifying most HER-2 overexpressing tumors, but not for confidently identifying most EGFR overexpressing tumors in clinical material. The clinical implications of such test performance characteristics are considered in greater detail later in this chapter. For present purposes it is sufficient to note that it is common clinical practice to identify HER-2 overexpression and to determine cell ploidy in primary breast cancers, but that EGFR levels are not determined on a routine basis. As a consequence, there is much that is not known regarding the relationship between HER-2 and EGFR in primary breast cancers, and the specific clinical behavior of EGFR-overexpressing breast cancers remains poorly understood.

DYNAMIC ASPECTS OF HER-2 AND EPIDERMAL GROWTH FACTOR RECEPTOR SIGNALING

TRANSIENT EFFECTS INVOLVING HER-2/EGFR SIGNALING

Given the complexity of HER-2 and EGFR interactions, it is difficult to track them all in one's mind as they change acutely in response to external stimuli such as growth factors or targeted therapeutic agents. However, computer simulations make it possible to sift through the complexity, and provide useful insights into the ways this system meshes together in quantitative and temporally coherent ways. Computer simulations also allow one to ask "what if" questions, making it easier to generate useful hypotheses for testing. Sometimes the insights gained from modeling seem simple and, in retrospect, self-evident. However, combinations of simple principles can also produce unanticipated and occasionally quite surprising results.

Hendriks *et al.* (2003) have modeled aspects of HER-2/EGFR activation and internal cellular receptor trafficking, and noted that the formation of the heterodimer appears to follow the law of mass action. In simple terms, this means that when HER-2 and EGFR are present in unequal proportions, it is the less abundant species that limits the formation of the heterodimer. In the breast cancer reference cell line studied by Hendriks *et al.* (2003), EGFR was in excess, and they concluded that HER-2 abundance would be a limiting factor in heterodimer formation.

If HER-2 were in excess, one might anticipate that it is the concentration of EGFR that would limit the formation of the heterodimer. Our own modeling studies support this premise. Consider the model shown in Figure 32.2A. This model takes into account the heterodimerization of EGFR and HER-2, internalization of the heterodimer, and return of the components of the heterodimer back to the cell membrane. In a tumor with a high ratio of HER-2 to EGFR (3:1, in this example), a constant level of each constituent is maintained under steady state conditions due to a balance between their respective rates of production and rates of loss. The application of an external stimulus, such as EGF (Figure 32.2B), for a brief period (e.g., minutes) transiently increases the rate of HER-2/EGFR heterodimer formation, and transiently decreases the availability of

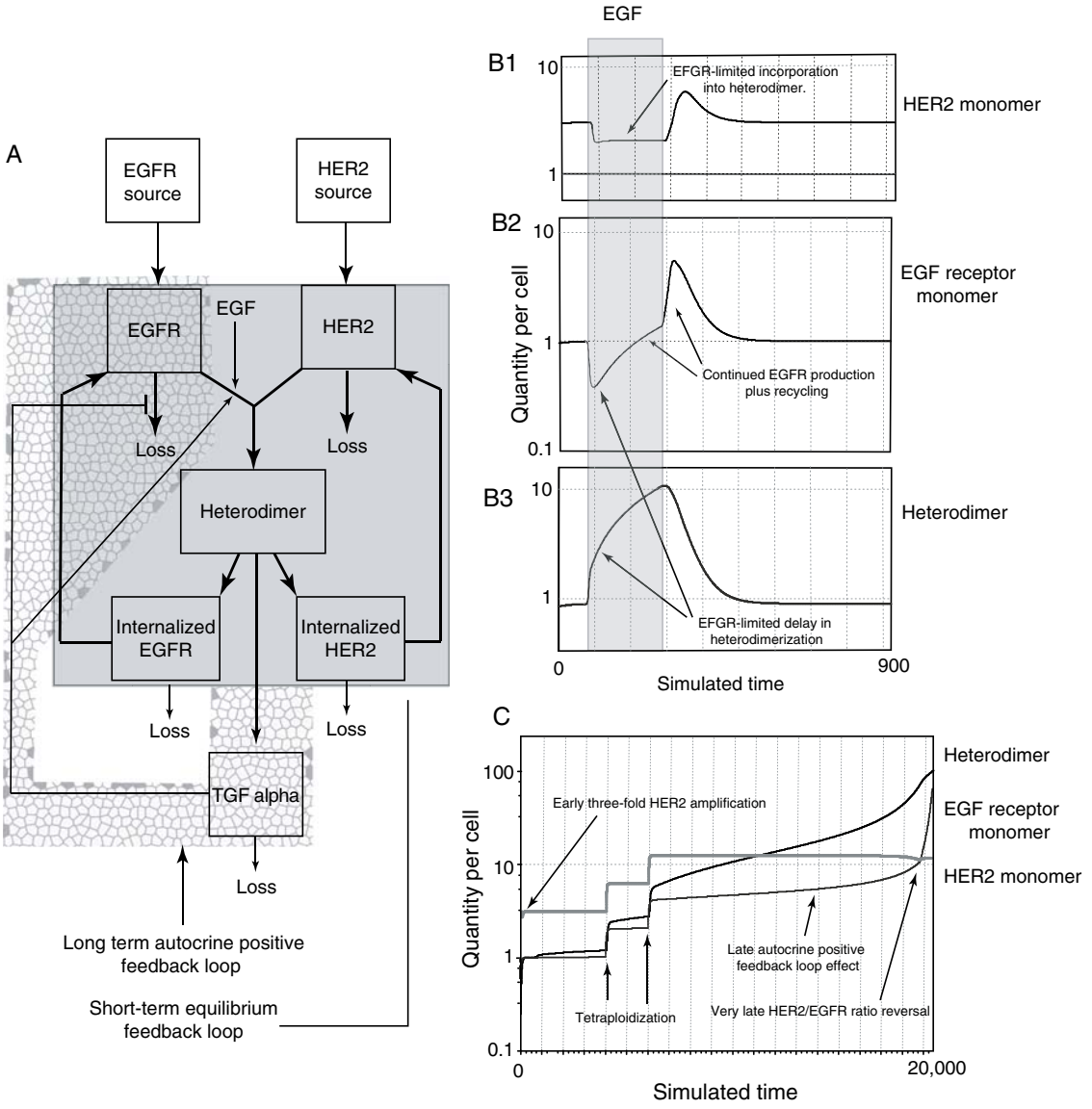

FIGURE 32.2. (A) A model for EGFR and HER-2 heterodimerization, internalization, and recycling to the cell membrane. Light gray region represents short term feedback loop that produces steady state equilibrium. Model also has the heterodimer producing TGF alpha, which forms a long term positive feedback loop by limiting EGFR degradation, and increasing heterodimer formation (cobblestoned area). (B) Simulated response to brief exposure to EGF (gray zone), under conditions where HER-2/EGFR ratio is 3:1. (B1) HER-2 monomer levels fall transiently to ~ 2, then overshoot, due to continued production and recycling of internalized HER-2, and finally return to equilibrium. (B2) EGFR monomer levels fall due to rapid heterodimer formation and internalization, overshoot due to continued EGFR production, and recycling to the cell membrane, and return to equilibrium levels. (B3) Heterodimer increases gradually, then falls after cessation of EGF signaling. (C) Simulated tumor progression following initial HER-2 amplification, and two rounds of tetraploidization. EGFR monomer accumulates as a result of autocrine TGF alpha positive feedback, slowly at first, and more rapidly in late stages of tumor evolution. It is not known if very late HER-2/EGFR ratio reversal actually occurs clinically.

EGFR and HER-2 monomers. The maximum amount of heterodimer formed is constantly limited by the amount of EGFR that is available for heterodimerization at any given time. The HER-2 monomer/EGFR monomer ratio falls initially from 3 to ~ 2. At intermediate and late time points (minutes to hours), under the conditions of this simulation the level of EGFR monomer recovers, overshoots the previous steady state level, and then gradually returns to baseline. The late changes are attributable to the recycling of internalized EGFR, as it works its way through its intracellular states, and returns to the cell membrane (Figure 32.2B). The levels of HER-2 and all other constituents of the system also return to baseline equilibrium.

If the HER-2/EGFR heterodimer plays a major role in HER-2 overexpressing tumors, and EGFR is limiting, why are EGFR inhibitors not being pursued more actively in breast cancer? The effects of these agents were relatively disappointing in early breast cancer clinical trials, and because of this other tumor types were accorded higher priority for study. There have been only a few relatively small published trials of gefitinib and erlotinib in breast cancer. Rates of clinical benefit were in the range of ~ 10%, based on unselected total patient populations. For comparison, if the numerator of clinical benefit rates for trastuzumab alone (35–50% of HER-2, 3+ patients) were applied to unselected patient populations, overall clinical benefit rates might be comparable to those of anti-EGFR therapies.

In a trial published by Baselga *et al.* (2005), data on multiple biomarkers were reported both in tumors and normal skin, including HER-2, EGFR, phosphorylated EGFR, TGF alpha, phosphorylated ERK, phosphorylated Akt (tumor only), Ki-67, and p27, all by immunohistochemistry. Statistically significant correlations were found between cell fractions stained for EGFR, and those of TGF alpha, phosphorylated EGFR, phosphorylated ERK, and Ki-67. Gefitinib inhibited EGFR and ERK phosphorylation in skin and tumor. These data could be interpreted as supporting a role for TGF alpha-mediated activation of an EGFR heterodimer, possibly with HER-2, with pretreatment activation of the ERK pathway, and with an effect on ERK signaling by gefitinib. Phospho-Akt, Ki-67, and p27 were not affected in the tumor samples, suggesting that upstream drug effects did little to alter key downstream signaling processes. The authors suggested that the lack of substantial clinical activity in this trial may have been due either to a suboptimal dose of drug, or to a lack of tumor dependence on EGFR. Even though this was not a successful trial clinically, the study provides a wealth of information on the effects of gefitinib on molecular signaling pathways in human breast cancers in vivo, which could be useful in planning future studies. For example, it might be of interest to study such patients before and after treatment with combined anti-HER-2 and anti-EGFR therapy, and to compare results in tumors with high and low HER-2/EGFR ratios.

EVOLUTION OF HER-2+ TUMORS

Gene expression microarray profiling studies have provided a sound molecular basis for subclassifying primary invasive human breast cancers. HER-2 overexpressing tumors have emerged as a distinct subset of predominantly ER and PR negative tumors. The loss of ER and PR reflects a relatively late biological stage in breast

cancer evolution, where estrogen ceases to play a significant role in the behavior of aggressive breast cancers.

Most of the salient molecular evolutionary changes that develop in human breast cancers, especially those that distinguish various cancer subtypes from one another, become established long before the development of invasive disease. HER-2 overexpression and aneuploidy first becomes clearly manifest at the stage of ductal carcinoma *in situ*. Therefore, much can be learned from studying the early and intermediate stages of tumor development, in order to better understand later stages of disease. To study these early changes, experimental tumor models were developed nearly 20 years ago, which utilized estrogen-dependent breast cancer cells that were subjected to prolonged estrogen deprivation (for months in cell culture), or were subjected to prolonged tamoxifen exposure. These early studies laid out a sequence of changes in hormonal responsiveness that consisted of decreased dependence on estrogen for continued growth, coupled with hypersensitivity to estrogen exposure, and increased expression of EGFR. This was followed later by loss of responsiveness to estrogen administration and loss of ER expression.

This experimental approach has been extended in recent years to better delineate the mechanistic aspects of changes in estrogen responsiveness at the molecular level (Nicholson *et al*., 2007). The state of decreased dependence on estrogen for growth is characterized by the overexpression of both HER-2 and EGFR, and by the overexpression of growth factors, particularly TGF alpha (Knowlden *et al*., 2003). An increase in ras/ERK signaling is also a prominent feature (Knowlden *et al*., 2003), although the PI3 kinase/Akt pathway is also active.

In these studies the increase in the level of HER-2 was expressed quantitatively as nearly twofold greater than that of the parent cell lines. The increase in EGFR was found to be nearly threefold (Knowlden *et al*., 2003). These last observations are of particular interest, because a near-doubling of the expression of two cell constituents with genes on two different chromosomes brings the development of aneuploidy to mind. Most primary HER-2 overexpressing breast cancers are aneuploid (Figure 32.1).

We had previously proposed that aneuploidy can arise by an episodic doubling of chromosome complement in the absence of cell division, which results in the establishment of a cell line with twice the previous chromosome complement during the course of subsequent "normal" cell divisions. In tumors with genetic instability, tetraploid cells can lose chromosomes randomly over time, producing aneuploid tumors containing fewer chromosomes than the newly established tetraploid stem line. There is evidence that such tumors can undergo multiple (two, and, less commonly, three) rounds of tetraploidization and chromosome loss ((Janocko *et al*., 2001; see fluorescence *in situ* hybridization data, Figure 32.3).

To account for the differential overexpression of HER-2, which eventually produces HER-2/EGFR ratios per cell that exceed 1, there must be additional mechanisms for increasing HER-2 alone. The coupling of HER-2 gene amplification with chromosomal aneuploidy could readily account for this phenomenon (see fluorescence *in situ* hybridization data in Figure 32.3).

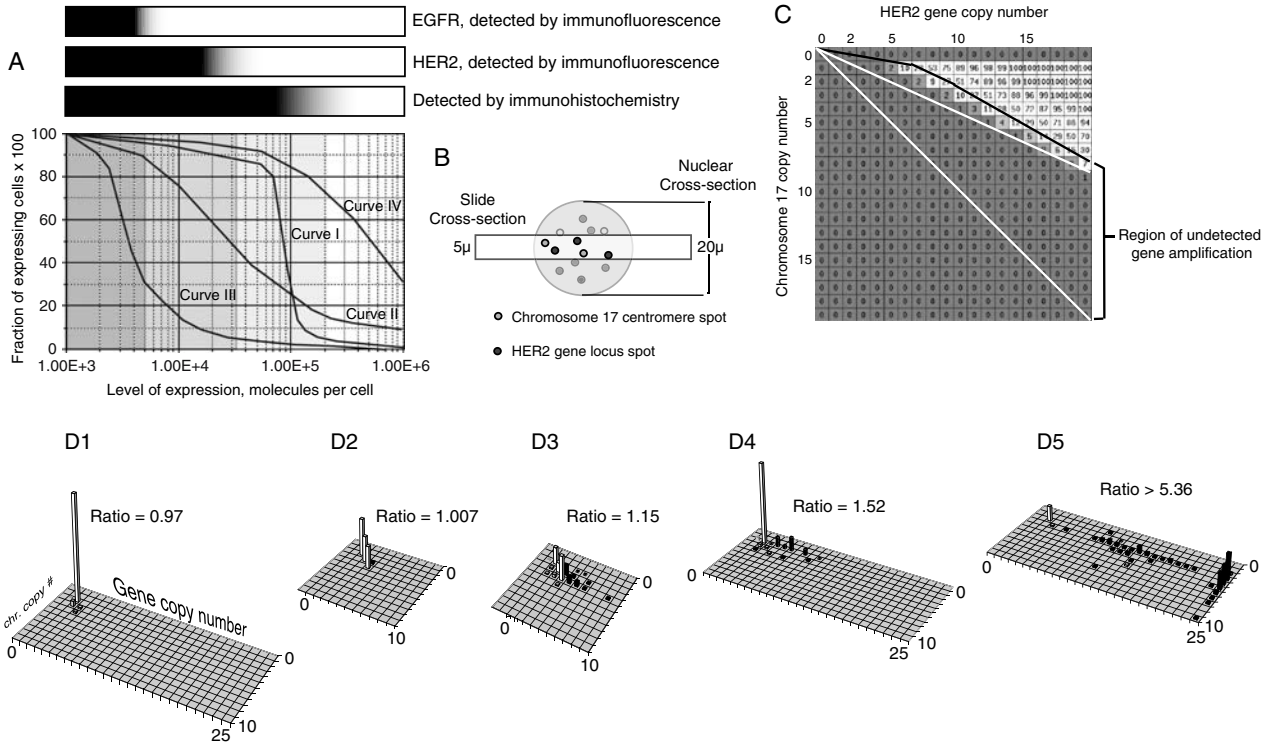

FIGURE 32.3. (A) Diagrammatic representation of profiles of positive cell fractions in individual tumor samples in relation to intracellular levels of expression (molecules per cell), and in relation to detectability by immunohistochemistry and immunofluorescence. Fractions of highly overexpressing cells may or may not reflect true mean expression levels in individual samples, and the latter are generally not detectable by IHC, especially inEGFR studies. (B) Cartoon of partial sectioning of cells in fluorescence *in situ* hybridization studies performed on paraffin embedded sections. (C) Modeling results of detectability of HER-2 amplification using Vysis counting criteria, and expression of results as ratios of HER-2 gene copy number to chromosome 17 centromere copy number. For each model cell, there is some probability the cell will be discarded because no chromosome signal is observed. Among those cells not discarded, there is then a binomial distribution of chromosome 17 signals and Her-2/neu signals. We can then analytically compute the probability that these signals, summed over forty trials, will give us a ratio of at least 2:1. These probabilities appear in the figure for different possible combinations of chromosome 17 and Her-2/neu copy numbers. White boxes exhibit probabilities of detection that exceed 50%. Region included between the white straight lines has ratios between 1 and 2. White and black lines encompass a region of cells that would not be scored as positive due to biases introduced by counting criteria. (D) Examples of fluorescence *in situ* hybridization data collected in smears of breast cancer cells that are not subject to partial sectioning artifacts. One hundred cells were counted per sample. (D1) Diploid sample, no HER-2 amplification. (D2) Pure polysomy. (D3) Aneuploidy with intermediate HER-2 amplification. (D4) Diploidy with modest HER-2 amplification. (D5) Aneuploidy with extensive HER-2 amplification.

Autocrine feedback loops. The finding that the tamoxifen-resistant state is accompanied not only by increased levels of both HER-2 and EGFR, but also by increased levels of growth factors that activate EGFR, particularly TGF alpha (Knowlden *et al*., 2003), raises the possibility that autocrine loops might also be a mechanism for increasing levels of EGFR in HER-2 overexpressing tumors. Autocrine loops are known to be active in breast cancer. TGF alpha is of special interest because it is expressed in a substantial proportion of clinical breast cancers, and the majority of high TGF alpha-expressing tumors coexpress EGFR (Normanno and Ciardiello, 1997).

To examine the potential role of growth factor-dependent autocrine signaling loops that might play a role in the long term progression of human breast cancers we turn once more to computer simulations, in order to ask relevant "what if" questions. In particular we wish to determine if, and under which conditions, simulations are capable of generating self-sustaining signaling through feedback loops. We find, for example, that prolonged increases in heterodimer formation can be produced when the heterodimer output signal generates a long lived product which, in turn, feeds back at very low rates by increasing the rate of heterodimer formation slightly and decreasing the rate EGFR monomer/homodimer degradation (Figure 32.2C). These particular conditions are consonant with what is known regarding the biological behavior of TGF alpha. TGF alpha is known to inhibit the downregulation of EGFR during cell signaling (Ouyang *et al*., 1999). Blockade of EGFR inhibits TGF alpha-induced growth of breast cancer cells (Arteaga *et al*., 1988), and suppresses tumorigenesis in MMTV/Neu + MMTV/TGF alpha bigenic mice (Lenferink *et al*., 2000). In the latter study ERK signaling was found to be downregulated, suggesting that targeted therapy against EGFR may have affected HER-2/EGFR heterodimer-mediated signaling through the ERK pathway. A permanent genomic increase in the rate of EGFR production, e.g., by tetraploidization or chromosome 7 trisomy, would compound the TGF alpha effect of decreasing the rate of EGFR destruction, and contribute further to EGFR overexpression. We would caution against reading too much into such modeling results, despite their consistency with actual data. There may be many other ways to produce the same type of behavior. Nonetheless, modeling exercises can be useful in developing more complex hypotheses, and these hypotheses can be subjected to critical testing.

Based on the information gathered up to this point, we hypothesize that there are two prominent evolutionary pathways that involve overexpression of HER-2 and/or EGFR. One is the HER-2+ pathway that is driven by genetic instability that results in HER-2 amplification and aneuploidy, where relatively modest increases in levels of EGFR "chase" more rapidly rising levels of HER-2. Thus, EGFR persistently may be rate limiting for heterodimer formation throughout most of the evolutionary history of HER-2+ tumors. The second hypothesized evolutionary pathway is driven by increasing levels of EGFR, with persistently lower levels of HER-2 in the same cells. Because much less is known regarding this pathway in breast cancer, we will probe its features in other EGFR-overexpressing tumors (see below). The distinction between these two

pathways may be of clinical importance because regimens consisting of combinations of agents that emphasize targeting signal-limiting components might differ for the two tumor types.

To draw this distinction, one might (1) probe the activities of these pathways more extensively in clinical tumor samples, and (2) test combinations of targeted agents that were designed purposefully to be pathway-specific in clinical studies. We now examine the performance characteristics of tests for the two critical biomarkers that would be central to such studies: HER-2 and EGFR.

TECHNICAL ASPECTS OF CLINICAL MEASUREMENTS OF HER-2 AND EPIDERMAL GROWTH FACTOR RECEPTOR

There is extensive literature on the use of different measurement techniques for determining levels of HER-2 expression/amplification in order to optimally select patients for treatment with trastuzumab. We shall not review this literature here. The collective clinicopathological experience with immunohistochemical methods and fluorescence *in situ* hybridization has been reviewed by the College of American Pathologists on clinical HER-2 testing. Their judgment is that neither immunohistochemistry nor fluorescence *in situ* hybridization has compelling overall advantages, and that both can be complementary in borderline cases.

In fact, there are disadvantages to both techniques that are linked directly to the use of paraffin embedded sections of formalin fixed pathological material. Formalin is a protein crosslinking fixative, which can mask antigenic epitopes and lead to reduced and more variable binding of antibodies to their targets, regardless of antibody specificity or binding affinity. Antigen retrieval methods commonly involve heating of the sample, which often leads to greater antibody binding. This also invariably introduces greater variability in staining which is difficult, if not impossible to control. Formalin can also penetrate tissues unevenly, leading to uneven staining. These properties of formalin fixed material have been emphasized by proponents of fluorescence *in situ* hybridization. However, formalin fixation could adversely affect both techniques. Fluorescence *in situ* hybridization studies are also commonly performed on formalin-fixed tissues, and formalin produces protein-DNA crosslinks in addition to protein-protein crosslinks.

It is not generally appreciated that the fluorescence *in situ* hybridization technique, as commonly practiced, is adversely affected by partial nuclear sectioning. That is, breast cancer cell nuclei have average diameters that are often much larger than the thickness at which paraffin embedded tissues are usually sectioned for microscopic study, resulting in irretrievable loss of signal. This, and several other commonly accepted conventions relating to data acquisition and analysis can conspire to limit the capability of fluorescence *in situ* hybridization to detect relatively small, but real increases in HER-2 gene copy number per cell.

Thus, both immunohistochemistry and fluorescence *in situ* hybridization may share a common shortcoming, namely, insufficient sensitivity to detect relatively small

increases in HER-2 amplification/overexpression that could be of biological significance. The recent finding that even HER-2 "negative" breast cancer patients can respond to trastuzumab in the adjuvant setting (Kim *et al*., 2007), brings the issue of poor detection sensitivity into sharp clinical focus, because it may result in the denial of trastuzumab to patients who might derive clinical benefit from this agent.

The problems of low detection sensitivity by immunohistochemistry and fluorescence *in situ* hybridization may also apply to the detection of increased EGFR expression, but are likely to be even more acute, because EGFR is commonly expressed and overexpressed at much lower levels than HER-2. Issues relating to the detection of minimally increased levels of HER-2 and EGFR are discussed in more quantitative terms in the next two sections.

TECHNICAL ASPECTS OF CLINICAL MEASUREMENTS OF HER-2 AND EPIDERMAL GROWTH FACTOR RECEPTOR: IMMUNOHISTOCHEMISTRY

As a practical matter, immunohistochemical methods appear to be relatively insensitive to levels of HER-2 protein overexpression that fall below 200,000 molecules per cell (Shackney and Shankey, 1997), although some antibodies may be much more sensitive than others, yet they are capable of identifying HER-2 expression in samples with much lower average levels of HER-2 expression. This may be due to the fact that levels of HER-2 protein expression in individual cells vary over a wide range within each sample. A detectable fraction of cells with levels of protein expression above 200,000 molecules per cell can often be found in samples with mean levels of expression of 100,000, 75,000, or even fewer molecules per cell.

Immunofluorescence measurements of HER-2 and EGFR by flow cytometry are far more sensitive than those obtained by immunohistochemistry, and can be quantitated objectively. We estimate that we can detect as few as 30,000 molecules of HER-2 per cell, and 5,000 molecules of EGFR per cell with reasonable confidence. Below these levels, nonspecific fluorescent antibody binding and cell autofluorescence become increasingly larger components of the fluorescent signal. Diagrammatic representations of typical profiles of fluorescence intensity within individual samples (Shackney *et al*., 1998) are shown in Figure 32.3A. The x axis is calibrated in molecules per cell on a logarithmic scale. Curve I in Figure 32.3A is representative of a fluorescence intensity profile in which most cells in the sample contain 75,000 fluorescent molecules per cell or less, but ~ 5% of the cells contain 200,000 molecules per cell or more. If such a sample were to be prepared for study by immunohistochemistry, it might be considered negative by commonly used criteria for HER-2 overexpression (minimum requirement of 10% of cells with positive staining), but positive for EGFR overexpression (minimum requirement of 1% of cells with positive staining). Small differences in the proportions of heavily stained cells could change the classification of a sample from negative to positive, even if the mean level of expression is lower (curve II, Figure 32.3A). On the other hand, samples in

which most cells exhibit high levels of HER-2 (curve IV, Figure 32.3A), would be scored as positive unambiguously under most conditions.

TECHNICAL ASPECTS OF CLINICAL MEASUREMENTS OF HER-2 AND EPIDERMAL GROWTH FACTOR RECEPTOR: FLUORESCENCE *IN SITU* HYBRIDIZATION

It is commonly held that fluorescence *in situ* hybridization has two major advantages over immunohistochemistry: objectivity in data acquisition, and accuracy of the measurements, because data acquisition involves the enumeration of discrete fluorescence-tagged chromosome centromeres and the enumeration of discrete fluorescence-tagged gene loci. However, a problem that arises in routine clinical fluorescence *in situ* hybridization studies is the effect of partial sectioning at 4–5 μm thickness of paraffin-embedded tumor samples. In breast cancer cells, nuclei have average diameters that are substantially larger than the thickness of the section (15–30 μm). This results in the exclusion from the analysis of a substantial proportion of the fluorescence *in situ* hybridization signals in each nucleus (see Figure 32.3B). Proponents of current fluorescence *in situ* hybridization data acquisition methods claim that, even in partially sectioned cells, the observed ratio of HER-2 signals to chromosome #17 copy number tends to approach the true ratio when a sufficiently large number of cells are counted. Hence, the use of ratio*s* when reporting fluorescence *in situ* hybridization results should be based on sectioned material. However, the absolute numbers of chromosome 17 and HER-2 signals/per cell are always underestimated in partially sectioned nuclei, regardless of the number of cells counted per sample. This makes it difficult to claim that fluorescence *in situ* hybridization data are truly quantitative, especially at the low end of the counting range, where the difficulties are greatest in making the crucial distinction between patients who may or may not benefit from trastuzumab therapy.

Modeling the effects of fluorescence *in situ* hybridization ratio criteria in formalin-fixed, paraffin embedded, partially sectioned cells. To better appreciate the impact of partial sectioning on fluorescence *in situ* hybridization results, we developed a simple spatial model, as shown in Figure 32.3B. The model assumes spherical nuclei, 20 μm in diameter, that are centered in a 5 μm histologic section, and counted and classified using standard Ventana/Vysis criteria. These criteria include ignoring cells with no signal, and cells that do not have at least one signal of chromosome 17 and one of HER-2 signal. For simplicity, chromosome 17 signals and HER-2 signals are assumed to be randomly distributed throughout the nucleus, independently of one another. The probability of observing any given signal is then equal to the fraction of the spherical nucleus volume that is included in the 5 μm section (37% given the above assumptions). Not surprisingly, tumors with HER-2: chromosome 17 ratios well above 2:1 are reliably detected as HER-2 amplifiers, while those below 2:1 are not. Less obvious, though, is that the counting method introduces a bias that adversely affects the detectability of amplification, especially in tumors with less than six chro-

mosome copies per cell (Figure 32.3C). We attribute this to the policy of discarding measurements in which the chromosome count is zero or one (in the absence of a HER-2 signal), biasing the model to overestimate chromosome number and underestimate the ratio. Furthermore, even if the ratio were accurately estimated, the test would not identify tumors with true HER-2 amplification with ratios between 1 and 2.

Ratios themselves (as distinct from absolute counts of HER-2 gene copy number in excess of chromosome #17 counts), have significant drawbacks. Consider a hypothetical sample with a true mean chromosome #17 copy number of 6.0, and a mean HER-2 gene copy number of 10.5, resulting in a true ratio of HER-2 gene copy per cell to chromosome #17 copies per cell of 1.75. This sample would be classified as negative by fluorescence *in situ* hybridization using Ventana/Vysis ratio criteria, despite the fact that both the absolute number of HER-2/neu copies per cell, and the *absolute number* of HER-2 copies per cell attributable purely to gene amplification are quite abnormal. It has been suggested that ratios between 1 and 2 might arise as a result of asynchronous normal gene replication, which could be mistaken for abnormal gene amplification However, given that G_1 fractions of most breast cancers are in the range of 85–95%, the potential overcounting error due to subsequent partial or total DNA replication in the same cell cycle is likely to be negligible. Thus, partial nuclear sectioning, the use of ratios, and the cutoff at a ratio of 2, all conspire to hide modest levels of gene amplification and to obscure polysomy. Both could be of potential clinical importance in identifying responders to anti-HER-2 therapy.

SURVEY OF FLUORESCENCE IN SITU HYBRIDIZATION PATTERNS IN SINGLE CELL SUSPENSIONS

We have accumulated fluorescence *in situ* hybridization data on >150 samples of primary human breast cancer that had been mechanically disaggregated into single cell suspensions, fixed in paraformaldehyde and methanol (for flow cytometry studies) and deposited on glass slides. This study will be presented in detail elsewhere. Here we provide some highlights that are pertinent to the issues at hand, because such cells are not subject to partial sectioning artifacts, and the data can be analyzed in several different ways for purposes of comparison.

Several distinct fluorescence *in situ* hybridization patterns were apparent in this data set (Figure 32.3D). One consisted almost entirely of cells that were disomic for chromosome 17, and also contained two copies of the HER-2 gene in the same cells (Figure 32.3D1). This group represented 60% of all samples. Another distinctive group of samples exhibited increased copy numbers of both chromosome 17 and HER-2, in equal proportions (pure polysomy, Figure 32.3D2). This group represented 19% of all samples. A third group of samples was disomic or aneusomic for chromosome 17, and contained cells with multiple copies of the HER-2 gene per cell that exceeded the number of copies of chromosome 17 in the same cells. These cases represented 21% of all samples. The disomic and low aneusomic group was heterogeneous, with some cases containing cells with a moderate excess of HER-2 gene copy number over chromosome number in the same cells, as shown in Figure 32.3D3 and D4. Some of the highly aneuploid tumors contained cells with a large excess of HER-2 gene copy numbers per cell, as shown in Figure 32.3D5. The ratios of mean HER-2

copy number per cell to mean chromosome copy number per cell varied among the cases with excess HER-2 gene copy numbers, depending both on the degree of polysomy/aneusomy and on the degree of gene amplification. It is apparent from these studies that there is still much to be done with regard to sorting patients for treatment or no treatment with trastuzumab, even under optimal fluorescence *in situ* hybridization counting conditions. The responsiveness to trastuzumab of patients with polysomic tumors, and patients whose tumors exhibit fluorescence *in situ* hybridization ratios that are between 1 and 2, require further study.

SUMMARY AND OVERVIEW OF METHODOLOGY

It seems clear that clinical studies involving HER-2 and EGFR have been hampered by problems associated with common methods of data gathering. Immunohistochemistry is a relatively insensitive and non-quantitative method for measuring HER-2. It is even less satisfactory for measuring EGFR, which is often expressed at lower levels than HER-2. ELISA, radioimmunohistochemistry, and immunofluorescence-based methods by microscopic imaging or flow cytometry, are all far more sensitive and quantitative than immunohistochemistry. Each of these methods has its advantages and drawbacks. All require sample handling and processing methods that differ from those used routinely in the clinical pathology laboratory. Among these methods, we favor those that involve measurements on whole cells, preferably calibrated in universal units of measurement (e.g., molecules/cell, or copy number/cell), so that levels of HER-2, EGFR, and other cell constituents can be compared quantitatively with one another.

When fluorescence *in situ* hybridization methods are performed on whole cells, they can be both quantitative and accurate, and their reporting need not be limited to using ratios of gene copy number to chromosome centromeres. We find that the absolute gene copy number in excess of the absolute chromosome copy number per cell (in whole cells) provides a much more balanced estimate of the relative contributions of ploidy and gene amplification than the ratio method. A major potential advantage of fluorescence *in situ* hybridization is that when data are reported in universal units of gene copy number per cell rather than as ratios, one can directly compare HER-2 fluorescence *in situ* hybridization results with EGFR fluorescence *in situ* hybridization results.

EXPRESSION OF HER-2 AND EPIDERMAL GROWTH FACTOR RECEPTOR

Gene amplification vs. aneuploidy. It is generally held that tumors with high levels of expression of HER-2 are driven by HER-2 gene amplification. However, it would seem reasonable to suppose that it is the phenotype, and not the genotype, that determines cell and tumor behavior. This hypothesis may be difficult to test in practice, because large-scale numerical chromosomal abnormalities (tetraploidy and aneuploidy) often are accompanied by structural chromosomal abnormalities (e.g., gene amplification), and the signaling effects of the two are not easily separable. The frequency of gene amplification of HER-2 by fluorescence *in situ* hybridization in nonlobular breast cancers increases with increasing stages of aneuploidy (Janocko *et al*., 2001). Given the frequent comingling

of the effects of numerical and structural chromosomal abnormalities, it would seem reasonable to group polysomic tumors that overexpress HER-2 protein together with the aneuploid tumors that amplify the HER-2 gene (and overexpress the protein) at modest levels, recognizing that truly high gene copy numbers and high protein overexpression are usually achieved only when the combined gene copy multiplier effects of ploidy changes and gene amplification come into play (Figure 32.3D4).

The effects of EGFR targeted agents in HER-2 overexpressing tumors. Preclinical studies have shown that anti-EGFR therapy is effective in HER-2 overexpressing breast cancers (Emlet *et al*., 2006). Moulder *et al*., (2001) showed that at low drug concentrations, ZD1839 (Iressa), an anti-EGFR small molecule, substantially reduced HER-2 phosphorylation in human breast cancer cell lines amplifying/overexpressing HER-2 that also express EGFR (BT474, SKBR3, MDA 361), but not in a HER-2 positive cell line that had no demonstrable EGFR. Indeed, the growth inhibitory and proapoptotic effects of ZD1839 in SKBR3 cells were greater than those of trastuzumab. Combined ZD1839 and trastuzumab therapy induced greater apoptosis than either agent alone. These results are consistent with the premise that the HER-2/EGFR heterodimer is biologically important, and that a strategy that targets both of its components makes sense. Based on our studies and those of others, the HER-2/EGFR ratio in BT474 cells and SKBR3 cells can be estimated to substantially exceed 1. If the studies of Moulder *et al*. (2001) were obtained under conditions in which HER-2 overexpression was dominant, EGFR might have been limiting for heterodimer formation.

Two other findings in the Moulder paper are of interest. First, ZD1839 was more effective in blocking TGF alpha-induced growth than was trastuzumab. We shall return to this later. Second, ZD1839 also interfered with the association of HER-2/HER-3 heterodimers, which was accompanied by the elimination of PI3 kinase activity. The authors hypothesized that the reduction in HER-2/HER-3 heterodimer formation may be a secondary effect of ZD1839-induced inactivation of EGFR /HER-2 heterodimers, perhaps by a dominant negative mechanism.

Using a panel of ten breast cancer cell lines, Moasser *et al*. (2001) found that the high HER-2 expressing lines (the same cell lines studied by Moulder *et al*. (2001)) were growth inhibited at lower drug concentrations of ZD1839 than the others, and that the cell lines with the highest levels of HER-2 were sensitive to the lowest concentrations of the drug. This further supports the premise that HER-2/EGFR heterodimers are biologically important, and that low EGFR levels may be limiting for heterodimer formation and biological effect in tumors with high levels of HER-2. In this study, Akt activity was reduced by the drug, as was HER-3 phosphorylation, and the association of HER-3 with the regulatory subunit of PI3 kinase.

In these studies, HER-2/HER-3 heterodimers emerged as another factor to be reckoned with in tumor responsiveness to EGFR inhibitors. Cell based analyses have shown that EGFR, HER-2, and HER-3 levels are often directly correlated with one another within individual cells, although HER-3 levels are generally much lower than those of HER-2 (Emlet *et al*., 2006). The importance of HER-3 lies in its predilection for activating the PI3 kinase/Akt signaling pathway.

HER-2 expression in EGFR-overexpressing tumors. Our studies suggest that EGFR overexpressing breast cancers (> 500,000 molecules per cell) represent ~ 20% of all cases. Within individual cells, HER-2 levels per cell are lower than those of EGFR by a factor of two or more (Shackney *et al*., 1998), over a range absolute values of HER-2 that extend down to 30–40,000 molecules per cell, the lower limits of sensitivity of HER-2 detection by flow cytometry (Figure 32.3). Robertson *et al*. (1996) and Tovey *et al*. (2006) examined EGFR expression in tumors with levels of HER-2 expression below 30,000 molecules per cell by the more sensitive technique of radioimmunohistochemistry. This group of tumors, representing 15% of all breast cancers, expressed the highest levels of EGFR that they observed, > 100,000 molecules per cell). These tumors were more aggressive clinically than tumors with intermediate levels of HER-2 expression (~ 30,000 to ~ 300,000 molecules per cell), and were as aggressive as high HER-2 overexpressing tumors (those expressing > 300,000 molecules per cell).

There are relatively few published studies on breast cancers that overexpress high levels of EGFR and low levels of HER2. DiGiovanna *et al*. (2005) evaluated EGFR and HER-2 overexpression by immunohistochemistry in over 800 patients with breast cancer, of which 15% expressed the former (DiGiovanna *et al*., 2005). They reported that 87% of breast cancer patients that overexpressed EGFR, co-overexpressed HER-2, and that tumors that expressed phosphorylated HER-2, coexpressed EGFR in 97% of cases. Co-overexpression of EGFR and HER-2, or overexpression of phosphorylated HER-2 were associated with poor survival.

To learn more about tumors with high levels of expression of EGFR and coexpression of HER-2, we turn to cancers of the upper aerodigestive tract. Levels of EGFR and TGF alpha mRNA'a are increased in ~ 90% of patients with cancers of the head and neck. The most aggressive squamous cell carcinomas of the head and neck feature levels of EGFR protein that are in the range of 5×10^6 molecules per cell, and levels of TGF alpha that vary in proportion to levels of EGFR in the same tumors (Rubin Grandis *et al*., 1998). This suggests that positive feedback loops may play a role in driving EGFR overexpression.

HER-2 is also overexpressed in about 5% of cancers of the head and neck, and is of prognostic value. The expression levels of this receptor and HER-3 have been correlated with HER-2 levels in individual tumors and a combination of all three has been shown to be of greater prognostic value than any single receptor (Xia *et al*., 1999). In cancer cell lines, increased expression levels of HER-2 and HER-3 were found to adversely affect response to gefitinib (Erjala *et al*., 2006). It appears, then, that many of the same general themes that have been encountered in breast cancer are also relevant in cancers of the head and neck, including the co-expression of multiple members of EGFR family in the same tumors, and the complexity of their interactions, particularly with respect to their responses to EGFR inhibitors.

One element that is especially prominent in head and neck tumors is the striking correlation between levels of TGF alpha expression and levels of EGFR in individual tumors (Rubin Grandis *et al*., 1998), prompting a closer look at the role of autocrine feedback loops in EGFR overexpressing cancers. We take TGF alpha

as a prime example, but it should be kept in mind that multiple growth factors are often overexpressed together, and their individual contributions may be difficult to untangle. The TGF alpha autocrine loop can be traced through its binding to EGFR, which causes a decrease in the rate of EGFR degradation (Ouyang *et al*., 1999). Our modeling studies suggest that this effect may set the stage for the gradual accumulation of cell membrane-associated EGFR in the presence of persistently high levels of TGF alpha (Figure 32.2A, outer feedback loop on left, and Figure 32.2C). TGF alpha induces HER-2/EGFR heterodimerization, albeit much less efficiently than EGF. Nevertheless, the HER-2/EGFR heterodimer plays an important role in TGF alpha signaling. Conditional expression of HER-2 leads to increased expression of TGF alpha (Xie *et al*., 1999), demonstrating the upstream role of HER-2 in the TGF alpha feedback loop. Tracing the heterodimer-driven signaling pathway further downstream, treatment with EGFR inhibitors induces inactive HER-2/EGFR heterodimers (Lenferink *et al*., 2000), and reduces ERK1/2 activation and cell proliferation *in vitro* and *in vivo* (Albanell *et al*., 2001) (Lenferink *et al*., 2000).

We suspect that autocrine positive feedback loops involving TGF alpha and, perhaps other EGFR ligands, might be driving cell proliferation in many head and neck cancers. In the absence of HER-2 amplification this produces predominantly EGFR overexpressing tumors, where HER-2 may be limiting for heterodimer formation. This issue might be addressed more directly by studies that permit quantitative comparisons among different molecular species in the same tumor.

Non-small cell lung cancer is also recognized as a predominantly EGFR-associated disease. Increased EGFR, measured by fluorescence *in situ* hybridization and immunohistochemistry has been shown to be predictive for clinical response to EGFR inhibitors (Hirsch *et al*., 2007).

Increased HER-2 is present in non-small cell lung cancers that have increased EGFR. HER-2 mRNA levels are directly correlated with levels of EGFR, and with levels of TGF alpha. Synchronous overexpression of EGFR and HER-2 is associated with poor survival (Onn *et al*., 2004).

In one of the few EGFR targeted therapy studies in which both EGFR and HER-2 were studied by fluorescence *in situ* hybridization, Daniele *et al*. (2007) showed that 9/10 patients with balanced increases in EGFR and HER-2 copies per cell responded to gefitinib (75% of all responders). Although the numbers are small, the data suggest that HER-2 may contribute to the predictive value of EGFR with regard to response to inhibitors of this receptor. Cappuzzo *et al*. (2005) showed that patients whose tumors exhibited increased HER-2 gene copy number and positive EGFR, had a significantly higher response rate to gefitinib and overall survival than patients who were negative for both receptors. In preclinical studies, Nakamura *et al*. (2005) showed that combined treatment with gefitinib and trastuzumab inhibited growth in A549 cells, which constitutively formed HER-2/EGFR heterodimers, whereas combination therapy failed to inhibit the growth of NCI-H23 cells, which did not form detectable HER-2/EGFR heterodimers.

Issues relating to signaling through HER-3, which were encountered earlier in relation to breast cancer and squamous

cell cancer of the head and neck, also appear to be relevant in non-small cell lung cancer. Hirata *et al*. (2005) found that two HER-2 transfected clones of a low EGFR expressing and low HER-2-expressing lung cancer cell line were much more sensitive to gefitinib than the parent line. In these cell lines, increased constitutive levels of HER-2/HER-3 heterodimers predominated, which associated with the regulatory subunit of PI3 kinase. Treatment with gefitinib reduced HER-2/HER-3 heterodimer levels, HER-3 activation, and Akt activation, but did not affect ERK signaling. It is unclear how gefitinib reduced HER-2/HER-3 heterodimers, unless one invokes additional effects, such as a direct effect of gefitinib on HER-2, a dominant negative effect of HER-2/EGFR heterodimer inactivation, or the effects of lateral signaling. Lateral signaling can be achieved through initial phosphorylation of HER-2 in HER-2/EGFR heterodimers, with subsequent dissociation of phospho-HER-2 and reassociation with HER-3, or through the formation of EGFR /HER-2/ HER-3 trimers (Graus-Porta *et al*., 1997). In any case, this study demonstrates a known relationship between heterodimers that contain HER-3 and downstream signaling through the PI3 kinase pathway, that is impacted by anti-EGFR therapy.

Engelman and Cantley (2006) have proposed a model in which the effectiveness of anti-EGFR therapy may depend on EGFR–driven signaling to HER-3, particularly on EGFR-driven lateral signaling to HER-3 through HER-2, subsequently inhibiting PI3 kinase signaling. This model was prompted by their previously published findings that the PI3 kinase pathway was downregulated in non-small cell lung cancer cell lines that were responsive to gefitinib and that HER-3 became associated with PI3 kinase only in these cell lines; significant HER-3 expression was not observed in resistant cell lines.

Signaling through the IGF-I receptor. In breast cancer, there is extensive crosstalk at multiple levels between members of EGFR family and membrane receptors that belong to other families, which can adversely affect response to EGFR inhibitors. A simplified diagram of downstream pathways that are responsive to EGFR dependent growth inhibition, and some of the pathways that may be involved in bypassing EGFR dependent growth inhibition is shown in Figure 32.4A. The phenomenon of adaptive switching from EGFR signaling to IGF-I receptor-mediated signaling has been reviewed recently by Jones *et al*. (2006). IGF-I receptor can form heterodimers with members of EGFR family, or crosstalk indirectly through src, a promiscuous intracellular kinase, which, itself, crosstalks with other signaling pathways.

IGF-I receptor-mediated Akt activation has been implicated both in trastuzumab resistance in breast cancer cells, and in the development of resistance to anti-EGFR agents. Other interactions in which EGFR and other members of its family participate include crosstalk with src, activation of the src/STAT pathway, the p38 pathway, and G-protein-coupled receptor pathways. These other pathways are not shown in Figure 32.4A, and are not considered further here.

OVERVIEW AND CONCLUSIONS

In this chapter, we have provided glimpses into the multifaceted cellular interactions

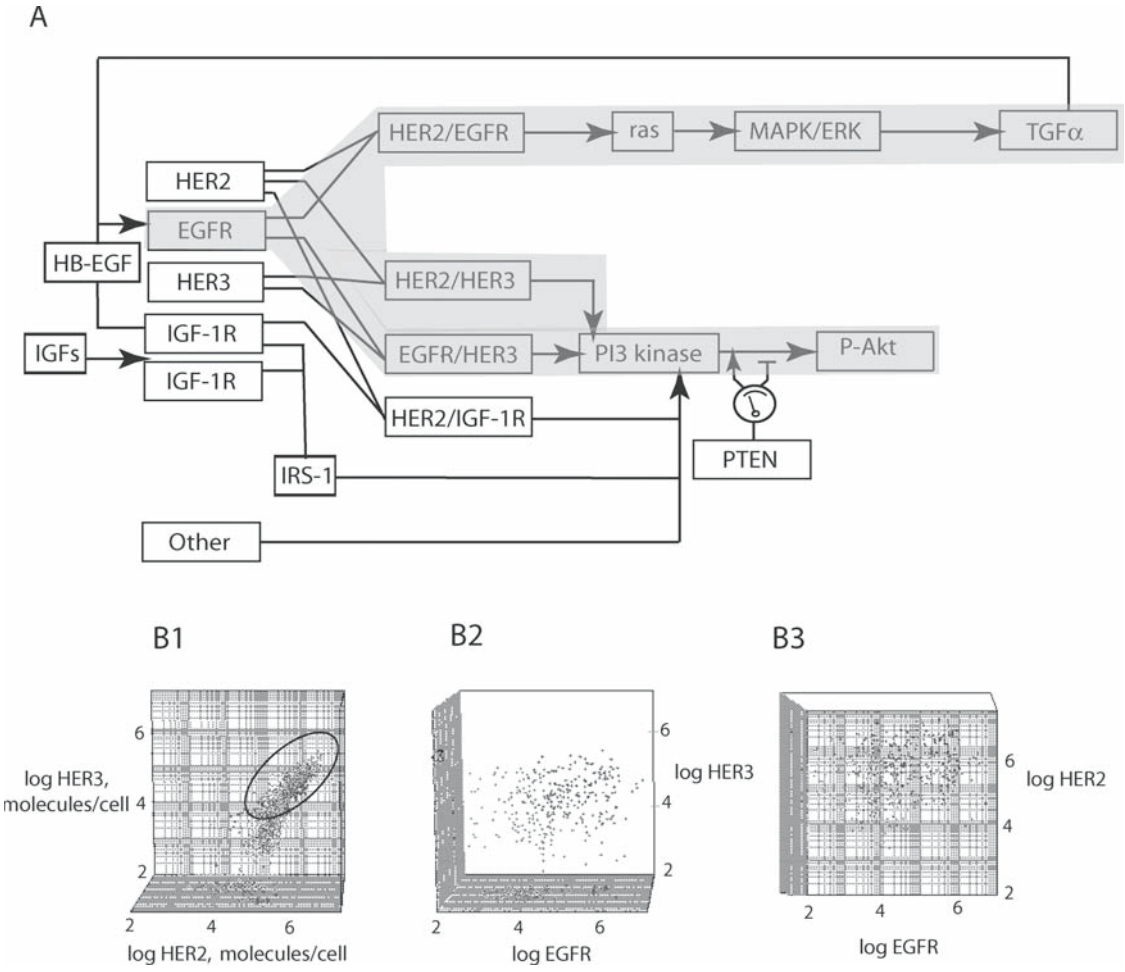

FIGURE 32.4. (A) Diagram of common EGFR pathways that are likely to be impacted by anti-Epidermal Growth Factor therapy. HER-2/*HER-3* heterodimer pathways may be affected by lateral signaling. IGF-I receptor pathways may evade agents targeted at EGFR. Many other relevant pathways are not shown. (B) Example of a quantitative multiparameter study of a primary breast cancer, in which cell DNA content (not shown), EGFR, HER-2, and *HER-3* were measured on each of several thousand cells in the sample. (B1) There is a direct relationship between the levels of HER-2 and *HER-3* expression in each cell, but not between intracellular levels ofEGFR and *HER-3* (B2) or between intracellular levels ofEGFR and HER-2 (B3)

in which EGFR and its family members are involved, in order to answer the question of whether or not HER-2 and/or EGFR expression levels can be used to identify patients who might respond to targeted therapies. It should be apparent that the original question has no simple answer(s). There is at present no single biomarker that can identify responders to HER-2 or EGFR inhibitors with confidence, nor should it reasonably be expected that one will be found in the future. Even measurements of HER-2 amplification/overexpression suffer from a 65% false-positive rate for predicting response to trastuzumab, and they have recently been shown to produce

disturbing false-negative results as well (Kim *et al*., 2007).

One must come to grips with such issues as the complexities of initial drug-tumor cell interactions, the plasticity of subsequent tumor cell responses, the heterogeneity of human breast cancers (and other solid tumors), and the deficiencies of the measurement tools that are currently being brought to bear on the study of solid tumors in the clinical setting. However, beyond these sobering realizations, there are other lessons that may be far more encouraging.

The importance of patient subsets. In retrospect, the early success of trastuzumab, and the perception of its success, were due to the fact that its introduction into the clinic was accompanied by a clinical test to identify the subset of patients that were likely to respond to the drug. This patient subsetting process ultimately made it possible to show a clear and lasting benefit of trastuzumab in the adjuvant setting, producing one of the most convincing demonstrations to date of the potential for cure of a subset of solid tumors by a targeted agent. Because EGFR inhibitors have not been subsetted during the course of drug development and clinical testing, apparent response rates appear to be low by comparison with HER-2.

How can one characterize the subset of patients with tumors that are potentially responsive to EGFR inhibitors? We propose the hypothesis that tumors in which EGFR levels exceed HER-2 levels be considered as EGFR driven and HER-2 limiting, whereas tumors in which HER-2 levels exceed EGFR levels be considered HER-2 driven and EGFR limiting. A combination of HER-2 and EGFR inhibitors might be useful in both types. However, in EGFR limiting tumors, anti-EGFR agents might be pursued more vigorously, perhaps by using a small molecule inhibitor in combination with an anti-EGFR antibody. Conversely, in the HER-2 limiting tumor subset, anti-HER-2 therapy might be pursued more vigorously with combined small molecule and antibody therapy. It would be interesting to determine which, if any of these tumor types might be more likely to respond to anti-TGF alpha therapy or to ERK pathway inhibitors.

Another subset involves HER-3 and its interactions. An example of a primary breast cancer that does not fit neatly into the first two subsets is shown in Figure 32.4B. In this multiparameter flow cytometry data set, HER-2, EGFR, HER-3, and cell DNA content (not shown) were measured simultaneously on each of several thousand cells. The mean numbers of molecules per cell of all three receptors were high. However, only the levels of HER-2 and HER-3 were correlated in individual cells (Figure 32.4B1); EGFR levels and HER-3 levels in individual cells appeared to vary randomly (Figure 32.4B2), as did the individual cellular levels of EGFR and HER-2 (Figure 32.4B3). The correlation between HER-2 and HER-3 was confined to the aneuploid cell component (data not shown). How might such a tumor respond to EGFR inhibitors? Would such a tumor respond to therapy targeting the Akt pathway? Is IGF-I receptor signaling of any clinical relevance in this tumor? Additional correlated cell-based measurements prior to treatment might provide answers to these questions.

The importance of methodology. In order to subset tumors properly, it is important to perform large numbers of quantitative measurements in units that permit comparisons not only among high and low values

of a particular molecular species, but that permit quantitative comparisons among different molecular species. The use of double fluorescence *in situ* hybridization studies would be a step in the right direction. Results might be improved further by using smears of cell suspensions obtained from needle biopsies of the surgical sample prior to formalin fixation. Separate fixation of cells in Carnoy's fixative for fluorescence *in situ* hybridization studies would produce better signals than formalin. Many of the molecular targets that are of potential interest, such as EGFR, HER-2, IGF-IR, Akt, ERK1/2, are subject to substantial post-translational alterations, which are best measured at the protein and phosphoprotein level. Again, measurements in universal units (e.g., mean molecules per cell) are required if one is to compare across molecular species.

Clinical study design. We believe that clinical studies of EGFR family inhibitors and other targeted agents (phase I trials excepted) should be driven by hypotheses that strive to match the complexity of the problems at hand. Such studies should include the collection of molecular data sets that are of high quality, and that are appropriately matched to the complexity of the hypotheses. It is these data sets, perhaps as much as patient outcomes, that are critical for planning the next generation of studies. Studies of this type may be required in order to achieve the goals of developing combinations of targeted agents that are optimized for specific tumor subsets, and the proper matching of these regimens to the patients who are most likely to benefit. Ultimately, this approach could increase the clinical success rates of pharmaceutical agents that are brought to market, and reduce the costs to patients of targeted agents.

REFERENCES

Albanell, J., Codony-Servat, J., Rojo, F., Del Campo, J. M., Sauleda, S., Anido, J., Raspall, G., Giralt, J., Rosello, J., Nicholson, R. I., Mendelsohn, J., and Baselga, J. 2001. Activated extracellular signal-regulated kinases: association with epidermal growth factor receptor/transforming growth factor alpha expression in head and neck squamous carcinoma and inhibition by anti-epidermal growth factor receptor treatments. *Cancer Res. 61*: 6500–6510.

Arteaga, C. L., Coronado, E., and Osborne, C. K. 1988. Blockade of the epidermal growth factor receptor inhibits transforming growth factor alpha-induced but not estrogen-induced growth of hormone-dependent human breast cancer. *Mol. Endocrinol. 2*: 1064–1069.

Baselga, J., Albanell, J., Ruiz, A., Lluch, A., Gascon, P., Guillem, V., Gonzalez, S., Sauleda, S., Marimon, I., Tabernero, J. M., Koehler, M. T., and Rojo, F. 2005. Phase II and tumor pharmacodynamic study of gefitinib in patients with advanced breast cancer. *J. Clin. Oncol. 23*: 5323–5333.

Cappuzzo, F., Varella-Garcia, M., Shigematsu, H., Domenichini, I., Bartolini, S., Ceresoli, G. L., Rossi, E., Ludovini, V., Gregorc, V., Toschi, L., Franklin, W. A., Crino, L., Gazdar, A. F., Bunn, P. A., Jr., and Hirsch, F. R. 2005. Increased HER2 gene copy number is associated with response to gefitinib therapy in epidermal growth factor receptor-positive non-small-cell lung cancer patients. *J. Clin. Oncol. 23*: 5007–5018.

Daniele, L., Macri, L., Schena, M., Dongiovanni, D., Bonello, L., Armando, E., Ciuffreda, L., Bertetto, O., Bussolati, G., and Sapino, A. 2007. Predicting gefitinib responsiveness in lung cancer by fluorescence in situ hybridization/chromogenic in situ hybridization analysis of EGFR and HER2 in biopsy and cytology specimens. *Mol. Cancer Ther. 6*: 1223–1229.

DiGiovanna, M. P., Stern, D. F., Edgerton, S. M., Whalen, S. G., Moore, D., and Thor, A. D. 2005. Relationship of epidermal growth factor receptor expression to ErbB-2 signaling activity and prognosis in breast cancer patients. *J. Clin. Oncol. 23*: 1152–1160.

Emlet, D. R., Schwartz, R., Brown, K. A., Pollice, A. A., Smith, C. A., and Shackney, S. E. 2006. HER2 expression as a potential marker for

response to therapy targeted to the EGFR. *Br. J. Cancer 94*: 1144–1153.

Engelman, J. A., and Cantley, L. C. 2006. The role of the ErbB family members in non-small cell lung cancers sensitive to epidermal growth factor receptor kinase inhibitors. *Clin. Cancer Res. 12*: 4372s–4376s.

Erjala, K., Sundvall, M., Junttila, T. T., Zhang, N., Savisalo, M., Mali, P., Kulmala, J., Pulkkinen, J., Grenman, R., and Elenius, K. 2006. Signaling via ErbB2 and ErbB3 associates with resistance and epidermal growth factor receptor (EGFR) amplification with sensitivity to EGFR inhibitor gefitinib in head and neck squamous cell carcinoma cells. *Clin. Cancer Res. 12*: 4103–4111.

Graus-Porta, D., Beerli, R. R., Daly, J. M., and Hynes, N. E. 1997. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. *Embo J. 16*: 1647–1655.

Hendriks, B. S., Opresko, L. K., Wiley, H. S., and Lauffenburger, D. 2003. Coregulation of epidermal growth factor receptor/human epidermal growth factor receptor 2 (HER2) levels and locations: quantitative analysis of HER2 overexpression effects. *Cancer Res. 63*: 1130–1137.

Hirata, A., Hosoi, F., Miyagawa, M., Ueda, S., Naito, S., Fujii, T., Kuwano, M., and Ono, M. 2005. HER2 overexpression increases sensitivity to gefitinib, an epidermal growth factor receptor tyrosine kinase inhibitor, through inhibition of HER2/HER3 heterodimer formation in lung cancer cells. *Cancer Res. 65*: 4253–4260.

Hirsch, F. R., Varella-Garcia, M., Cappuzzo, F., McCoy, J., Bemis, L., Xavier, A. C., Dziadziuszko, R., Gumerlock, P., Chansky, K., West, H., Gazdar, A. F., Crino, L., Gandara, D. R., Franklin, W. A., and Bunn, P. A., Jr. 2007. Combination of EGFR gene copy number and protein expression predicts outcome for advanced non-small-cell lung cancer patients treated with gefitinib. *Ann. Oncol. 18*: 752–760.

Janocko, L. E., Brown, K. A., Smith, C. A., Gu, L. P., Pollice, A. A., Singh, S. G., Julian, T., Wolmark, N., Sweeney, L., Silverman, J. F., and Shackney, S. E. 2001. Distinctive patterns of Her-2/neu, c-myc, and cyclin D1 gene amplification by fluorescence in situ hybridization in primary human breast cancers. *Cytometry 46*: 136–149.

Jones, H. E., Gee, J. M., Hutcheson, I. R., Knowlden, J. M., Barrow, D., and Nicholson, R. I. 2006. Growth factor receptor interplay and resistance in cancer. *Endocr. Relat. Cancer 13*: S45–S51.

Kim, C., Jeong, J., Geyer, C., Romond, E., Mejia-Mejia, O., Mamounas, E., Wckerham, D., Constantino, J., and Wolmark, N. 2007. Benefit from adjuvant trastuzumab may not be confined to patients with IHC 3+ and/or FISH-positive tumors. *J. Clin Oncol, 25*: 5 s.

Knowlden, J. M., Hutcheson, I. R., Jones, H. E., Madden, T., Gee, J. M., Harper, M. E., Barrow, D., Wakeling, A. E., and Nicholson, R. I. 2003. Elevated levels of epidermal growth factor receptor/c-erbB2 heterodimers mediate an autocrine growth regulatory pathway in tamoxifen-resistant MCF-7 cells. *Endocrinology 144*: 1032–1044.

Lenferink, A. E., Simpson, J. F., Shawver, L. K., Coffey, R. J., Forbes, J. T., and Arteaga, C. L. 2000. Blockade of the epidermal growth factor receptor tyrosine kinase suppresses tumorigenesis in MMTV/Neu + MMTV/TGF-alpha bigenic mice. *Proc. Natl. Acad. Sci. U. S. A. 97*: 9609–9614.

Moasser, M. M., Basso, A., Averbuch, S. D., and Rosen, N. 2001. The tyrosine kinase inhibitor ZD1839 ("Iressa") inhibits HER2-driven signaling and suppresses the growth of HER2-overexpressing tumor cells. *Cancer Res. 61*: 7184–7188.

Moulder, S. L., Yakes, F. M., Muthuswamy, S. K., Bianco, R., Simpson, J. F., and Arteaga, C. L. 2001. Epidermal growth factor receptor (HER1) tyrosine kinase inhibitor ZD1839 (Iressa) inhibits HER2/neu (erbB2)-overexpressing breast cancer cells in vitro and in vivo. *Cancer Res. 61*: 8887–8895.

Nakamura, H., Takamori, S., Fujii, T., Ono, M., Yamana, H., Kuwano, M., and Shirouzu, K. 2005. Cooperative cell-growth inhibition by combination treatment with ZD1839 (Iressa) and trastuzumab (Herceptin) in non-small-cell lung cancer. *Cancer Lett. 2301*: 33–46.

Nicholson, R. I., Hutcheson, I. R., Jones, H. E., Hiscox, S. E., Giles, M., Taylor, K. M., and Gee, J. M. 2007. Growth factor signalling in endocrine and anti-growth factor resistant breast cancer. *Rev. Endocr. Metab. Disord. 8*: 241–253

Normanno, N., and Ciardiello, F. 1997. EGF-related peptides in the pathophysiology of the mammary gland. *J. Mammary Gland Biol. Neoplasia 2*: 143–151.

Onn, A., Correa, A. M., Gilcrease, M., Isobe, T., Massarelli, E., Bucana, C. D., O'Reilly, M. S., Hong, W. K., Fidler, I. J., Putnam, J. B., and Herbst, R. S. 2004. Synchronous overexpression of epidermal growth factor receptor and HER2-neu protein is a predictor of poor outcome in patients with stage I non-small cell lung cancer. *Clin. Cancer Res. 10*: 136–143.

Ouyang, X., Gulliford, T., Huang, G., and Epstein, R. J. 1999. Transforming growth factor-alpha short-circuits downregulation of the epidermal growth factor receptor. *J. Cell Physiol. 179*: 52–57.

Robertson, K. W., Reeves, J. R., Smith, G., Keith, W. N., Ozanne, B. W., Cooke, T. G., and Stanton, P. D. 1996. Quantitative estimation of epidermal growth factor receptor and c-erbB-2 in human breast cancer. *Cancer Res. 56*: 3823–3830.

Rubin Grandis, J., Melhem, M. F., Gooding, W. E., Day, R., Holst, V. A., Wagener, M. M., Drenning, S. D., and Tweardy, D. J. 1998. Levels of TGF-alpha and EGFR protein in head and neck squamous cell carcinoma and patient survival. *J. Natl. Cancer Inst. 90*: 824–832.

Shackney, S., Emlet, D. R., Pollice, A., Smith, C., Brown, K., and Kociban, D. 2006. Guidelines for improving the reproducibility of quantitative multiparameter immunofluorescence measurements by laser scanning cytometry on fixed cell suspensions from human solid tumors. *Cytometry B Clin. Cytom. 70*: 10–19.

Shackney, S. E., and Shankey, T. V. 1997. Common patterns of genetic evolution in human solid tumors. *Cytometry 29*: 1–27.

Shackney, S. E., Pollice, A. A., Smith, C. A., Janocko, L. E., Sweeney, L., Brown, K. A., Singh, S. G., Gu, L., Yakulis, R., and Lucke, J. F. 1998. Intracellular coexpression of epidermal growth factor receptor, Her-2/neu, and p21ras in human breast cancers: evidence for the existence of distinctive patterns of genetic evolution that are common to tumors from different patients. *Clin. Cancer Res. 4*: 913–928.

Tovey, S. M., Reeves, J. R., Stanton, P., Ozanne, B. W., Bartlett, J. M., and Cooke, T. G. 2006. Low expression of HER2 protein in breast cancer is biologically significant. *J. Pathol. 210*: 358–362.

Xia, W., Lau, Y. K., Zhang, H. Z., Xiao, F. Y., Johnston, D. A., Liu, A. R., Li, L., Katz, R. L., and Hung, M. C. 1999. Combination of EGFR, HER-2/neu, and HER-3 is a stronger predictor for the outcome of oral squamous cell carcinoma than any individual family members. *Clin. Cancer Res. 5*: 4164–4174.

Xie, W., Chow, L. T., Paterson, A. J., Chin, E., and Kudlow, J. E. 1999. Conditional expression of the ErbB2 oncogene elicits reversible hyperplasia in stratified epithelia and up-regulation of TGFalpha expression in transgenic mice. *Oncogene 18*: 3593–3607.

33
Young Breast Cancer Patients Undergoing Breast-Conserving Therapy: Role of BRCA1 and BRCA2

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INTRODUCTION

Breast cancer is the most common cancer in women and the second leading cause of cancer-related death. In 2005, approximately 211,000 American women were diagnosed with invasive breast cancer and 40,000 died from the disease. 11,000 of these diagnoses were made in women under the age of 40 (American Cancer Society). BRCA1 and BRCA2 are tumor suppressor genes that are involved in multiple cellular processes including DNA repair and transcriptional regulation in response to DNA damage, chromosomal stability and cell-cycle regulation (Kiyotsugu and Yoshio, 2004). Unlike classical tumor suppressor genes; however, mutations in BRCA1 and BRCA2 are almost never seen in sporadic breast cancers.

BRCA1 and BRCA2

Inherited predisposition to breast and ovarian cancer accounts for between 5 and 10% of all breast cancer cases (Claus *et al*., 1996). Mutations in the BRCA1 and BRCA2 genes are thought to account for the largest proportion of hereditary breast cancer and explain ~ 10% of breast cancer cases in women diagnosed under the age of 35 (Malone *et al*., 2000). The lifetime risk of breast cancer for BRCA1 or 2 mutation carriers is estimated by several studies to be between 45% and 80% (Antoniou *et al*., 2003). In 1990, genetic studies provided initial evidence that connected the risk of early-onset breast cancer in some families to chromosome 17q21 (Hall *et al*., 1992). Soon after, linkage to this same locus was established in families with hereditary breast and ovarian cancer syndrome (Narod *et al*., 1991). Miki *et al*. (1994) subsequently cloned the BRCA1 gene on chromosome 17q21. Since its discovery, BRCA1 has been intensely researched. Analyses of thousands of samples in research and clinical contexts have identified many different germline mutations. BRCA2 was cloned in 1995 after chromosome 13q12-13 was linked to families with both early-onset breast

cancer and male breast cancer (Wooster *et al*., 1994, 1995). BRCA2 contributes to fewer cases of early-onset breast cancer in the United States when compared to BRCA1, but confers an estimated 6 to 10% lifetime risk of breast cancer in male mutation carriers (Thompson *et al*., 2001). Germline mutations in BRCA2 are associated with increased risk of several other cancers in addition to breast and ovarian cancer, including cancers of the prostate, pancreas, stomach, gallbladder, and bile duct, as well as malignant melanoma (van Asperen *et al*., 2005). BRCA3 has yet to be identified; however, a growing list of genes have been shown to confer either substantial increases in breast cancer risk in small numbers of patients or small increases in risk in larger population groups. These genes are currently being studied.

BRCA Related Breast Cancer

BRCA1 and BRCA2 associated breast cancers differ from sporadic breast cancers in both clinical and pathologic characteristics. Mutation carriers have a younger age of cancer onset, with tumors occurring on average 1 decade earlier than those in non-mutation carriers (Gayther *et al*., 1997). Furthermore, BRCA1 related lesions possess worse histologic factors than sporadic tumors. Studies have demonstrated a greater degree of aneuploidy, higher nuclear and histologic grade, and higher proliferation indices in BRCA1-related lesions (The Breast Cancer Linkage Consortium, 1997; Eisinger *et al*., 1996; Johannsson *et al*., 1997; Karp *et al*., 1997; Marcus *et al*., 1996). BRCA1 tumors are also more frequently estrogen and progesterone receptor negative than BRCA2 associated and non-hereditary breast cancers (Johannsson *et al*., 1997; Karp *et al*., 1997; Verhoog *et al*., 1998). BRCA2 tumors are predominantly high grade invasive ductal carcinomas. Despite their high histologic grade, BRCA2 tumors demonstrate a luminal phenotype and nearly 90% are ER and/or PR positive (Bane *et al*., 2007). Compared to sporadic cancers, both BRCA1 and BRCA2 tumors are less often HER2 positive (Robson *et al*., 1998).

Although BRCA linked breast cancers are associated with worse histological features and reduced levels of estrogen and progesterone receptor expression, survival analyses have been controversial. Most studies have found survival rates in BRCA patients to be equivalent to those in patients with sporadic breast cancer. However, a few studies have demonstrated reduced survival in BRCA carriers and two analyses showed BRCA1 mutation to be an independent negative prognostic factor in breast cancer. Unfortunately, many of these studies did not control for adjuvant therapeutic intervention or eliminate age as a confounding factor. In addition, most studies addressed BRCA1 and BRCA2 related cancers together rather than as two separate entities (Liebens *et al*., 2006).

Studies addressing local recurrence in BRCA patients have also been inconsistent. Though the majority of reviews demonstrate no significant difference between genetic and sporadic cases, a few have shown increased ipsilateral breast tumor recurrence in BRCA patients. Most studies reporting an increased recurrence rate were small in number and none addressed the issue of adjuvant therapy. Furthermore, the increase in local recurrence did not adversely affect overall survival. In contrast, BRCA carriers do appear to have a

higher probability of developing ipsilateral new primaries (Liebens *et al*., 2006; Pierce *et al*., 2006). Studies looking at contralateral breast cancer risk have consistently shown a higher incidence of tumors in BRCA patients than in the general population. The average risk of developing a contralateral breast cancer ranges from 0.5% to 0.8% per year in the sporadic patient. This number increases to 2–3% per year in a woman with an inherited predisposition to carcinoma based on BRCA1 or 2 mutation (Liebens *et al*., 2006; Metcalfe *et al*., 2004).

Breast-Conserving Therapy Versus Mastectomy

In the early 1990s, an NIH consensus committee declared breast-conserving therapy, or lumpectomy followed by radiation, to be the preferred surgical treatment of early-stage breast cancer (NIH Consensus Conference, 1991). This conclusion was based on seven prospective, randomized clinical trials which all confirmed that survival after breast-conserving therapy is equivalent to that after mastectomy alone (Arriagada *et al*., 1996; Blichert-Toft *et al*., 1992; Fisher *et al*., 1995; Jacobsen *et al*., 1995; Sarrazin *et al*., 1989; van Dongen *et al*., 1992; Veronesi *et al*., 1994). Further studies have shown outstanding local control of the disease as well as good cosmetic outcome following breast-conserving therapy (Barnett *et al*., 1996; Fine *et al*., 1994; Watterson *et al*., 1995).

Breast-Conserving Therapy in BRCA Carriers

When deciding whether breast-conserving therapy is appropriate for BRCA carriers, it is important to consider the risk of ipsilateral tumor recurrence, new ipsilateral primary, contralateral disease and overall survival after lumpectomy and radiation versus mastectomy in these patients. Since a higher local tumor recurrence rate has not been consistently demonstrated in BRCA patients following breast-conserving therapy and survival is equivalent, it is reasonable to consider this treatment option for mutation carriers. However, prior to making any decisions regarding therapy, patients must be counseled about the possibility of an ipsilateral new primary as well as contralateral disease, and consequently, the risk of needing further surgery and eventual mastectomy. In addition, patients should understand the other widely accepted treatment options with regards to their cancer, including unilateral and bilateral mastectomy, with and without reconstruction.

Many mutation carriers are diagnosed with breast cancer prior to having undergone genetic testing. In a study performed by our group looking at women under the age of 39 at diagnosis who underwent breast-conserving therapy before genetic testing was available, 8 of 89 (9%) were found to be carriers of a deleterious mutation (Golshan *et al*., 2006). The dilemma posed to young women who have not been identified as BRCA1 or BRCA2 mutation carriers before their cancer diagnosis is whether or not to pursue genetic counseling and testing, when available, in a timely fashion prior to surgery. Most patients who are newly diagnosed with breast cancer present with early stage disease. Often the surgeon will evaluate a patient and discuss the treatment options of breast-conserving therapy versus mastectomy without addressing the role of genetic mutations. The overwhelming task

of local therapy management often precludes meaningful time spent gathering family history and discussing the implications of genetic testing. Although the 4–6 week delay in obtaining test results for a patient will not alter overall survival, the emotional effect of this delay often prevents testing from being performed prior to surgery. In addition, patients are dealing with the burden of a new cancer diagnosis, and the upheaval and informational overload make it especially difficult to proceed with genetic counseling at this time. One study noted that almost one quarter of patients eligible for testing at the time of diagnosis declined genetic counseling and/or testing (Schwartz *et al*., 2005). Given the potential implications of systemic therapy such as prophylactic oophorectomy and/or adjuvant chemoradiation in BRCA mutation carriers, it is important to readdress the issue of genetic testing once local therapy has been completed.

In an interesting study of women at risk for BRCA1 or BRCA2 mutation, genetic testing was offered at the time of diagnosis to patients with a 10% or greater chance of being a carrier. In the group of women who tested positive for a deleterious mutation, half opted for bilateral mastectomy as opposed to a quarter of the sporadic group. A substantial proportion of these women chose bilateral mastectomy despite negative genetic testing and sometimes even prior to test results being available. The most important factor influencing surgical decision-making was the recommendation of the surgeon (Schwartz *et al*., 2004). Other studies have confirmed the higher rate of bilateral mastectomy in this patient population (Weitzel *et al*., 2003).

However, if no survival advantage is seen with more extensive surgery, then serious consideration should be given to close surveillance and risk reduction in preference to bilateral mastectomy. Long-term adverse effects of bilateral mastectomy such as need for reconstruction and change in body image and sexuality should be discussed with the patient prior to surgery.

In contrast, women who choose breast conservation should know of the higher rate of contralateral and new ipsilateral tumors, the complexity introduced into reconstruction with a local recurrence and history of radiation therapy, and the possible eventual need for mastectomy. Close surveillance will include a combination of interval physical exams, mammography and breast magnetic resonance imaging (MRI) (Warner *et al*., 2004). Recently, the American Cancer Society published guidelines recommending annual screening MRI in BRCA mutation carriers and those at increased risk of developing breast cancer (Saslow *et al*., 2007). Although MRI is a more sensitive test, it is less specific, often detecting abnormalities that lead to benign biopsies. The preferred interval of imaging for women with a personal history of breast cancer has yet to be determined as some centers recommend alternating modalities every 6 months while others combine yearly mammography with breast MRI. The added anxiety of this close surveillance along with the inherent risk of benign breast biopsies are factors that must be considered in this young patient population.

Chemoprevention using either a selective estrogen receptor modulator (SERM) or bilateral salpingo-opherectomy may provide additional risk reduction. Potential adverse effects of SERMs, including thromboembolic events, uterine cancer,

loss of the ability for future pregnancy, and decrease in bone mineral density are risks that must be addressed. Tamoxifen has been shown to reduce the risk of contralateral disease in BRCA mutation carriers by more than 50% when given as treatment for the original breast cancer (Gronwald *et al*., 2006). Bilateral prophylactic oophorectomy has also been demonstrated to reduce breast cancer risk by approximately 50% when performed in younger premenopausal women (Rebbeck *et al*., 2002).

Algorithm: For Surgical Decision Making

Our algorithm for determining whether breast-conserving therapy should be used in young woman with breast cancer starts with a detailed family history of the paternal and maternal lineage in relation to all carcinomas (Figure 33.1). Patients with a 10% or greater chance of harboring a deleterious mutation based on statistical models such as BRCAPRO will be referred for genetic counseling. We next review the pathologic phenotype of the

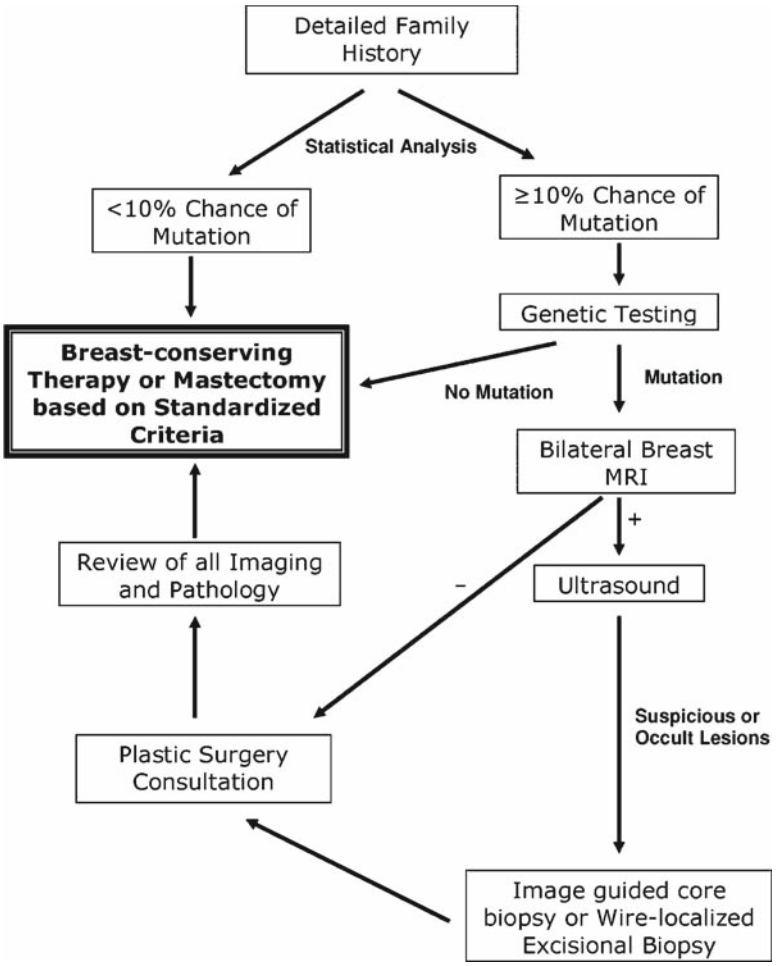


FIGURE 33.1. Algorithm: for surgical decision making

breast carcinoma as well as all imaging. Bilateral breast MRI is recommended to mutation carriers or women under the age of 40 in order to look more closely for multicentric and/or contralateral disease. Any additional lesion that is deemed worrisome by MRI is further evaluated with ultrasound. Ultrasound guided core biopsy or MRI guided core biopsy of worrisome lesions is performed prior to definitive local therapy. In the interim, patients consult with a dedicated breast reconstruction surgeon to review reconstruction options should mastectomy be necessary. For large tumors, neoadjuvant therapy options are discussed. We also address fertility issues and the timing of prophylactic oopherectomy early in the management of patients' breast carcinoma.

Once all test results are obtained, a follow-up appointment is made to discuss the surgical management of the patient's cancer. Patients are deemed appropriate candidates for breast-conserving therapy according to the standard criteria, i.e. they do not have lesions greater than 5 cm, a contraindication to radiation, multicentric disease, small breast to tumor ratio, poor compliance or central lesions that involve the nipple. After this comprehensive preoperative testing and counseling, patients are assisted in making an informed decision on breast conservation versus mastectomy. Adjuvant therapy recommendations follow based on pathologic findings. Patients are followed closely at intervals of 3–4 months for the first 3 years and every 6 months thereafter.

The care of young breast cancer patients, particularly those who are genetic mutation carriers, requires a multidisplinary team of specialists including breast and reconstruction surgeons, medical and radiation oncologists, genetic counselors, reproductive endocrinologists, and social workers. Proper identification and management of women with an inherited predisposition to breast cancer would not only affect the cancer patient herself, but also family members who may require additional screening and risk assessment. Breast-conserving therapy with close postoperative surveillance is a reasonable option for the carefully selected patient.

REFERENCES

No authors listed. NIH Consensus Conference 1991. Treatment of early-stage breast cancer. *JAMA 265*: 391–395.

No authors listed. The Breast Cancer Linkage Consortium 1997. Pathology of familial breast cancer: Differences between breast cancers in carriers of BRCA1 or BRCA2 mutations and sporadic cases. *The Lancet 349*: 1505–1510.

American Cancer Society. Breast Cancer Facts & Figures 2005–2006. Atlanta: American Cancer Society.

Antoniou, A., Pharoah, P.D., Narod, S., Risch, H.A., Eyfiord, J.E., Hopper, J.L., Loman, N., Olsson, H., Johannsson, O., Borg, A., Pasini, B., Radice, P., Manoukian, S., Eccles, D.M., Tang, N., Olah, E., Anton-Culver, H., Warner, E., Lubinski, J., Gronwald, J., Gorski, B., Tulinius, H., Thorlacius, S., Eerola, H., Nevanlinna, H., Syriakoski, K., Kallioniemi, O.P., Thompson, D., Evans, C., Peto, J., Lalloo, F., Evans, D.G., and Easton D.G. 2003. Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case Series unselected for family history: a combined analysis of 22 studies. *Am. J. Hum. Genet. 73*: 709.

Arriagada, R., Le, M.G., Rochard, F., and Contesso, G. for the Institute Gustave Roussy Breast Cancer Group 1996. Conservative treatment versus mastectomy in early breast cancer: patterns of failure with 15 years of follow-up data. *J. Clin. Oncol. 14*: 1558–1564.

Bane, A.L., Beck, J.C., Bleiweiss, I., Buys, S.S., Catalano, E., Daly, M.B., Giles, G., Godwin, A.K., Hibshoosh, H., Hopper, J.L., John, E.M.,

Layfield, L, Longacre, T., Miron, A., Senie, R., Southey, M.C., West, D.W., Whittemore, A.S., Wu, H., Andrulis, I.L., and O'Malley, F.P. 2007. BRCA2-mutation-associated breast cancers exhibit a distinguishing phenotype based on morphology and molecular profiles from tissue microarrays. *Am. J. Surg. Pathol. 31*: 121–128.

Barnett, G.R. and Gianoutsos, M.P. 1996. The latissimus dorsi added flap for natural tissue breast reconstruction: report of 15 cases. *Plast. Reconstr. Surg. 97*: 63–70.

Blichert-Toft, M., Rose, C., Andersen, J.A., Overgaard, M., Axelsson, C.K., Andersen, K.W., and Mouridsen, H.T. 1992. Danish randomized trial comparing breast conservation therapy with mastectomy: six years of life-table analysis. *J. Natl. Cancer Inst. Monogr. 11*: 19–25.

Claus, E.B., Schildkraut, J.M., Thompson, W.D., and Risch, N.J. 1996. The genetic attributable risk of breast and ovarian cancer. *Cancer 77*: 2318–2324.

Eisinger, F., Stoppa-Lyonnet, D., Longy, M., Kerangueven, F., Noguchi, T., Bailly, C., Vincent-Salomon, A., Jacquemier, J., Birnbaum, D., and Sobol, H. 1996. Germline mutation at BRCA1 affects the histoprognostic grade in hereditary breast cancer. *Cancer Res. 56*: 471–474.

Fine, N.A., Orgill, D.P., and Pribaz J.J. 1994. Early clinical experience in endoscopic-assisted muscle flap harvest. *Ann. Plast. Surg. 33*: 465–469.

Fisher, B., Anderson, S., Redmond, C.K., Wolmark, N., Wickerham, D.L., and Cronin, W.M. 1995. Reanalysis and results after 12 years of follow-up in a randomized clinical trial comparing total mastectomy with lumpectomy with or without irradiation in the treatment of breast cancer. *N. Engl. J. Med. 333*: 1456–1461.

Gayther, S.A., Pharoah, P.D., and Ponder, B.A. 1997. The genetics of inherited breast cancer. *J. Mamm. Gland Biol. Neoplasia 3*: 365–376.

Golshan, M., Miron, A., Nixon, A.J., Garber, J.E., Cash, E.P., Iglehart, J.D., Harris, J.R. and Wong, J.S. 2006. The prevalence of germline BRCA1 and BRCA2 mutations in young women with breast cancer undergoing breast-conserving therapy. *Am. J. Surg. 192*: 58–62.

Gronwald, J., Tung, N., Foulkes, W.D., Offit, K., Gershoni, R., Daly, M., Kim-Sing, C., Olsson, H., Ainsworth, P., Eisen, A., Saal, H., Friedman, E., Olopade, O., Osborne, M., Weitzel, J., Lynch, H., Ghadirian, P., Lubinski, J., Sun, P., and Narod, S.A, Hereditary Breast Cancer Clinical Study Group 2006. Tamoxifen and contralateral breast cancer in BRCA1 and BRCA2 carriers: an update. *Int. J. Cancer 118*: 2281–2284.

Hall, J.M., Lee, M.K., Newman, B., Morrow, J.E., Anderson, L.A., Huey, B., and King, M.C. 1992. Linkage of early-onset familial breast cancer to chromosome 17q21. *Science 250*: 1684–1689.

Jacobsen, J.A., Danforth, D.N., Cowan, K.H., d'Angelo, T., Steinberg, S.M., Pierce, L., Lippman, M.E., Lichter, A.S., Glatstein, E., Okunieff, P. 1995. Ten-year results of a comparison of conservation with mastectomy in the treatment of stage I and II breast cancer. *N. Engl. J. Med. 332*: 907–911.

Johannsson, O.T., Idvall, I., Anderson, C., Borg, A., Barkardottir, R.B., Egilsson, V., Olsson, H. 1997. Tumour biological features of BRCA1-induced breast and ovarian cancer. *Eur. J. Cancer 33*: 362–371.

Karp, S.E., Tonin, P.N., Begin, L.R., Martinez, J.J., Zhang, J.C., Pollak, M.N., and Foulkes, W.D. 1997. Influence of BRCA1 mutations on nuclear grade and estrogen receptor status of breast carcinoma in Ashkenazi Jewish women. *Cancer 80*: 435–441.

Kiyotsugu, Y. and Yoshio, M. 2004. Role of BRCA1 and BRCA2 as regulators of DNA repair, transcription, and cell cycle in response to DNA damage. *Cancer Sci. 95*: 866–871.

Liebens, F.P., Carly, B., Pastijn, A., and Rozenberg, S. 2006. Management of BRCA1/2 associated breast cancer: a systematic qualitative review of the state of knowledge in 2006. *Eur. J. Cancer 43*: 238–257.

Malone, K.E., Daling, J.R., Neal, C., Suter, N.M., O'Brien, C., Cushing-Haugen, K., Jonasdottir, T.J., Thompson, J.D., and Ostrander, E.A. 2000. Frequency of BRCA1/BRCA2 mutations in a population-based series of young breast carcinoma cases. *Cancer 88*: 1393–1402.

Marcus, J.N., Watson, P., Page, D.L., Narod, S.A., Lenoir, G.M., Tonin, P., Linder Stephenson, L., Salerno, G., Conway, T.A., and Lynch, H.T. 1996. Hereditary breast cancer: pathobiology, prognosis and BRCA1 and BRCA2 gene linkage. *Cancer 77*: 697–709.

Metcalfe, K., Lynch, H., Ghadirian, P., Tung, N., Olivotto, I., Warner, E., Olopade, O.I., Eisen, A.,

Weber, B., McLennan, J., Sun, P., Foulkes, W.D., and Narod, S.A. 2004. Contralateral breast cancer in BRCA1 and BRCA2 mutation carriers. *J. Clin. Oncol. 22*: 2328–2335.

Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P.A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L.M., Ding, W., Bell, R., Rosenthal, J., Hussey, C., Thanh, T., Mcclure, M., Frye, C., Hattier, T., Phelps, R., Haugen-Strano, A., Katcher, H., Yakumo, K., Gholami, Z., Shaffer, D., Stone, S., Bayer, S., Wray, C., Bogden, R., Priya, D., Ward, J., Tonin, P., Narod, S., Bristow, P.K., Norris, F.H., Helvering, L., Morrison, P., Rosteck, P., Mei, L., Barrett, J.C., Lewis, C., Neuhausen, S., Cannon-Albright, L., Goldgar, D., Wiseman, R., Kamb, A., Skolnick, M.H. 1994. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science 266*: 66–71.

Narod, S.A., Feunteun, J., Lynch, H.T., Watson, P., Conway, T., Lynch, J., and Lenoir, G.M. 1991. Familial breast-ovarian cancer locus on chromosome 17q12-q23. *Lancet 338*: 82–83.

Pierce, L.J., Levin, A.M., Rebbeck, T.R., Pierce LJ, Ben-David, M.A., Friedman, E., Solin, L.J., Harris, E.E., Gaffney, D.K., Haffty, B.G., Dawson, L.A., Narod, S.A., Olivotto, I.A., Eisen, A., Whelan, T.J., Olopade, O.I., Isaacs, C., Merajver, S.D., Wong, J.S., Garber, J.E., and Weber, B.L. 2006. Ten-year multi-institutional results of breast-conserving surgery and radiotherapy in BRCA1/2-associated stage I/II breast cancer. *J. Clin. Oncol. 24*: 2437–2443.

Rebbeck, T.R., Lynch, H.T., Neuhausen, S.L., Narod, S.A., van't Veer, L., Garber, J.E., Evans, G., Isaacs, C., Daly, M.B., Matloff, E., Olopade, O.I., Weber, B.L. 2002. Prevention and Observation of Surgical End Points Study Group. Prophylactic oophoerectomy in carriers of BRCA1 or BRCA2 mutations. *N. Engl. J. Med. 346*: 1616–1622.

Robson, M., Rajan, P., Rosen, P.P., Gilewski, T., Hirschaut, Y., Pressman, P., Haas, B., Norton, L., and Offit, K. 1998. BRCA-associated breast cancer: absence of a characteristic immunophenotype. *Cancer Res. 58*: 1839–1842.

Sarrazin, D., Lê, M.G., Arriagada, R., Contesso, G., Fontaine, F., Spielmann, M., Rochard, F., Le Chevalier, T., and Lacour, J. 1989. Ten-year results of a randomized trial comparing a conservative treatment to mastectomy in early breast cancer. *Radiother. Oncol. 14*: 177–184.

Saslow, D., Boetes, C., Burke, W., Harms, S., Leach, M.O., Lehman, C.D., Morris, E., Pisano, E., Schnall, M., Sener, S., Smith, R.A., Warner, E., Yaffe, M., Andrews, K.S., and Russell, C.A. 2007. American cancer society guidelines for breast screening with MRI as an adjunct to mammography. *CA Cancer J. Clin. 57*: 75–89.

Schwartz, M.D., Lerman, C., Brogan, B., Peshkin, B.N., Halbert, C.H., DeMarco, T., Lawrence, W., Main, D., Finch, C., Magnant, C., Pennanen, M., Tsangaris, T., Willey, S., and Isaacs, C. 2004. Impact of BRCA1/ BRCA2 counseling and testing in newly diagnosed breast cancer patients. *J. Clin. Oncol. 22*: 1823–1829.

Schwartz, M.D., Lerman, C., Brogan, B., Peshkin, B.N., Isaacs, C., DeMarco, T., Halbert, C.H., Pennanen, M., and Finch, C. 2005. Utilization of BRCA1/BRCA2 mutations testing in newly diagnosed breast cancer patients. *Cancer Epidemiol. Biomarkers Prev. 14*: 1003–1007.

Thompson, D., Easton, D., and the Breast Cancer Linkage Consortium. 2001. Variation in cancer risks, by mutation position, in BRCA2 mutation carriers. *Am. J. Hum. Genet. 68*: 410–419.

van Asperen, C.J., Brohet, R.M., Meijers-Heijboer, E.J., Hoogerbrugge, N., Verhoef, S., Vasen, H.F., Ausems, M.G., Menko, F.H., Gomez Garcia, E.B., Klijn, J.G., Hogervorst, F.B., van Houwelingen, J.C., van't Veer, L.J., Rookus, M.A., and van Leeuwen, F.E.; Netherlands Collaborative Group on Hereditary Breast Cancer. 2005. Cancer risks in BRCA2 families: estimates for sites other than breast and ovary. *J. Med. Genet. 42*: 711–719.

van Dongen, J.A., Bartelink, H., Fentiman, I.S., Lerut, T., Mignolet, F., Olthuis, G., van der Schueren, E., Sylvester, R., Winter, J., and van Zijl, K. 1992. Randomized clinical trial to assess the value of breast-conserving therapy in stage I and II breast cancer: EORTC 10801 trial. *J. Natl. Cancer Inst. 11*: 15–18.

Verhoog, L.C., Brekelmans, C.T.M., Seynaeive, C., van den Bosch, L.M., Dahmen, G., van den Bosch, L.M., Dahmen, G., van Geel, A.N., Tilanus-Linthorst, M.M., Bartels, C.C., Wagner, A., van den Ouweland, A., Devilee, P., Meijers-Heijboer, E.J., Klijn, J.G. 1998. Survival and

tumor characteristics of breast cancer patients with germline mutations in BRCA1. *Lancet 351*: 316–321.

Veronesi, U., Luini, A., Galimberti, V., and Zurrida, S. 1994. Conservation approaches for the management of stage I/II carcinoma of the breast: Milan Cancer Institute trials. *World J. Surg. 18*: 70–75.

Warner, E., Plewes, D.B., Hill, K.A., Causer, P.A., Zubovits, J.T., Jong, R.A., Cutrara, M.R., DeBoer. G., Yaffe, M.J., Messner, S.J., Meschino, W.S., Piron, C.A., Narod, S.A. 2004. Surveillance of BRCA1 and BRCA2 mutation carriers with magnetic resonance imaging, ultrasound, mammography, and clinical breast examination. *JAMA 292*: 1317–1325.

Watterson, P.A., Bostwick, J., Hester, T.R., Bried, J.T., and Taylor, G.I. 1995. TRAM flap anatomy correlated with a 10-year clinical experience with 556 patients. *Plast. Reconstr. Surg. 95*: 1185–1194.

Weitzel, J.N., McCaffer, S.M., Nedelcu, R., MacDonald, D.J., Blazer, K.R., Cullinane, C.A. 2003. Effect of genetic cancer risk assessment on surgical decisions at breast cancer diagnosis. *Arch. Surg. 138*: 1323–1328.

Wooster, R., Neuhausen, S.L., Mangion, J., Quirk, Y., Ford, D., Collins, N., Nguyen, K., Seal, S., Tran, T., Averill, D., Fields, P., Marshall, G., Narod, S., Lenoir, G.M., Lynch, H., Feunteun, J., Devilee, P., Cornelisse, C.J., Menko, F.H., Daly, P.A., Ormiston, W., McManus, R., Pye, C., Lewis, C.M., Cannon-Albright, L.A., Peto, J., Ponder, B.A.J., Skolnick, M.H., Easton, D.F., Goldgar, D.E., Stratton, M.R. 1994. Location of a breast cancer susceptibility gene, BRCA2, to chromosome 13q12-13. *Science 265*: 2088–2090.

Wooster, R., Bignell, G., Lancaster, J., Swift, S., Seal, S., Mangion, J., Collins, N., Gregory, S., Gumbs, C., and Micklem, G. 1995. Identification of the breast cancer susceptibility gene BRCA2. *Nature 378*: 789–792.

34
Radiation Therapy for Older Women with Early Breast Cancer

Benjamin D. Smith

INTRODUCTION

Older women comprise a rapidly growing and unique subgroup of women with early breast cancer. Because the aging process spans both a chronologic and physiologic continuum, a precise cutpoint that defines a person as "older" cannot be readily defined. Nevertheless, as 65 years of age marks a common age for retirement and eligibility for Medicare benefits in the United States, this chapter will use the term "older" to denote women age ≥ 65 years. The term "early breast cancer" commonly includes invasive breast cancer with size ≤ 5 cm with 0–3 involved regional lymph nodes (stages I and II in the American Joint Commission on Cancer Sixth Edition as reported by Greene *et al*. (2002)).

Early breast cancer diagnosed in older women merits attention as an important and unique entity due to both tumor and patient factors. For example, breast tumors in older women tend to have more favorable biologic characteristics and growth patterns. With respect to patient factors, physiologic changes associated with the aging process may influence the effectiveness and tolerability of cancer-directed treatment. Furthermore, the burden of comorbid illness associated with the aging process increases the competing risk of death from non-cancer causes, and may thus attenuate the potential benefit of cancer-directed therapy. Finally, psychosocial factors, such as diminished autonomy, may limit a patient's access to certain treatment options and thereby influence treatment selection.

For women with early breast cancer, the benefits of radiation therapy can be conceptualized into three discrete categories. First, radiation therapy may be used to improve local control of disease in the breast and/or chest wall. Second, radiation therapy may be used to improve regional control, particularly in patients who have not received surgical treatment of their regional lymph nodes. Finally, because radiation lowers the risk of local-regional recurrence, the third benefit of radiation is a potential improvement in overall survival.

EPIDEMIOLOGY

Data from the National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER) program indicate the following age distribution for incident breast cancer cases: 12% age 20–44 years, 47% age 45–64 years, 36% age 65–84 years, and 5% age ≥ 85 years. When stratified by age, the incidence of breast cancer cases is highest among women age 65–84 years, at 513 cases per 100,000.

In the future, the absolute number of older women diagnosed with early breast cancer in the United States will increase dramatically as the population continues to age. Data from the US Census Bureau forecast that, from 2010 through 2050, the number of women (age 65–84 years) diagnosed will double, and the number of women age ≥ 85 years will quadruple. Assuming constant incidence rates, the annual incidence of breast cancer in women age 65–84 will approach 180,000 cases per year by 2030, surpassing all other age groups.

NATURAL HISTORY

Older women with early breast cancer experience a lower risk of local recurrence than younger women with early breast cancer. This fact was most clearly demonstrated in the European Organization for the Research and Treatment of Cancer (EORTC) randomized clinical trial reported by Bartelink *et al*. (2001). In this prospective, randomized trial that included 5,318 women age ≤ 70 years with stage I or II breast cancer, age at diagnosis was the most significant clinical-pathologic predictor of local recurrence in multivariate analysis. Among patients treated with conventional whole breast radiation to 50 Gy, the 5-year risk of local recurrence was 19.5% for age ≤ 40 years, 9.5% for age 41–50 years, 4.2% for age 51–60 years, and 4.0% for age 60–70 years (Figure 34.1). However, because this trial excluded women age > 70 years, it was unable to determine whether women over the age of 70 years experience an even lower risk of local recurrence than women under the age of 70 years. To address this question, Smith *et al*. (2006) used data from the population-based SEER-Medicare cohort to compare the risk of local recurrence among 10,969 women age ≥ 66 years with stage I breast cancer. Among patients treated with conservative surgery without radiation, the 8-year risk of local recurrence was 13% for those age 66–69 years compared to 8% for those age ≥ 70 years, suggesting that the risk of local recurrence continues to decrease with increasing age.

In addition to the influence of age on risk of local recurrence, age also appears to influence risk of mortality. Diab *et al*. (2000) reported relative survival as a function of age at diagnosis, tumor size, and

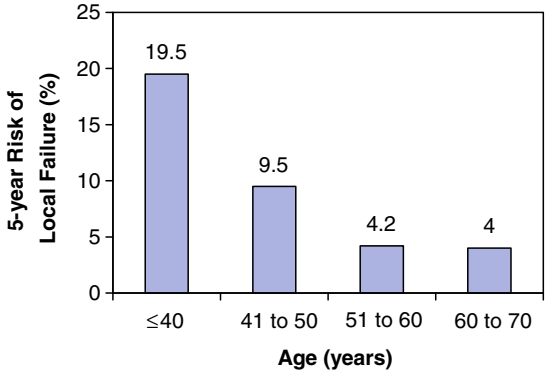


FIGURE 34.1. Relationship between age at diagnosis and 5-year risk of local failure among 2,657 women treated with 50 Gy in 2 Gy/fraction in the EORTC boost trial. (From Bartelink *et al*., 2001.)

nodal status for 256,287 women age ≥ 55 years reported in the SEER registry. Relative survival indicates the survival of patients with breast cancer divided by the survival of a matched cohort of non-cancer patients. Hence, relative survival is a population-based method used to approximate cause-specific survival. For example, a 5-year relative survival of 90% suggests that 5-year cancer-specific mortality is approximately 10%, provided that the cancer cases and non-cancer controls are adequately matched. In the study reported by Diab *et al*. (2000), relative survival for patients with node-negative breast cancer varied significantly with age at diagnosis. For patients diagnosed between ages 55–69 years, 8-year relative survival ranged from 91–97%, and was significantly less than 100%. In contrast, for node-negative patients diagnosed at age 70 years or older, relative survival ranged from 101–119%, indicating that older women age ≥ 70 years with node-negative breast cancer experience long-term survival which meets or exceeds the survival of similar older women who do not have breast cancer. This observation suggests that node-negative breast cancer is a relatively infrequent cause of death among women age ≥ 70 years. Further, important differences in mortality may exist between older women with node-negative breast cancer and older women without breast cancer, and surprisingly, these differences actually favor older women with breast cancer.

PATHOLOGY

Data from several large cohort studies suggest that breast cancers diagnosed in older women have more favorable biologic characteristics than breast cancers diagnosed in younger women. For example, in a cohort of 50,828 women from the San Antonio breast cancer database, Diab *et al*. (2000) found that older age was associated with the following favorable characteristics: ER positivity, low S-phase fraction, normal intracellular p53 levels, and absence of epidermal growth factor and HER2-neu overexpression. These findings have been confirmed in smaller Italian cohorts reported by Daidone *et al*. (2003) and Molino *et al*. (2006). In addition, Diab *et al*. (2000) reported a higher prevalence of more favorable histologies, such as invasive lobular and mucinous carcinomas, in older women.

Microscopic growth pattern also appears to vary with patient age. For example, Imamura *et al*. (2000) reported the maximum distance of intraductal spread away from the primary invasive tumor in 253 women with breast cancer. The mean maximum spread varied significantly with age, measuring 10.5 mm for age ≤ 39 years, 8.3 mm for age 40–64 years, and 5.3 mm for age ≥ 65 years. This observation suggests that wider lumpectomy margins are required for younger patients, and may help to explain the observed association between increased age at diagnosis and lower risk of local recurrence.

RADIATION THERAPY FOR LOCAL CONTROL

Radiation Following Conservative Surgery. Historically, radiation therapy to the whole breast has been recommended for all patients treated with conservative surgery. A meta-analysis reported by Vinh-Hung and Verschraegen (2004) concluded that radiation therapy confers a threefold reduction in the relative risk of local

failure. Further, the Early Breast Cancer Trialists Collaborative Group meta-analysis reported by Clarke *et al*. (2005) concluded that the relative benefit of radiation therapy was similar regardless of patient age at diagnosis, tumor grade, tumor size, and hormone receptor expression.

However, despite the finding of a consistent relative benefit in local control derived from radiation, the absolute benefit of radiation may vary substantially as a function of the underlying risk of local failure. For example, if radiation lowers the risk of local recurrence from 30% to 10% in population A and from 3% to 1% in population B, the relative benefit of radiation is the same for each population, but the absolute benefit is a 20% risk reduction in population A versus a 2% risk reduction in population B. Thus, many investigators have attempted to prospectively identify subgroups of patients in whom the risk reduction conferred by radiation is sufficiently low that radiation therapy can be withheld. One important early trial that attempted to omit radiation was recently reported by Lim *et al*. (2006). This study included 87 patients with unicentric stage I invasive breast cancer, resected with negative margins at a minimum of 1 cm, without lymphovascular space invasion. All patients were treated with conservative surgery without adjuvant radiation therapy, endocrine therapy, or chemotherapy. At a median follow up exceeding 7 years, 23% of patients experienced a local recurrence, including 22% of the patients age ≥ 70 years. A very important conclusion from this carefully executed trial is that conservative surgery alone is insufficient therapy, even for carefully selected patients.

Rather than simply withholding radiation, more recent trials have attempted to replace radiation therapy with adjuvant endocrine therapy. In the context of older women, the most notable trial was the C9343, reported by Hughes *et al*. (2004) for the Cancer and Leukemia Group B. This trial included only women age ≥ 70 years with small (≤ 2 cm), estrogen receptor positive or unknown tumors treated with marginally negative lumpectomy followed by 5 years of adjuvant tamoxifen (Table 34.1). Patients were randomized to no radiation therapy or radiation therapy (45 Gy in 1.8 Gy/fraction to the whole breast followed by an electron boost of 14 Gy in 2 Gy/fraction; intentional radiation of the regional lymph nodes was not allowed). At 5 years, the risk of local-regional recurrence was 4% in the no radiation group versus 1% in the radiation group. Although this difference was statistically significant, the authors' questioned the clinical relevance of this small difference, particularly in light of the observation that many local recurrences could be salvaged with additional conservative surgery, and that radiation did not improve overall survival. Subsequently, Carlson *et al*. (2005) updated the National Comprehensive Cancer Network clinical guidelines to state that "radiation therapy may be omitted" in patients who meet the C9343 entry criteria.

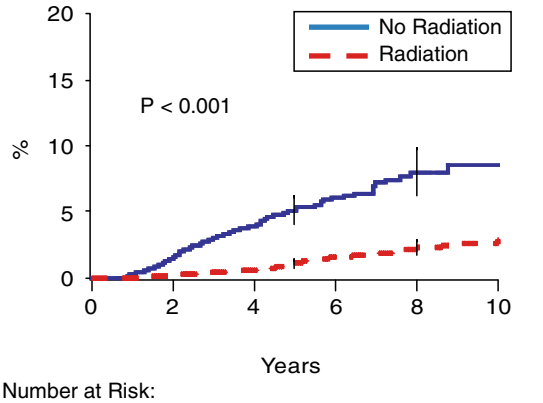
Despite its importance, the C9343 trial has left several important unanswered questions. For example, because only 5-year data were reported, the long-term impli-

TABLE 34.1. Entry criteria for the cancer and leukemia group B C9343 randomized clinial trial. (From Hughes *et al*., 2004.)

1. Clinical stage T1 N0 M0 invasive breast cancer
2. Age 70 years or older
3. Margin-negative conservative surgery
4. Estrogen receptor positive (or unknown)

cations of withholding radiation are still unknown. To address this issue, Smith *et al.* (2006) used data from the SEER-Medicare cohort to estimate the 8-year risk of local recurrence among 8,724 C9343-eligible older women treated in the community setting between 1992 through 1999. In this retrospective cohort study, 73% of patients received radiation therapy following conservative surgery. Because local recurrence is not specifically reported by SEER-Medicare, the authors used a combined outcome, defined as either a subsequent mastectomy reported by Medicare claims or a second ipsilateral invasive breast cancer reported by SEER, as a surrogate for local recurrence. At 5 years, the risk of this combined, surrogate outcome was nearly identical to the risk of local-regional recurrence reported in the C9343 trial, with 5% of patients treated with conservative surgery alone experiencing the combined outcome, compared to 1% among patients treated with conservative surgery plus radiation. At 8 years, the risk of the combined outcome was 8% among patients treated with conservative surgery alone compared to 2% among patients treated with conservative surgery plus radiation (Figure 34.2). Because the risk of the combined outcome continued to increase past 5 years, these results suggest that patients with a longer life expectancy derive a larger absolute risk reduction from radiation.

In this population of older women with a higher prevalence of comorbid illness, tools to predict life expectancy are needed to identify patients who are most likely or least likely to live long enough to derive a clinical benefit from radiation therapy. To address this issue, Smith *et al.* (2006) estimated the number needed to treat (NNT)



Number at Risk:

No RT	2,364	2,006	1,317	676	266	80
RT	6,360	6,009	4,494	2,500	1,099	307

FIGURE 34.2. Kaplan-Meier curves indicate risk of a second breast cancer event (either second ipsilateral breast cancer or subsequent mastectomy) among older women who would have been eligible for C9343, diagnosed with breast cancer between 1992 through 1999 and reported in the SEER-Medicare data. (Reproduced with permission from Smith *et al.*, 2006). RT = radiation therapy. Error bars = 95% confidence intervals

with radiation to prevent one recurrence as a function of age at diagnosis and comorbid illness. A lower NNT indicates a greater benefit from radiation, whereas a higher NNT indicates a smaller benefit from radiation. As shown in Table 34.2, women age 70–74 years without significant comorbidity experience an 8-year survival of 84%, and the NNT is 21. In contrast, for a woman age 80–84 with moderate to severe comorbidity (Charlson score = 2–9), 8-year survival is 29%, and the NNT is 61, indicating that 61 women would need to be treated with radiation in order to prevent just one recurrence.

Given the relatively small benefit of radiation for C9343-eligible older women, Smith *et al.* (2006) also sought to determine whether radiation might be more beneficial for C9343-ineligible women. As shown

TABLE 34.2. Number needed to treat to prevent one second breast cancer event[a]. (Used with permission from Smith *et al*., 2006.)

Age	Comorbidity score	N	Eight-year survival[b] (95% confidence interval)	Adjusted number needed to treat[c] (95% confidence interval)
70–74	0	2,188	84 (83–86)	21 (16–31)
	1	640	72 (68–76)	24 (18–36)
	2–9	226	47 (40–55)	37 (28–55)
75–79	0	1,721	79 (76–81)	22 (17–33)
	1	572	62 (58–67)	28 (21–42)
	2–9	262	43 (36–51)	41 (31–60)
80–84	0	1,096	61 (57–64)	29 (22–43)
	1	388	47 (40–53)	38 (28–56)
	2–9	218	29 (21–36)	61 (46–90)
85 and older	0	661	33 (29–38)	53 (40–78)
	1	316	18 (13–24)	97 (73–143)
	2–9	178	14 (7.2–21)	125 (94–185)

[a] The combined outcome of second ipsilateral breast cancer reported by Surveillance, Epidemiology, and End Results (SEER) data and/or subsequent mastectomy reported by Medicare claims.
[b] Expected survival calculated with Kaplan-Meier method.
[c] At 8 years follow up, the unadjusted number needed to treat was 18 (95% CI 13–26).

in Figure 34.3, women who would have been ineligible for C9343 due to younger age (66–69 years), larger tumor size (2.1–5.0 cm), or estrogen receptor negativity experience a higher risk of local recurrence, and, consequently, derive a larger absolute risk reduction when treated with radiation therapy. These results suggest that the C9343 eligibility criteria truly identify the lowest risk subgroup of older women, among whom the harm associated with omission of radiation is minimized.

In summary, even older women who satisfy the C9343 entry criteria, and therefore comprise the *lowest* risk patient population, still derive a small but measurable local control benefit from radiation therapy. Older women age 70–79 years in good health are most likely to benefit from radiation given their relatively long life expectancy and, in this author's opinion, should be evaluated by a radiation oncologist and counseled regarding the risks and benefits of radiation in this setting. Older women who decline radiation should be reminded that their risk of local relapse is unacceptably high unless they are compliant with adjuvant endocrine therapy. Finally, for those older women with early breast cancer who do not satisfy the C9343 entry criteria, the absolute risk reduction conferred by radiation is more sizeable, and radiation should be considered a standard component of treatment.

Radiation Following Mastectomy. Radiation therapy is rarely indicated for older women with early breast cancer treated with mastectomy. Although a positive margin may provide sufficient justification for post-mastectomy radiation (PMRT), even certain older women with positive margins may not need PMRT. For example, in a cohort of 41 women with positive chest wall margins treated with mastectomy without PMRT, Truong *et al*. (2004) reported a 0% risk of local-regional recurrence among women with T1, grade 1 or 2 tumors without lymphovascular space invasion. Similarly, in a study of 34 women with early breast

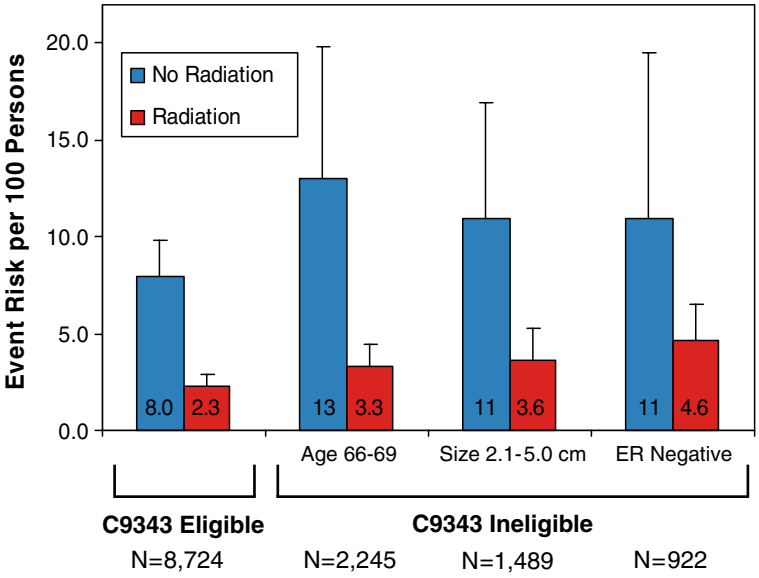


FIGURE 34.3. Eight-year risk of a second breast cancer event (either second ipsilateral breast cancer or subsequent mastectomy) among older women with early breast cancer reported in the SEER-Medicare data. (Reproduced with permission from Smith *et al.*, 2006). Eight-year event risk without and with radiation therapy is reported for four mutually exclusive patient groups, including patients eligible for trial C9343, patients ineligible for trial C9343 because of age 66–69 years, patients ineligible for trial C9343 because of tumor size 2.1–5.0 cm, and patients ineligible for trial C9343 because of estrogen receptor-negative status. Patients in each of the ineligible groups, including age 66–69 years, tumor size 2.1–5.0 cm, and estrogen receptor-negative status, meet all the other criteria for trial C9343. ER = estrogen receptor. Error bars = 95% confidence intervals

cancer treated with mastectomy without radiation who were found to have tumor within 1 cm of the deep margin, a 0% risk of chest wall recurrence for women age > 50 years was reported (Freedman *et al.*, 1998) These results suggest that PMRT is rarely required following mastectomy for early breast cancer in older women, even potentially in the setting of close or positive margins.

RADIATION THERAPY FOR REGIONAL CONTROL

Following a Negative Sentinel Lymph Node Biopsy Without Completion Axillary Dissection. For older women with a negative sentinel lymph node biopsy, axillary radiation is not generally considered a standard of care. However, data from 516 patients with T1 breast cancer reported in a randomized trial by Veronesi *et al.* (2003) indicate that, in the hands of experienced surgeons, the false-negative rate for sentinel lymph node biopsy is approximately 9%. Thus, nearly 1 in 10 patients who have a negative sentinel lymph node biopsy harbor occult axillary metastasis. Nevertheless, patients with a negative sentinel lymph node biopsy who do not undergo axillary lymph node dissection have not been shown to experience a significant risk of axillary failure in clinical studies. Receipt of breast radiation may contribute to the low rates of axillary failure

in this group of patients. As reported by several groups including Schlembach *et al.* (2001), 80% or more the axillary lymph node dissection clips are often covered by tangent portals used to irradiate the breast. The possible benefit of breast radiation with respect to control of occult axillary disease should be carefully considered, especially when omission of breast radiation is contemplated for older women who meet the C9343 entry criteria.

Following a Negative Axillary Lymph Node Dissection. For patients who undergo a traditional level I/II axillary lymph node dissection that reveals no axillary nodal spread, the risk of axillary failure is very low and axillary radiation, either through a separate third field or with modified tangent portals, is not indicated. For example, in the National Surgical Adjuvant Breast and Bowel Project (NSABP) trial B-04 reported by Fisher *et al.* (2002), the risk of axillary failure as the first site of recurrence was only 1% among clinically node-negative patients who underwent axillary dissection without radiation.

Following a Positive Sentinel Lymph Node Biopsy Without Axillary Lymph Node Dissection. Therapeutic options for this patient group include axillary radiation or observation. To assist with clinical decision making, a validated nomogram initially reported by Van Zee *et al.* (2003) (http://www.mskcc.org/mskcc/html/15938.cfm) can be used to estimate the risk of additional positive axillary lymph nodes. Hwang *et al.* (2007), reported that 196 patients with positive sentinel lymph nodes did not undergo completion axillary lymph node dissection. In this cohort of generally low-risk patients, the median size of the sentinel node metastasis was 1 mm and the median number of positive axillary lymph nodes was 1. As predicted by the Van Zee *et al.* (2003) nomogram, the median risk of additional positive axillary lymph nodes in the MD Anderson cohort was less than 10%. Only 58% of the patients in this cohort received radiation therapy. At a median follow up of nearly 2.5 years, no patients had experienced an axillary failure, and only one patient had experienced a supraclavicular failure. Thus, the early results of this study suggest that the risk of regional nodal failure for selected patients with a positive sentinel node who do not undergo completion axillary lymph node dissection is quite low. Although radiation therapy to the axilla is probably the most oncologically conservative option, patients with a low (< 10%) predicted risk of having additional positive axillary lymph nodes may not need axillary radiation, and further clinical data are needed to clarify this issue.

Following a Positive Sentinel Lymph Node Biopsy with Completion Axillary Lymph Node Dissection. For older women with 1–3 involved regional lymph nodes, axillary radiation is rarely indicated following complete level I/II axillary dissection. Although extracapsular extension has been suggested as a possible indication for axillary radiation following axillary dissection, recent data from 933 node-positive patients reported by Gruber *et al.* (2005) revealed that the presence of extracapsular extension was not correlated with local-regional control after adjusting for the number of positive lymph nodes. Therefore, extracapsular extension should not be considered an indication for axillary radiation among older women with early breast cancer. A possible indication for axillary radiation would be gross residual disease in the axilla, but such patients rarely have "early" breast cancer.

Following No Surgical Management of the Axilla. Therapeutic options for this patient group include axillary radiation or observation. The NSABP B-4 randomized trial, reported by Fisher *et al*. (2002), demonstrated that, for younger patients with a clinically negative axilla, the risk of axillary recurrence as the first site of failure was 19% for patients treated with axillary observation, compared to 3% for patients treated with axillary radiation. Of note, women were accrued to this trial between 1971 through 1974, in an era when the vast majority of breast cancers were detected via palpation, and none of the patients received adjuvant systemic therapy. Nevertheless, this trial served to justify the need for axillary treatment in the management of invasive breast cancer.

However, given underlying differences in the biology of early breast cancer in older women as compared to younger women, coupled with advances in adjuvant systemic therapy, the International Breast Cancer Study Group sought to determine whether axillary observation is a viable treatment option for older women with early breast cancer. This study, reported by Rudenstam *et al*. (2006), randomized 475 women age ≥ 60 years with clinical stage T1-3 N0 breast to axillary lymph node dissection or observation. All patients were treated with either total mastectomy or conservative surgery followed by adjuvant tamoxifen. Only 33% of patients received radiation to the breast, and axillary radiation was not intentionally administered. In this trial, 28% of patients undergoing axillary lymph node dissection were found to have involved axillary lymph nodes. Nevertheless, among patients who did not undergo axillary lymph node dissection, the risk of axillary recurrence as the first site of failure was only 3% with a median follow up of 6.6 years. Further, no significant differences in either disease-free or overall survival were noted in the two treatment arms. The results of this trial suggest that axillary observation is a reasonable treatment option for many older women with early breast cancer. However, given its low risk of morbidity, axillary radiation may be considered as an alternative to axillary dissection, particularly for patients at risk for axillary spread due to larger tumor size, or for patients with estrogen receptor negative tumors that will not respond to adjuvant endocrine therapy.

RADIATION THERAPY TO IMPROVE SURVIVAL

The benefit of radiation therapy with respect to local-regional control translates into a small, but measurable, survival benefit. For example, the Early Breast Cancer Trialists Collaborative Group meta-analysis reported by Clarke *et al*. (2005) concluded that, through 15 years of follow up, one life is saved for each four local-regional recurrences prevented by radiation. The following example applies this "four to one rule" to younger women with early breast cancer. If the 15-year risk of local-recurrence is 30% without radiation versus 10% with radiation, then 20 recurrences are prevented for each 100 women treated, and correspondingly five lives (= 20/4) are saved for each 100 women treated. Thus, for younger women at a relatively high risk for recurrence, radiation confers a meaningful benefit with respect to overall survival. However, for older women with early breast cancer, the long-term risk of local-regional recurrence for C9343-eligible patients is likely as low as approximately

8–10% without radiation. If the four to one rule is applied to this situation, the anticipated survival benefit from radiation is much lower, on the order of 1.5–1.9%, assuming that the competing risk of death from other causes is negligible. However, because the competing risk of non-cancer death among older women is more substantial, the small potential overall survival benefit derived from radiation therapy may not be clinically meaningful.

RADIATION TECHNIQUES

Treatment Planning. Conventional whole breast radiation for older women does not significantly differ from whole breast radiation used to treat younger women. General treatment planning issues with respect to whole breast radiation are discussed in other chapters of this text and will not be duplicated here. One potential unique aspect of treatment planning that may be particularly relevant to older women is the use of prone breast radiation. During the aging process, certain women may develop large, pendulous breasts. If treated with conventional whole breast radiation using supine techniques, such women require treatment of a large portion of the skin of the inframammary fold and upper abdominal wall. Such women frequently develop painful moist desquamation during the course of radiotherapy due to the apposition of the breast to the inframammary fold and upper abdominal wall. However, if such patients are treated in the prone position on a custom-made breast board that enables the treated breast to extend away from the chest wall, the skin of the inframammary and abdominal wall can be largely spared. For example, data from Memorial Sloan-Kettering Cancer Center, recently reported by Stegman *et al*. (2007), reported that the risk of grade 2 or higher acute dermatitis among 245 women treated with prone breast radiation was only 18%, which compares favorably to the supine position, where the risk of grade 2 or higher dermatitis as reported by Pommier *et al*. (2004) exceeds 60%.

Dose and Fractionation. There is no clear consensus regarding the optimal radiotherapy dose and fraction size for older women with early breast cancer. The largest clinical trial of radiotherapy in the published literature, reported by Bartelink *et al*. (2001), used 50 Gy in 2 Gy/fraction, followed by randomization to either no boost or an electron boost to deliver 16 Gy in 2 Gy/fraction to the lumpectomy bed. Given its size and favorable results, the doses used in this trial are thought to define a reasonable standard of care for women of all ages with breast cancer.

Recently, interest has grown in using hypofractionated radiotherapy, in which a lower total dose is given, but the size of each individual fraction is increased. This approach has particular appeal for older women who may experience greater difficulty with transportation to and from the radiotherapy facility. For example, a randomized trial reported by Whelan *et al*. (2002) randomized patients to 50 Gy in 2 Gy/fraction versus 42.5 Gy in 2.65 Gy/fraction. This trial included women with pathologic stage T1-2 N0 breast cancer treated with margin-negative lumpectomy and axillary lymph node dissection. With a median follow up of nearly 6 years, the 5-year risk of local recurrence was 3.2% in the conventional arm and 2.8% in the hypofractionated arm. Furthermore, there was a trend toward lower risk of late skin

and soft tissue toxicity in the hypofractionated arm. Building on the results of this trial, investigators in the United Kingdom began the Standardization of Radiotherapy Trials (START), initially reported in abstract form by Dewar *et al*. (2007) at the Annual Meeting of the American Society of Clinical Oncology. This trial included 4,451 women completely excised stage T1-3 N0-1 breast cancer. With a median follow up of 5 years, local control was equivalent in the conventional and hypofractionated radiation treatment arms, and furthermore cosmesis was slightly improved in the hypofractionated arms. Thus, there is growing evidence to support hypofractionated radiotherapy for the treatment of early breast cancer, although some physicians await additional follow up before abandoning conventional fractionation.

Boost Versus No Boost. A separate issue concerns the utility of a radiotherapy boost, typically 10–16 Gy in 2 Gy/fraction, in the treatment of older women with early breast cancer. In an update of the EORTC trial boost trial with 10 years follow up, Bartelink *et al*. (2007) found that the risk of local failure was 7.3% in the no boost arm versus 3.8% in the boost arm among patients age 60–70 years. The small local control benefit derived from the boost came at the expense of an increased risk of moderate to severe soft tissue fibrosis which impaired long-term cosmesis. In a separate analysis of this trial, Antonini *et al*. concluded that omission of the boost among patients age 60–70 years would result in a 0.6% increase in the total risk of local failure among the study population, while sparing a boost to approximately 1/3 of patients. Thus, there is a small benefit from boost radiation among women ages 60–70 years, at the expense of impaired cosmesis. For women over the age of 70 years, no data exist to define the benefit of a boost.

Partial Breast Radiation. Partial breast radiation offers several potential advantages in the treatment of older women with early breast cancer. First, by delivering only high doses of radiation to the tumor bed plus margin, the volume of normal tissue exposed to high doses of radiation is decreased, which should theoretically lower the risk of both acute and chronic toxicity. Second, most partial breast radiation is delivered in an accelerated fashion, such as 3.85 Gy delivered twice daily for a total of ten treatments, resulting in a dramatic reduction in the overall treatment time, thereby enhancing patient convenience and compliance, particularly for older women. Partial breast radiation can be delivered using three-dimensional conformal external beam techniques, temporary interstitial brachytherapy using a catheter-based approach, or with intracavitary brachytherapy using the Mammosite® balloon. Given the appeal of partial breast radiation, coupled with multiple single institution studies that have demonstrated a low risk of local recurrence with acceptable morbidity, the Radiation Therapy and Oncology Group (RTOG) and the NSABP launched a prospective, randomized trial (RTOG 04-13, NSABP B-39) to compare conventional whole breast radiation to accelerated partial breast radiation using either of these three techniques. As accelerated partial breast radiation represents a substantial departure from the historical standard of conventional whole breast radiation, and may carry with it an increased risk of in-breast failure or late morbidity, it is the opinion of this author that partial

breast radiation therapy should be conducted in the setting of prospective protocols until clinical trial results are mature.

RADIATION TOXICITY

Data from the C9343 randomized trial reported by Hughes *et al*. (2004) provide the most complete data on toxicity of breast radiation, specifically in older women, from both a physician and patient perspective. As compared to women who received tamoxifen alone, women who received radiation plus tamoxifen consistently rated breast pain as worse through 4 years of follow up. Women treated with radiation also rated fibrosis or retraction as worse through 2 years follow up, but these differences resolved by 4 years follow up. Physicians rated overall cosmesis, breast edema, skin color changes, and fibrosis or retraction as worse in the radiation plus tamoxifen group through 2 years follow up, but these differences resolved at 4 years follow up. Other toxicities of radiation therapy, such as rib fracture and pneumonitis, are not known to differ in older versus younger women and are discussed elsewhere in this text.

In conclusion, older women with early breast cancer generally experience favorable oncologic outcomes, and the challenge for clinicians is to develop treatment paradigms that maximize oncologic outcome and quality of life, while minimizing the number of older women who receive unnecessary treatment. Future studies will undoubtedly continue to refine our understanding of risk factors for local and regional recurrence in this group of patients, and also provide improved tools for predicting risk of non-cancer death. Such knowledge should be of assistance in determining which patients will derive a substantial benefit from radiation, and which patients can be effectively treated with adjuvant endocrine therapy alone or other non-radiation based strategies.

REFERENCES

Bartelink, H., Horiot, J.C., Poortmans, P., Struikmans, H., Van den Bogaert, W., Barillot, I., Fourquet, A., Borger, J., Jager, J., Hoogenraad, W., Collette, L., and Pierart, M. 2001. Recurrence rates after treatment of breast cancer with standard radiotherapy with or without additional radiation. *N. Engl. J. Med. 345*: 1378–1387.

Bartelink, H., Horiot, J.C., Poortmans, P.M., Struikmans, H., Van den Bogaert, W., Fourquet, A., Jager, J.J., Hoogenraad, W.J., Bing Oei, S., Warlam-Rodenhuis, C.C., Pierart, M., and Collette, L. 2007. Impact of a higher radiation dose on local control and survival in breast-conserving therapy of early breast cancer: 10-year results of the randomized boost versus no boost EORTC 22881-10882 trial. *J. Clin. Oncol.* [Jun 18 epub ahead of print].

Carlson, R.W., Anderson, B.O., Burstein, H.J., Cox, C.E., Edge, S.B., Farrar, W.B., Goldstein, L.J., Gradishar, W.J., Hayes, D.F., Hudis, C., Jahanzeb, M., Ljung, B.M., Marks, L.B., McCormick, B., Nabell, L.M., Pierce, L.J., Reed, E.C., Silver, S.M., Smith, M.L., Somlo, G., Theriault, R.L., Ward, J.H., Winer, E.P., and Wolff, A.C. 2005. Breast cancer. *J. Natl. Compr. Cancer Netw. 3*: 238–289.

Clarke, M., Collins, R., Darby, S., Davies, C., Elphinstone, P., Evans, E., Godwin, J., Gray, R., Hicks, C., James, S., MacKinnon, E., McGale, P., McHugh, T., Peto, R., Taylor, C., and Wang, Y. 2005. Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15-year survival: an overview of the randomised trials. *Lancet 366*: 2087–2106.

Daidone, M.G., Coradini, D., Martelli, G., and Veneroni, S. 2003. Primary breast cancer in elderly women: biological profile and relation with clinical outcome. *Crit. Rev. Oncol. Hematol. 45*: 313–325.

Dewar, J.A., Haviland, J.S., Agrawal, R.K., Bliss, J.M., Hopwood, P., Magee, B., Owen, J.R., Sydenham, M.A., Venables, K., and Yarnold, J.R. 2007. Hypofractionation for early breast cancer: first results of the UK standardisation of breast radiotherapy (START) trials. *J. Clin. Oncol. 25* (June 20 Suppl): LBA518.

Diab, S.G., Elledge, R.M., and Clark, G.M. 2000. Tumor characteristics and clinical outcome of elderly women with breast cancer. *J. Natl. Cancer Inst. 92*: 550–556.

Fisher, B., Jeong, J.H., Anderson, S., Bryant, J., Fisher, E.R., and Wolmark, N. 2002. Twenty-five-year follow-up of a randomized trial comparing radical mastectomy, total mastectomy, and total mastectomy followed by irradiation. *N Engl J Med. 347*: 567–575.

Freedman, G.M., Fowble, B.L., Hanlon, A.L., Myint, M.A., Hoffman, J.P., Sigurdson, E.R., Eisenberg, B.L., Goldstein, L.J., and Fein, D.A. 1998. A close or positive margin after mastectomy is not an indication for chest wall irradiation except in women aged fifty or younger. *Int J Radiat. Oncol. Biol. Phys. 41*: 599–605.

Greene, F.L., Page, D.L., Fleming, I.D., Fritz, A.G., Balch, C.M., Haller, D.G., and Morrow, M. 2002. *AJCC Cancer Staging Handbook, Sixth Edition*. New York, Springer.

Gruber, G., Bonetti, M., Nasi, M.L., Price, K.N., Castiglione-Gertsch, M., Rudenstam, C.M., Holmberg, S.B., Lindtner, J., Golouh, R., Collins, J., Crivellari, D., Carbone, A., Thurlimann, B., Simoncini, E., Fey, M.F., Gelber, R.D., Coates, A.S., and Goldhirsch, A. 2005. Prognostic value of extracapsular tumor spread for locoregional control in premenopausal patients with node-positive breast cancer treated with classical cyclophosphamide, methotrexate, and fluorouracil: long-term observations from International Breast Cancer Study Group Trial VI. *J. Clin. Oncol. 23*: 7089–7097.

Hughes, K.S., Schnaper, L.A., Berry, D., Cirrincione, C., McCormick, B., Shank, B., Wheeler, J., Champion, L.A., Smith, T.J., Smith, B.L., Shapiro, C., Muss, H.B., Winer, E., Hudis, C., Wood, W., Sugarbaker, D., Henderson, I.C., and Norton, L. 2004. Lumpectomy plus tamoxifen with or without irradiation in women 70 years of age or older with early breast cancer. *N. Engl. J. Med. 351*: 971–977.

Hwang, R.F., Gonzalez-Angulo, A.M., Yi, M., Buchholz, T.A., Meric-Bernstam, F., Kuerer, H.M., Babiera, G.V., Tereffe, W., Liu, D.D., and Hunt, K.K. 2007. Low locoregional failure rates in selected breast cancer patients with tumor-positive sentinel lymph nodes who do not undergo completion axillary dissection. *Cancer* [June 22 epub ahead of print].

Imamura, H., Haga, S., Shimizu, T., Watanabe, O., Kinoshita, J., Nagumo, H., Kajiwara, T., and Aiba, M. 2000. Relationship between the morphological and biological characteristics of intraductal components accompanying invasive ductal breast carcinoma and patient age. *Breast Cancer Res. Treat. 62*: 177–184.

Lim, M., Bellon, J.R., Gelman, R., Silver, B., Recht, A., Schnitt, S.J., and Harris, J.R. 2006. A prospective study of conservative surgery without radiation therapy in select patients with Stage I breast cancer. *Int. J. Radiat. Oncol. Biol. Phys. 65*: 1149–1154.

Molino, A., Giovannini, M., Auriemma, A., Fiorio, E., Mercanti, A., Mandara, M., Caldara, A., Micciolo, R., Pavarana, M., and Cetto, G.L. 2006. Pathological, biological and clinical characteristics, and surgical management, of elderly women with breast cancer. *Crit. Rev. Oncol. Hematol. 59*: 226–233.

Pommier, P., Gomez, F., Sunyach, M.P., D'Hombres, A., Carrie, C. and Montbarbon, X. 2004. Phase III randomized trial of Calendula officinalis compared with trolamine for the prevention of acute dermatitis during irradiation for breast cancer. *J. Clin. Oncol. 22*: 1447–1453.

Rudenstam, C.M., Zahrieh, D., Forbes, J.F., Crivellari, D., Holmberg, S.B., Rey, P., Dent, D., Campbell, I., Bernhard, J., Price, K.N., Castiglione-Gertsch, M., Goldhirsch, A., Gelber, R.D., and Coates, A.S. 2006. Randomized trial comparing axillary clearance versus no axillary clearance in older patients with breast cancer: first results of International Breast Cancer Study Group Trial 10–93. *J. Clin. Oncol. 24*: 337–344.

Schlembach, P.J., Buchholz, T.A., Ross, M.I., Kirsner, S.M., Salas, G.J., Strom, E.A., McNeese, M.D., Perkins, G.H., and Hunt, K.K. 2001. Relationship of sentinel and axillary level I-II lymph nodes to tangential fields used in breast irradiation. *Int. J. Radiat. Oncol. Biol. Phys. 51*: 671–678.

Smith, B.D., Gross, C.P., Smith, G.L., Galusha, D.H., Bekelman, J.E., and Haffty, B.G. 2006. Effectiveness of radiation therapy for older women with early breast cancer. *J. Natl. Cancer Inst. 98*: 681–690.

Stegman, L.D., Beal, K.P., Hunt, M.A., Fornier, M.N., and McCormick, B. 2007. Long-term clinical outcomes of whole-breast irradiation delivered in the prone position. *Int. J. Radiat. Oncol. Biol. Phys. 68*: 73–81.

Truong, P.T., Olivotto, I.A., Speers, C.H., Wai, E.S., Berthelet, E., and Kader, H.A. 2004. A positive margin is not always an indication for radiotherapy after mastectomy in early breast cancer. *Int. J. Radiat. Oncol. Biol. Phys. 58*: 797–804.

Van Zee, K.J., Manasseh, D.M., Bevilacqua, J.L., Boolbol, S.K., Fey, J.V., Tan, L.K., Borgen, P.I., Cody, H.S., III., and Kattan, M.W. 2003. A nomogram for predicting the likelihood of additional nodal metastases in breast cancer patients with a positive sentinel node biopsy. *Ann. Surg. Oncol. 10*: 1140–1151.

Veronesi, U., Paganelli, G., Viale, G., Luini, A., Zurrida, S., Galimberti, V., Intra, M., Veronesi, P., Robertson, C., Maisonneuve, P., Renne, G., De Cicco, C., De Lucia, F., and Gennari, R. 2003. A randomized comparison of sentinel-node biopsy with routine axillary dissection in breast cancer. *N. Engl. J. Med. 349*: 546–553.

Vinh-Hung, V., and Verschraegen, C. 2004. Breast-conserving surgery with or without radiotherapy: pooled-analysis for risks of ipsilateral breast tumor recurrence and mortality. *J. Natl. Cancer Inst. 96*: 115–121.

Whelan, T., MacKenzie, R., Julian, J., Levine, M., Shelley, W., Grimard, L., Lada, B., Lukka, H., Perera, F., Fyles, A., Laukkanen, E., Gulavita, S., Benk, V., and Szechtman, B. 2002. Randomized trial of breast irradiation schedules after lumpectomy for women with lymph node-negative breast cancer. *J. Natl. Cancer Inst. 94*: 1143–1150.

35
Acute Side Effects of Radiotherapy in Breast Cancer Patients: Role of DNA-Repair and Cell Cycle Control Genes

Xiang-Lin Tan, Odilia Popanda, and Jenny Chang-Claude

INTRODUCTION

It is a basic clinical observation that large variability exists regarding the incidence and severity of normal tissue reactions during radiotherapy (RT) even within a group of identically treated patients. Severity of damage to normal tissue after therapeutic RT is predominantly influenced by factors related to radiation exposure, which, however, is not sufficient to explain fully the patient-to-patient variability (Perez and Brady, 1992). There is considerable evidence that both patient and treatment related factors as well as intrinsic factors of individual radiosensitivity could influence the variability of side effects observed (Bentzen and Overgaard, 1994; Rogers *et al*., 2000). Genetic factors, however, might play a central role in the patient-related variability in response to RT (Andreassen *et al*., 2002). Genetic variability is largely determined by single nucleotide polymorphisms (SNPs) which cover the entire genome and can easily be determined by high throughput methods. Some SNPs can modify the amino acid structure and thus, the function of the encoded protein. Establishing a profile of specific marker SNPs related to development of RT-related side effects for a patient should be useful to predict this patient's risk to suffer from side effects.

Selection of Possible Marker Genes and SNPs

The most important cellular target molecule of ionizing radiation (IR) is thought to be DNA where damage can be induced by direct ionization of DNA or indirectly *via* the generation of free radicals. These various types of DNA damage can be repaired by the cellular repair machinery and can induce cell cycle arrest or delay in G1, S, and G2 as well as apoptosis (Maity *et al*., 1994). The mechanisms that allow cells to detect DNA damage and to respond by cell cycle arrest in a compartment in which the damage can be repaired are called DNA-damage cell cycle 'checkpoints' (reviewed by Paulovich *et al*., 1997).

Thus, the avalanche of knowledge in cellular biochemistry and molecular biology has had a great impact on the current perspective on studies of DNA damage and cell cycle response after IR and its application in radiation oncology.

In our studies summarized here (Chang-Claude *et al*., 2005; Tan *et al*., 2006; Popanda *et al*., 2006), we analyzed the effects of genetic variation on the development of acute side effects of irradiation. Because of their central function in the damage response, we decided to concentrate on genes involved in DNA repair and cell cycle control. In addition, the choice of a SNP in our study was limited mainly by two criteria. There had to be published evidence that the SNP can modify protein function and the frequency of the variant allele had to be higher than 5% to allow sufficient statistical power in our study.

DNA Repair Genes and Clinical Radiation Reaction

Radiation-Induced DNA Damage and DNA Repair

Upon irradiation several types of DNA damages have been identified occurring at different rates (Dahm-Daphi *et al*., 1998). Thus, the damaged DNA requires the concerted action of a number of DNA-repair enzymes to restore genomic integrity. The individual DNA repair capacity consists of several pathways: base-excision repair (BER), nucleotide-excision repair (NER), homologous recombination, end joining, mismatch repair and telomere metabolism (Hoeijmakers, 2001). BER is the main system for repairing single-stranded lesions and removing small base damages, including oxidative DNA lesions. NER is used to excise so-called bulky DNA adducts as part of the oligonucleotide. However, other forms of DNA damage, such as IR-induced oxidative damage, are also repaired by means of NER. For DNA double-strand break (DSB) repair, there are two distinct and complementary mechanisms: homologous recombination and non-homologous end-joining (NHEJ), both of which have been the subjects of recent reviews (Karran, 2000). DSBs are considered to be particularly biologically important because their repair is intrinsically more difficult than that of other types of DNA damage. BER of single strand breaks as well as NHEJ and homologous recombination of DSBs are considered to be the most important pathways involved in the repair of radiation-induced DNA damage.

DNA Repair Genes XRCC1 and APE1 and Their Genetic Variants

X-ray repair cross-complementing group 1 (*XRCC1*) and Apurinic/ apyrimidinic endonuclease factor 1 (*APE1*) genes are important players in BER. *XRCC1* gene shows three relatively common polymorphisms in codon 194 (Arg/Trp), 280 (Arg/His) and 399 (Arg/Gln) affecting the amino acid sequence (Shen *et al*., 1998) (Table 35.1). The $Arg^{399}Gln$ polymorphism has been extensively investigated, and the presence of the variant allele has been shown to be associated with measurable reduced DNA repair capacity as assessed by the persistence of DNA aflatoxin B_1 adducts (Lunn *et al*., 1999) and DNA damage measured by ^{32}P post-labeling of bulky DNA adducts (Duell *et al*., 2000), and elevated levels of sister chromatid exchanges (Abdel-Rahman and EL Zein, 2000). Several epidemiological studies have assessed the association

of the Arg[399]Gln and Arg[194]Trp polymorphism with cancer incidence (Hung *et al*., 2005), with both increases and decreases in cancer frequencies being found depending on the tumor type and levels of environmental exposures. The polymorphism in codon 280, however, has been rarely studied. The most common substitution of *APE1* is Asp[148]Gln in exon 5 (Table 35.1). Although it does not result in reduced endonuclease activity, the number of variant alleles in *APE1* Asp[148]Glu and *XRCC1* Arg[399]Gln was significantly associated with prolonged cell-cycle delay in G2 phase after irradiation (Hu *et al*., 2001).

DNA Repair Gene XPD and its Genetic Variants

The xeroderma pigmentosum complementation group D (*XPD*) gene is an essential member of the NER pathway. Elevated chromatid aberration frequency found in lymphocytes containing a mutated XP gene after exposure to IR suggests a role for NER proteins in the repair of IR-induced damage (Parshad *et al*., 1993). There are two common genetic polymorphisms causing amino acid changes in codon 312 (Asp/Asn) and in codon 751 (Lys/Gln) (Shen *et al*., 1998) (Table 35.1), and there is evidence that subjects homozygous for the variant genotypes of

Table 35.1. Polymorphisms studied and primers and probes used for genotyping.

Gene (EMBL Acc. No.)	Polymorphisms (dbSNP ID)	Sequence of primers or probes (5′–3′)[a]
XRCC1(L34079)	C26304T/Arg[194]Trp (rs1799782)	F: TACTGACCTTGCGGGACCTTA R: TACCCTCAGACCCACGAGTCTA S: TGTCTTGTTGATCCAGCTGAAGAAG-FITC A: LC Red640-AGCCCCCGGCCTCAGAGAGTTGGp
	G27466A/Arg[280]Trp (rs25489)	F: GCTGGGGCCTGGATTG R: GCACCACTACCACACCCTGAA S: TGCCAGCTCCAACTCATACCCC- FITC A: LC Red640-CCACAGCCCCAGTCCCTGCCCGp
	G28152A/Arg[399]Gln (rs25487)	F: CCCCAAGTACAGCCAGGTC R: TGTCCCGCTCCTCTCAGTAG S: CCCTCCCAGAGGTAAGGCC-FITC A: LC Red640-CACACGCCAACCCTGCTCCTTATp
APE1 (M92444)	T2197G/Asp[148]Gln (rs3136820)	F: CTTGATTGCTTTCCCTTTTCTTA R: CGCTGCCGGTACTCCA S: LC Red640-TGCTCCTCCTCGCCTATAGAAATGAp A: CACAATCACCCGGCCTTCCTGATC-FITC
XPD (L47234)	G23591A/Asp[312]Asn (rs1799793)	F: GACAGACGAGCAGCGCC R: AAGCCCAGGAAATGCTCG S: CCCGTGCTGCCCAACGAA-FITC A: LC Red640-TGCTGCAGGGTGAGCCCCGACCp
	A35931C/Lys[751]Gln (rs13181)	F: GCTCAGCCTGGAGCAGCTA R: GTGAGAAATGTCACCTGACTTCAT S: LC Red640-CAATCTGCTCTATCCTCTGCAGCGTCp A: CCCACCCGCCCCACTCAGAGCTGCT-FITC
XRCC2 (AC003109)	G31579A/Arg[188]His (rs3218536)	F: TTGATATGCTCCGGCTAGTTA R: CTGCCATGCCTTACAGAGATAA S: CTTGTAAATGACTATCACCTGGTTCTT-FITC A: LC Red640-TGCAACGACACAAACTATAATGCAGAA AGCp
XRCC3 (AF037222)	C18067T/Thr[241]Met (rs861539)	F: TGAATAAGAAGGTCCCCGTACT R: CTTGGTGCTCACCTGGTTGAT

(continued)

TABLE 35.1. (continued)

Gene (EMBL Acc. No.)	Polymorphisms (dbSNP ID)	Sequence of primers or probes (5′–3′)[a]
NBS1 (AB013139)	G11122C/Glu[185]Gln (rs1805794)	F: TTTTATGGATGTAAACAGCCTCT
		R: AAACCTTCCATTAATAATACCGAA-3′
		A: [LC Red640-]AGAAGCAGCCTCCACAAATTGAAAGGTp
		S: TGAATTCCTGAAAGCAGTTCAGTCC[-FITC]
TP53 (U94788)	C429G/Arg[72]Pro (rs1042522)	F: GATGCTGTCCCCGGACGA-3′
		R: AGGGGCCGCCGGTGTAG-3′
		S: [LC Red640-]TCCCCCCGTiiCCCCTGCACCp
		A: CCAGATGAAGCTCCCAGAATGCCAGAGGCT[FITC]
	*p53*PIN3[b]	F: CTGAAAACAACGTTCTGGTA
		R: AAGGGGGACTGTAGATGGGTG
p21 (AF497972)	C6829A/Ser[31]Arg (rs1801270)	F: GGCGCCATGTCAGAACCGGC
		R: CCAGACAGGTCAGCCCTTGG
		S: CATCACAGTCGCGTCTCAGC[-FITC]
		A: [LC Red640-]GCTCGCTGTCCACTGGGCCGAAp

[a]Mutation site is shaded; F, forward primer; R, reverse primer; S, sensor; A, anchor.
[b]*p53*PIN3, a single tandem repeat of 16 nucleotides in intron 3 of the *TP53* gene.

XPD have suboptimal DNA repair capacity for benzo[a]pyrene adducts and ultraviolet DNA damage (Spitz *et al*., 2001).

DNA Repair Genes XRCC2, XRCC3 and NBS1, and Their Genetic Variants

Several syndromes associated with increased radiation sensitivity are caused by deficiencies in genes of DSBs repair, leading to the hypothesis that the individual repair capacity for these lesions should be an important determinant of the devotement of clinical radiation reaction. *XRCC2*, *XRCC3*, and Nijmegen breakage syndrome group 1 (NBS1) genes are involved in DSBs repair pathway. A common polymorphism at codon 241 in exon 7 of the *XRCC3* gene that results in a Thr to Met amino acid substitution has been described, and a relatively rare polymorphism in the *XRCC2* gene was identified, which results in an Arg[188]His amino acid substitute (Table 35.1). *XRCC2* [188]His variant allele was shown to induce a subtle effect of the cell's ability to survive after treatment by cross-linking reagent (Rafii *et al*., 2002). One previous study suggested that *XRCC3* [241]Thr allele increased the risk of subcutaneous fibrosis as well as telangiectasia after RT (Andreassen *et al*., 2003). However there was no information regarding *XRCC2* Arg[188]His and *NBS1* Glu[185]Gln polymorphisms and the development of clinical radiation reactions.

Cell Cycle Control Genes *TP53* and *p21,* and Clinical Radiation Reaction

A common cellular response to DNA-damaging agents is the activation of cell cycle checkpoints. The DNA damage induced by IR initiates signals that can ultimately activate either temporary checkpoints to permit time for genetic repair or irreversible growth arrest that results in cell death (necrosis or apoptosis). One of the key proteins in cell cycle control is derived from the tumor suppressor gene *TP53* that coordinates DNA repair with cell cycle progression and apoptosis. In response to IR, p53 protein is activated after phosphorylation by a variety of DNA damage-induced

kinases, including Ataxia telangiectasia-mutated and the DNA-dependent protein kinases (Banin *et al*., 1998). Activated p53 protein has various downstream targets including genes involved in cell-cycle regulation, apoptosis, and DNA repair. p21, as a universal inhibitor of cyclin-dependent kinases (CDKs), is a critical cell-cycle checkpoint gene, regulated tightly by p53. As soon as DNA is damaged by radiation, binding of p53 protein induces transcription of downstream gene p21, which stops cells from entering into the S-phase (el Deiry *et al*., 1994). p21, together with p53, is directly involved in G1/S checkpoint control in response to IR.

Many polymorphisms have been identified in *TP53* gene, the most studied being *TP53* Arg to Pro exchange at codon 72 (*TP53* Arg^{72}Pro) and *p53*PIN3. *TP53* Pro^{72}Arg polymorphism is located in a proline-rich region of the gene homologous to a SH3 binding domain that is required for the growth suppression activity of p53. *p53*PIN3 polymorphism consists of a single repeat of 16 nucleotides in intron 3 of the *TP53* gene. At least four polymorphisms have been described for *p21*, the most studied being *p21* Ser > Arg at codon 31 (*p21* Ser^{31}Arg), which is located in a highly conserved region of this gene (Table 35.1). Most studies have focused on the association between these three polymorphisms and cancer susceptibility; however, the findings have remained inconclusive. In addition, the *TP53* ^{72}Arg allele has been reported to be associated with a much stronger capacity to induce apoptosis than the ^{72}Pro variant (Pim and Banks, 2004). There was a strong hypothesis to analyze the association between these three polymorphisms and the development of normal tissue reactions to RT.

With this investigation, we used a prospective epidemiologic study among breast cancer patients receiving RT after breast-conserving surgery to address the role of the polymorphisms in DNA repair and cell cycle control genes in predicting the development of acute side effects during or shortly after RT. Our results are likely to make an important contribution to the future of individualized treatment plans for women with breast cancer.

MATERIALS AND METHODS

Study Subjects and Data Collection

An appropriate study design and good epidemiological practice are crucial for drawing valid conclusions from study data (Twardella and Chang-Claude, 2002). Prospective studies are less prone to misclassification of clinical radiosensitivity and selection bias because clinical/epidemiologic data can be collected in a standardized manner and biologic material (blood or biopsies) can be collected prior to radiotherapy. We, therefore, conducted a prospective study of unselected breast cancer patients who were treated homogeneously (Twardella *et al*., 2003). Briefly, we recruited female breast cancer patients receiving primary RT of the breast after breast conserving surgery, who were Caucasians, regardless of age, at RT units of four hospitals (Women'' Clinic in Heidelberg, St. Vincentius Clinic in Karlsruhe, City Hospital of Karlsruhe, and University Hospital of Mannheim) from June 1998 to February 2001. Women were excluded if they were currently or ever treated with chemotherapy to avoid confounding of side effect data. A total

of 478 patients fulfilling study criteria participated with complete clinical and epidemiologic information. Blood samples collected before starting RT were available for 446 patients (average age, 60.3 ± 9.0 years). They were included in this analysis. The study was approved by the Ethical Committee of the University of Heidelberg and informed consent was obtained from all patients enrolled.

Participants completed a self-administered questionnaire at the first visit before starting RT, which elicited information on demographic factors, medical history, family history of cancer, and lifestyle. They were requested to return the questionnaire at one of the following visits during RT. Assessment and documentation of side effects was performed by mainly one physician at the Women's Clinic in Heidelberg and by different physicians at the other clinics using the standardized side effects documentation sheet of the study. Clinical data on tumor characteristics and therapy regime were abstracted from patient records.

Clinical radiation reactions developing in the skin within the radiation field of the breast were used to measure clinical radiosensitivity. Toxicity observed in portals of lymph node radiation was excluded. Side effects were documented four times during the study: (1) before the beginning of RT, and at a cumulative dose of (2) 36–42 Gy, (3) 44–50 Gy, and (4) about 60–66 Gy (end of RT). The severity of acute side effects was assessed using a modified classification system based on the common toxicity criteria v2.0 (CTC) of the NIH, USA (Reference: http://ctep.cancer.gov/reporting/ctc.html). For more detailed categorization of skin reactions, grade 2a was defined as tender/bright erythema or moderate edema, grade 2b as severe erythema and grade 2c as at least one moist desquamation or interruption of RT due to toxicity (Tan *et al*., 2006). Acute side effects of grade 2c or above were considered to indicate the occurrence of acute skin toxicity after RT in our study (Twardella *et al*., 2003). Of 446 patients, 77 presented with acute toxicity of grade > 2c.

All the patients were administered a common breast-radiation treatment including CT-based planning, simulation, verification and quality assurance, and received conformal tangential irradiation with lateral and medial wedge fields. The standard RT regime included irradiation of the whole breast followed by a boost therapy at three hospitals, either 50 Gy given in 5 × 2.0 Gy fractions per week or 50.4 Gy in 5 × 1.8 Gy fractions per week. At the fourth radiology department, 56 Gy of whole breast irradiation were applied in 5 × 2.0 Gy fractions per week without boost therapy. To account for differences in fractionation and overall treatment time, the biologically effective radiation dose (BED) was calculated using the formula:

$$BED = nd\left(1+\frac{d}{\alpha/\beta}\right)-\frac{\gamma}{\alpha}\left(T-T_0\right),$$

given the number of fractions n, the fraction size of d, an α/β-ratio of 10 Gy for acute skin reactions, a time factor γ/α of 0.7 Gy per day, the overall treatment time of T and a starting time for compensatory proliferation T_0 of 21 days (Twardella *et al*., 2003). The average BED by censoring was 54.0 ± 4.8 Gy with range 35.5–64.5 Gy.

Genotyping Methods

DNA-Isolation and Storage

Blood samples (15 ml) were collected using standard venipuncture techniques before starting RT. Genomic DNA was purified from lymphocytes extracted from whole blood using QIAamp DNA Blood Midi Kit according to the manufacturer's instructions. The DNA concentration is adjusted to 10 ng DNA/µl by TE buffer for genotyping. All DNA preparations were stored at 4°C until used.

Genotyping

XRCC3 Thr^{241}Met polymorphism was analyzed by PCR-RFLP as previously described (Popanda *et al*., 2004). The digestion patterns observed after digestion with the restriction enzyme *Nla*III were as follows: 37, 105 and 63 bp for homozygote CC (Thr/Thr), 37 and 168 bp for homozygote TT (Met/Met) and 37, 63, 105 and 168 bp for heterozygote CT (Thr/ Met). A 16 bp duplication polymorphism in *TP53* (*p53*PIN3 A1/A2) was detected using a standard PCR protocol. The amplification of the 16 bp duplication polymorphism resulted in a 119 bp or 135 bp fragment (Wang-Gohrke *et al*., 1998). The PCR products observed were as follows: 119 bp for homozygous wild-type (A1/A1), 135 bp for homozygous variant (A2/A2), and 119 and 135 bp for heterozygote (A1/A2), where A2 allele carries a 16 bp duplication.

Detection of *XRCC1* Arg1^{194}Trp, Arg^{280}His and Arg^{399}Gln, *APE1* Asp^{148}Gln, *XPD* Asp^{312}Asn and Lys^{751}Gln, *XRCC2* Arg^{188}His, *NBS1* Glu^{185}Gln, *TP53* Arg^{72}Pro and *p21* Ser^{31}Arg polymorphisms was performed by rapid capillary PCR followed by melting curve analysis using fluorescence labeled hybridization probes in a LightCycler (Roche Diagnostics, Mannheim, Germany). The melting point analysis uses fluorescence resonance energy transfer for detecting a polymorphic site. Therefore, the 3′-end of the sensor probe was labeled with FITC and the 5′-end of the anchor probe (which was placed 1 nucleotide downstream of the sensor probe) was labeled with LC Red 640 and the 3′-end was phosphorylated. The sensor was designed for a perfect match either to the wild-type or the variant allele sequence. Thus, in the allele with the sequence deviating from the sensor, a 1-nucleotide mismatch between sensor and target DNA sequence was formed and caused destabilization of the hybrid yielding to a melting point (T_m) shift of 5–10°C. All PCR primers and probes for melting point analysis are given in Table 35.1. Probes were designed and prepared by Tib Molbiol (Berlin, Germany). Details of genotyping method have been described in our recent reports (Chang-Claude *et al*., 2005; Popanda *et al*., 2006; Tan *et al*., 2006).

Quality Control of Genotyping

For all polymorphisms, a negative control containing all reagents but water instead of the DNA template was included to each amplification set. The melting curves were evaluated by two independent observers who were blinded to the analysis of the clinical data. In addition, 10% of randomly selected samples were repeated independently to verify genotyping results and 100% concordance was found. For each polymorphism, PCR fragments of the homozygous common allele, the homozygous variant

allele, and the heterozygous samples were sequenced to confirm genotypes using ABI PRISM® 377 DNA Sequencer automatically analyzes.

Statistical Methods

Deviation from Hardy-Weinberg equilibrium for each polymorphism was tested by comparing the observed and expected genotype frequencies using the Chi-squared test with 1df (degree of freedom). The effect of the genetic variants on risk of developing acute skin toxicity of RT was evaluated by Cox proportional hazards model using procedure PHREG of SAS®, Release 8.2 (SAS Institute Inc Cary NC USA). The acute skin toxicity was defined as the occurrence of at least one moist desquamation or interruption of RT due to toxicity (CTC score 2c or above), and patients who developed acute skin toxicity were considered as 'sensitive patients' in data analysis. The association between genetic polymorphisms and the development of acute skin toxicity was assessed using hazard ratios (HRs) and 95% confidence intervals (CIs). In our population, the risk of acute skin toxicity increased with BED received (Twardella *et al.*, 2003); therefore, we modeled the occurrence of acute skin toxicity after RT in relation to the BED. In this way, we adjusted for differences in radiation dose when skin toxicity of grade 2c or more was recorded and for the total dose received. In the model we included the variables, hospital, photon beam energy for whole breast irradiation and boost irradiation in the model to adjust for differences by treating hospital.

Genotypes were analyzed as categorical variables, differentiating between homozygous wild-type, heterozygous, and homozygous variant genotypes; and the homozygous wild-type as reference group. Because of the small percentage of individuals among our subjects with homozygous variants (< 10%) for some polymorphisms (e.g., *XRCC1* $Arg^{194}Trp$), the heterozygous and homozygous variants were grouped together and compared with the homozygous wild-type. The genotype therefore was modeled as dichotomous variable (carrier *versus* non-carrier of variant alleles). Higher body mass index (BMI) was significantly associated with an increased risk for acute skin toxicity (HR = 1.08 per $1\,kg/m^2$; 95% CI, 1.04–1.12; $P < 0.001$), which was in accordance with our previous results (Twardella *et al.*, 2003). BMI (as a continuous variable) was therefore included as a possible confounder in all models. Effect modification by BMI was investigated by separate analysis for the two groups subdivided at the median BMI of 24.9, i.e., normal weight ($BMI \leq 25.0$) and overweight/obese ($BMI > 25.0$). Interaction between BMI group and the genotypes were measured by using multiplicative terms in the model including the main effect and evaluated by the likelihood ratio tests.

Linkage disequilibrium between different markers in *XRCC1*, *XPD*, and *TP53* was estimated using the 'Estimating Haplotypes' program (Terwilliger and Ott, 1994). Haplotypes for *XRCC1*, *XPD* and *P53* were reconstructed using the PHASE V2.0 online software (Stephens and Donnelly, 2003). The haplotype-specific risks to the development of acute skin toxicity after RT were investigated by Cox proportional hazards model using the most probable haplotype pairs yielded by the PHASE software. The potential combined effects of genotypes to the risk of developing acute skin toxicity after RT were explored in several ways (see Results).

RESULTS

Overall, the variant allele frequencies were 0.37 for *XRCC1* ^{399}Gln, 0.06 for *XRCC1* ^{280}His and ^{194}Trp, 0.48 for *APE1* ^{148}Glu, 0.37 for *XPD* ^{312}Asn, 0.38 for *XPD* ^{751}Gln, 0.41 for *XRCC3* ^{241}Met, 0.07 for *XRCC2* ^{188}His and NBS1 ^{185}Gln, 0.24 for TP53 ^{72}Pro and 0.14 for *p53*PIN3 A2 in our population. All the genotype distributions were in Hardy-Weinberg equilibrium.

Genotype-Specific Risks to Acute Skin Toxicity of Radiotherapy

We found no significant association between the genetic variants of *XRCC1*, *APE1*, and *XPD* Lys^{751}Gln and the development of acute skin toxicity after RT although the HRs were generally below one (Figure 35.1A). Compared to non-carriers, carriers of the *XPD* ^{312}Asn had a non-significantly decreased risk of developing acute skin toxicity (HR 0.42; 95% CI, 0.17–1.05; P = 0.06). The HRs of individuals carrying heterozygous or homozygous variants for *XRCC2* Arg^{188}His and *NBS1* Glu185Gln were decreased, and increased for *XRCC3* Thr^{241}Met, compared to carriers of homozygous wild type. But they were not found to be statistically significant (Figure 35.1A). No significant association was found between *TP53* and *p21* polymorphisms and the development of acute skin toxicity after RT, although the HRs of acute skin toxicity associated with heterozygous or homozygous variants for both *TP53* Arg^{72}Pro and *p53*PIN3 were generally decreased, compared to homozygous wild types (Figure 35.1A).

Further analysis stratified for patients with normal weight and with overweight yielded differences (Figure 35.1B and C). Compared to non-carriers, carriers of the *XRCC1* ^{399}Gln had a decreased risk of developing acute skin toxicity in normal weight patients (HR 0.51; 95% CI, 0.22–1.19), but not in overweight patients (HR 1.28; 95% CI, 0.72–2.27) ($P_{\text{interaction}} = 0.08$). There was a similar differential risk for carriers of *APE1* ^{148}Glu, with a HR of 0.49 (95% CI, 0.21–1.15) for normal weight patients and 1.05 (95% CI, 0.55–2.00) for overweight patients ($P_{\text{interaction}} = 0.14$). Compared to non-carriers, carriers of the *XPD* ^{751}Gln had a non-significantly decreased risk of developing acute skin toxicity in normal weight patients (HR 0.48; 95% CI, 0.20–1.14; $P_{\text{interaction}} = 0.09$). No statistically significant association was found between the development of acute skin toxicity and either *XRCC1* Arg^{194}Trp, *XRCC1* Arg^{280}His *XRCC2* Arg^{188}His, *NBS1* Glu^{185}Gln or *XRCC3* Thr^{241}Met polymorphism ($P_{\text{interaction}} > 0.10$) in normal or overweight patients. *TP53* ^{72}Pro carriers had a decreased risk of developing acute skin toxicity after RT in normal weight patients (HR 0.46; 95% CI, 0.18–1.18) but not in overweight patients (HR 1.07; 95% CI, 0.61–1.89) ($P_{\text{interaction}} = 0.14$), compared to non-carriers. No significant association between *p21* Ser^{31}Arg or *p53*PIN3 polymorphisms and the risk of developing acute skin toxicity was found either in normal weight or overweight patients. Compared to non-carriers, the HR of *p21* ^{31}Arg carrier was increased in normal weight patients and decreased in overweight patients, the two-way interaction between *p21* Ser^{31}Arg and BMI was however not statistically significant ($P_{\text{interaction}} = 0.32$).

Haplotype-Specific Risks to Acute Skin Toxicity of Radiotherapy

A strong association (linkage disequilibrium) was found among the three *XRCC1* polymorphisms, the two *XPD* polymorphisms

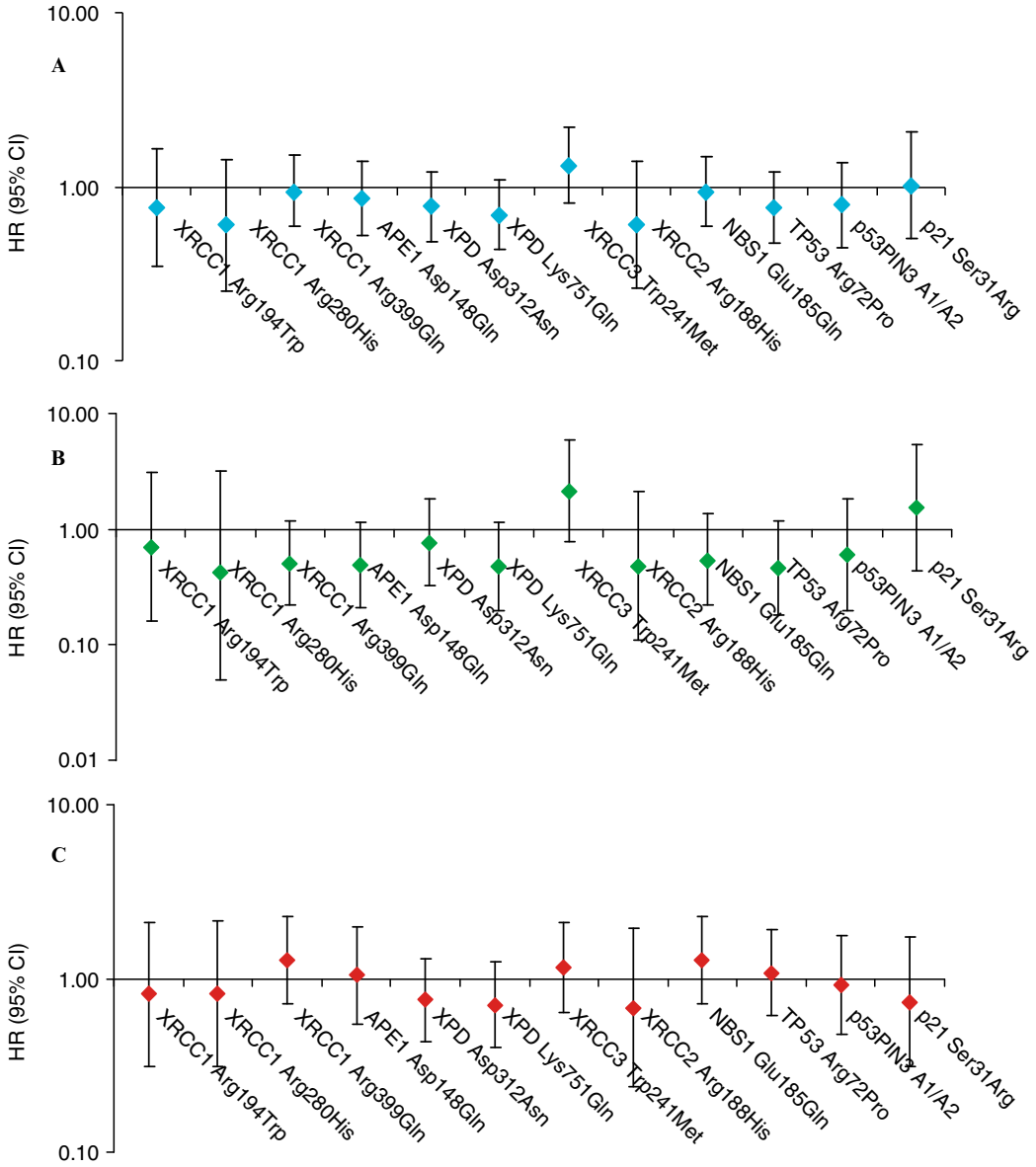


FIGURE 35.1. Association between polymorphisms and the risk of acute toxicity (A) in all participants, (B) in normal weight patients, and (C) in overweight patients. Hazard ratios (HR) and 95% confidence intervals are shown for the comparison of carriers of the variant allele compared to individuals homozygous for the wild type allele

and the two polymorphisms in *TP53* ($P < 0.001$). A total of 5 *XRCC1* haplotypes were found, but the haplotype CAA (^{194}Arg^{280}His^{399}Gln) occurred in only one patient (Table 35.2). The rare variants, *XRCC1* ^{194}Trp, *XRCC1* ^{280}His, were not found to occur on the same haplotype. The results showed that the haplotype effects reflect that of the ^{399}Gln allele. Thus, compared to the common wild type CGG (^{194}Arg^{280}Arg^{399}Arg) haplotype, the CGA (^{194}Arg^{280}Arg^{399}Gln) haplotype solely

yielded a borderline significant association with the risk of acute skin toxicity after RT in normal weight women (HR 0.55; 95% CI, 0.29–1.05) but not in overweight women (HR 1.14; 95% CI, 0.76–1.72) ($P_{interaction}$ = 0.12). None of the four *XPD* haplotypes showed a clear association with the development of acute skin toxicity of RT (Table 35.2). Four haplotypes for the two *TP53* polymorphisms were reconstructed (Table 35.2). Compared to the common CA1 (^{72}ArgA1) haplotype, both GA1 (^{72}ProA1) and GA2 (^{72}ProA2) haplotypes had a decreased risk of developing acute skin toxicity after RT in normal weight patients. HR of GA1 (^{72}ProA1) was 0.48 (95% CI, 0.17–1.37) in normal weight patients and 1.53 (95% CI, 0.89–2.63) in overweight patients ($P_{interaction}$ = 0.06). HR of GA2 (^{72}ProA2) was 0.48 (95% CI, 0.15–1.57) in normal weight women and 0.92 (95% CI, 0.48–1.74) in overweight women ($P_{interaction}$ = 0.39). This result showed that the haplotype effects reflect that of the ^{72}Pro allele.

Combined Effects of Genotypes on the Risk of Acute Skin Toxicity After Radiotherapy

Combined Effects of XRCC1 Arg^{399} Gln and APE1 Asp^{148}Gln

Both *XRCC1* and *APE1* are involved in the BER pathway. The results of *XRCC1* haplotype analysis showed that the haplotype effects reflected that of the ^{399}Gln allele. We therefore explored the effects of combined genotypes of *XRCC1* ^{399}Gln and *APE1* ^{148}Glu (Table 35.3). Compared to homozygous carriers of the wild-type allele in both genes, carrying either the *XRCC1* ^{399}Gln or the *APE1* ^{148}Glu allele was associated

Table 35.2. Association of haplotypes of *XRCC1, XPD* and *TP53* with the risk of developing acute skin toxicity after radiotherapy in breast cancer patients stratified by BMI.

		Normal weight (BMI ≤ 25)			Overweight (BMI > 25)		
Haplotype	Number	Patients	Sensitive patients	Adjusted HR (95% CI)[a]	Patients	Sensitive patients	Adjusted HR (95% CI)[a]
XRCC1							
CGG	466	235	29	1.00	231	55	1.00
CGA	325	173	14	0.55 (0.29–1.05)	152	42	1.14 (0.76–1.72)
CAG	51	24	1	0.34 (0.05–2.54)	27	6	1.01 (0.42–2.41)
CAA	1	0	0	NC[b]	1	0	NC
TGG	49	26	2	0.55 (0.13–2.37)	23	6	0.85 (0.33–2.17)
XPD							
GA	501	265	33	1.00	236	62	1.00
GC	66	31	1	0.36 (0.05–2.68)	35	10	0.93 (0.47–1.84)
AA	52	30	3	0.83 (0.25–2.75)	22	2	0.28 (0.07–1.17)
AC	273	132	9	0.55 (0.26–1.16)	141	34	0.85 (0.55–1.31)
TP53							
CA1	661	336	38	1.00	325	76	1.00
CA2	17	10	1	1.01 (0.14–7.47)	7	3	2.21 (0.68–7.19)
GA1	104	56	4	0.48 (0.17–1.37)[c]	48	18	1.53 (0.89–2.63)
GA2	110	56	3	0.48 (0.15–1.57)	54	11	0.92 (0.48–1.74)

[a] Adjusted for BMI, hospital (four clinics), photon beam quality for whole breast (three categories) and for boost irradiation (no boost and five categories).

[b] NC, not calculated.

[c] P = 0.06 for interaction with BMI.

TABLE 35.3. The combined effects of polymorphisms in base excision repair genes (*XRCC1* and *APE1*), DNA double strand break repair (*NBS1* and *XRCC3*), and cell cycle control genes (*TP53* and *p21*) on the risk of developing acute skin toxicity after radiotherapy.

Combined genotypes		Normal weight (BMI ≤ 25)			Overweight (BMI > 25)		
		No. of patients	Sensitive patients	Adjusted HR (95% CI)[a]	No. of patients	Sensitive patients	Adjusted HR (95% CI)[a]
XRCC1	*APE1*						
Arg399Gln	Asp148Gln						
Arg/Arg	Asp/Asp	20	6	1.00	32	6	1.00
Arg/Arg	Gln carrier	64	5	0.20 (0.06–0.71)[b]	64	14	1.17 (0.44–3.16)
Gln carrier	Asp/Asp	40	4	0.19 (0.05–0.73)[b]	29	7	1.57 (0.51–4.84)
Gln carrier	Gln carrier	104	8	0.19 (0.06–0.56)[c]	92	27	1.39 (0.56–3.45)
NBS1	*XRCC3*						
Glu185Gln	Thr241Met						
Glu/Glu	Met carrier	64	12	1.00	54	11	1.00
Glu/Glu	Thr/Thr	44	3	0.44 (0.12–1.58)[b]	32	9	1.59 (0.65–3.93)
Gln carrier	Met carrier	77	5	0.55 (0.18–1.62)[b]	92	27	1.80 (0.86–3.77)
Gln carrier	Thr/Thr	41	2	0.29 (0.06–1.32)[b]	39	7	1.01 (0.38–2.68)
TP53	*p21*						
Arg72Pro	Ser31Arg						
Arg/Arg	Arg carrier	15	2	1.00	23	4	1.00
Arg/Arg	Ser/Ser	115	14	0.71 (0.15–3.28)	103	26	1.39 (0.48–4.05)
Pro carrier	Arg carrier	13	1	0.52 (0.05–6.07)	7	2	1.09 (0.19–6.39)
Pro carrier	Ser/Ser	85	6	0.33 (0.07–1.73)[c]	84	22	1.44 (0.49–4.28)

[a] Adjusted for BMI, hospital (four clinics), photon beam quality for whole breast (three categories) and for boost irradiation (no boost and five categories).
[b] $P < 0.05$ for interaction with BMI.
[c] $P < 0.01$ for interaction with BMI.

with a significantly decreased risk of development of acute skin toxicity after RT in patients with normal weight but not with overweight patients. Carriers of both variant alleles showed the greatest risk reduction (HR 0.19; 95% CI, 0.06–0.56), and this was also significantly different from the hazard ratio of 1.39 (95% CI, 0.56–3.45) among overweight patients ($P_{\text{interaction}} = 0.009$).

Combined Effects of NBS1 Glu^{185} Gln and XRCC3 Thr^{241}Met

XRCC2, *XRCC3* and *NBS1* are involved in the DSBs repair pathway. Because of a low variant allele frequency of *XRCC2* ^{188}His (0.07), the *XRCC2* Arg^{188}His polymorphism was not combined with *NBS1* Glu^{185}Gln and *XRCC3* Thr^{241}Met polymorphisms. According to the results of the single gene analysis, *XRCC3* ^{241}Met variant allele was associated with an increased risk of acute skin toxicity after RT, and *NBS1* ^{185}Gln variant allele with a decreased risk of acute skin toxicity. Therefore, the combined effects of their genotypes were investigated using individuals carrying the Glu/Glu in *NBS1* and the Met carrier in *XRCC3* as reference. There are no significant combined effects of *NBS1* Glu^{185}Gln and *XRCC3* Thr^{241}Met genotypes on the development of acute skin toxicity, although the HRs are generally associated with a decreased risk in normal weight patients (Table 35.3).

Combined Effects of TP53 Arg^{72}Pro and p21 Ser^{31}Arg

TP53 and *p21* are required for cell cycle control. *TP53* haplotype effects reflect that of the ^{72}Pro allele. The potential combined

protective effects of *TP53* ^{72}Pro and *p21* ^{31}Ser allele for the development of acute skin toxicity after RT were investigated using individual carriers of Arg/Arg in *TP53* and Arg carriers in *p21* as reference (Table 35.3). Among normal weight women, there was a suggestive trend (P_{trend} = 0.10) in risk associated with the genotype combinations; *TP53* ^{72}Pro carriers who were homozygote for the *p21* ^{31}Ser allele had the lowest risk of acute skin toxicity. However, their risk could not be shown to differ significantly from that in overweight patients with the same genotype combination ($P_{interaction}$ = 0.16).

Joint Effects of the Genotypes in DNA Repair Genes and TP53 Arg72Pro

Furthermore, we investigated the relative effects by including all the genetic polymorphisms simultaneously in a multivariate model. In the subgroup of normal weight patients, we observed that the carriers of the variant allele in *APE1* Asp^{148}Gln (HR 0.28, P = 0.01), *XRCC1* Arg^{399}Gln (HR 0.39, P = 0.07), *XPD* Lys^{751}Gln (HR 0.32, P = 0.07), or *TP53* Arg^{72}Pro (HR 0.35, P = 0.10) were associated with a reduced risk of developing acute skin toxicity compared to their corresponding non-carriers of the variant allele. The other genetic variants did not show an association.

To explore the potentially synergistic effects to the risk of acute skin toxicity after RT, we evaluated an association with the number of variant alleles in the four polymorphisms (*XRCC1* ^{399}Gln, *APE1* ^{148}Glu, *XPD* ^{751}Gln and *TP53* ^{72}Pro) (Table 35.4). Only one sensitive patient carried no variant allele in any of these four polymorphisms; therefore, patients carrying zero to one variant allele were grouped as reference. We found a significant decrease in risk of developing acute skin toxicity with two or more of the variant alleles in women with normal weight. A significant decrease in the risk of developing acute skin toxicity (HR 0.32; 95% CI, 0.11–0.94) was already seen for those with two variant alleles. A more strongly significantly decreased risk of acute skin toxicity was found both in patients with three variant alleles (HR 0.13; 95% CI, 0.03–0.50) and in patients with four or more variant alleles (HR 0.14; 95% CI, 0.05–0.48) for normal weight subgroup, but not for overweight subgroup ($P_{interaction}$

TABLE 35.4. The association between the number of variant alleles in putative risk genotypes and the risk of acute skin toxicity after radiotherapy investigated by Cox proportional hazard model stratified by BMI.

	Normal weight (BMI ≤ 25)			Overweight (BMI > 25)		
No. of variant alleles[b]	No. of patients	Sensitive patients	Adjusted HR (95% CI)[a,e]	No. of patients	Sensitive patients	Adjusted HR (95% CI)
0–1	30	8	1.00	35	5	1.00
2	54	7	0.32 (0.11–0.94)[c]	50	15	2.27 (0.81–6.37)
3	66	3	0.13 (0.03–0.50)[d]	57	16	1.59 (0.57–4.41)
4–7	74	4	0.14 (0.04–0.48)[d]	74	18	1.46 (0.53–4.01)

[a] Adjusted for BMI, hospital (four clinics), photon beam quality for whole breast (three categories) and for boost irradiation (no boost and five categories).

[b] The variant alleles were defined as *XRCC1* ^{399}Gln, *APE1* ^{148}Glu, *XPD* ^{751}Gln, and *TP53* ^{72}Pro.

[c] P < 0.05 for interaction with BMI.

[d] P < 0.01 for interaction with BMI.

[e] P < 0.001, test for trend in normal weight patients.

< 0.01). As there was a significant trend ($P_{trend} < 0.001$) in the subgroup of normal weight patients, our data suggested that there was a dose-response relationship between the number of the variant alleles and reduction of HR for developing acute skin toxicity in patients with normal weight.

DISCUSSION

Overall, no main effects of any of the variants investigated were observed in the breast cancer patients as a whole. Among patients with normal weight, however, carriers of the *XRCC1* ^{399}Gln variant, the *APE1* ^{148}Glu variant, the *XPD* ^{751}Gln variant and *TP53* ^{72}Pro had a decreased risk of acute skin toxicity after RT (HR = 0.51, 0.49, 0.48 and 0.46, respectively). This protective effect was not found among overweight or obese patients. The *XRCC1, XPD* and *TP53* haplotype analysis provided no strong evidence for the involvement of a risk haplotype but confirmed the genotype results. There was however clear evidence of combined effects of the *XRCC1* ^{399}Gln and the *APE1* ^{148}Glu variant alleles, which were differential for normal and overweight women. Among normal weight women, we found that the carriers of either one or both of the *XRCC1* ^{399}Gln and *APE1* ^{148}Glu variant alleles were protected against the development of acute skin toxicity after RT when compared to those carrying neither the *XRCC1* codon 399 nor the *APE1* codon 148 variant allele. Furthermore, when considering the number of variant alleles in *XRCC1* Arg^{399}Gln, *APE1* Asp^{148}Gln, *XPD* Lys^{751}Gln and *TP53* Arg^{72}Pro, we found a strong significant decrease in the risk of developing acute skin toxicity with two or more of the variant alleles in normal weight patients.

Polymorphisms in DNA Repair Genes and the Risk of Acute Side Effects After Radiotherapy

Effects of the Polymorphisms in BER Genes XRCC1 and APE1

The only other study that investigated the association between the *XRCC1* polymorphisms and acute toxicity reported a non-significantly increased risk of developing an adverse response to RT associated with the *XRCC1* ^{194}Trp allele (Moullan *et al*., 2003). Although as in our study, they found no association with haplotype analysis, they observed one particular genotype combination involving this allele and the *XRCC1* ^{399}Gln allele to be associated with significantly increased risk of clinical radiation reactions. Our results may differ because of the different definitions of clinical radiosensitivity used in the two studies. We included only the occurrence of acute skin reactions during and immediately after RT while they included both acute and late skin reactions occurring within 2 years of follow-up. Therefore, their observations may be predominantly driven by the effect of inefficient DNA repair related to late reaction of RT. Indeed, in human fibroblasts, reduced DNA repair as an indicator of cellular radiosensitivity has been found to be associated with various late rather than acute normal tissue responses after RT (Brock *et al*., 1995). Early and late reactions are not necessarily related and they may be influenced differently by genetic predisposition (Bentzen and Overgaard, 1991).

Several reports indicate that the variant alleles of the repair polymorphisms examined may truly affect DNA repair function. The presence of the *XRCC1* ^{194}Arg and ^{399}Gln alleles was demonstrated to be associated with increased mutagen sensitivity after bleomycin treatment (Wang *et al*., 2003). Duell *et al*. (2000) reported elevated sister chromatid exchange frequencies and higher levels of polyphenol adducts in *XRCC1* ^{399}Gln/Gln homozygous. Abdel-Rahman and El-Zein (2000) noted that individuals with ^{399}Gln allele had significantly more sister chromatid exchanges in response to the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) than did those with the Arg/Arg genotype. In addition, the variant alleles in *XRCC1* ^{399}Gln and/or *APE1* ^{148}Glu were associated with a prolonged cell-cycle G2 delay in response to IR (Hu *et al*., 2001). But the relationship between IR-induced cell cycle arrest and tissue response is complex and still not well understood. It is therefore not easy to explain our findings of *XRCC1* ^{399}Gln, which is associated with reduced DNA repair activity, to be protective against the development of acute side effects. However, our observations are supported by studies of the development of therapy-related acute myeloblastic leukemia, in which a protective effect of the *XRCC1* ^{399}Gln allele was also observed (Seedhouse *et al*., 2002). It is evident that the completeness of DNA repair after exposure to IR is not only dependent upon the level of damage. The efficiency of the repair machinery depends also on the time out for a damaged cell, through cell cycle arrest, to ensure effective repair, other wise the damaged cell will be eliminated by cell death. There might be an increase in cell death in insufficiently repaired cells, thus reducing the risk of adverse reactions. This may be particularly important when the functional and structural integrity of DNA repair genes is damaged by IR. To confirm our findings, further functional studies of the *XRCC1* and *APE1* polymorphisms will be necessary.

Effects of the Polymorphisms in NER Gene XPD

From the present study, it is unclear how the exchange of amino acids at codon 312 and 751 of the *XPD* gene influences the development of clinical radiation toxicity. One possible explanation is that polymorphism affects DNA repair capacity. However, not all studies have yielded consistent results on association between *XPD* polymorphisms and DNA repair activity. Lunn *et al*. (2000) reported *XPD* ^{751}Lys/Lys wild-type genotype was associated with the decreased DNA repair proficiency and the Asp^{312}Asn did not appear to affect DNA repair activity. Spitz *et al*. (2001) reported that *XPD* ^{312}Asn/Asn homozygous was associated with less optimal DNA repair capacity in cultured cells that have been transfected with benzo(*a*)pyrene diol epoxide-treated plasmids. It is difficult to explain the seemingly conflicting data regarding the effect of *XPD* polymorphisms on DNA repair capacity. A hypothesis presented first by Lunn *et al*. (2000) and later by Spitz *et al*. (2001) suggests that the variant allele may have different effects on different DNA repair pathways. Therefore, the use of varying DNA damaging agents and varying assays to assess DNA repair may lead to disparate results. The observed effects of *XPD* polymorphisms on the development of clinical radiation toxicity cannot

be completely understood because of the currently limited information available on the functional significance of the *XPD* polymorphisms.

Effects of the Polymorphisms in DSBs Repair Genes XRCC2, XRCC3 and NBS1

We did not observe a clear main effect of the genetic variants in the DSBs repair genes *XRCC3*, *XRCC2* and *NBS1* on the development of acute skin toxicity after RT. To our knowledge, this is the only study to investigate the association between *XRCC2* Arg^{188}His and *NBS1* Glu^{185}Gln polymorphisms and acute side effects of radiotherapy. For *XRCC3* Thr^{241}Met, the Thr/Thr genotype was reported to be correlated with increased risk of late effects in irradiated breast cancer patients, such as s.c. fibrosis and telangiectasia (Andreassen *et al*., 2003). This finding was not confirmed in another study of late-adverse radiotherapy effects in gynecologic tumors (De Ruyck *et al*., 2005). Our results weakly implicated the *XRCC3* ^{241}Met allele as risk allele and may differ from the other studies because we focused on acute side effects rather than late effects of radiotherapy.

All three polymorphisms investigated were predicted to have a maximal effect on cellular, and possibly clinical, function using an algorithm based on allele frequency, potential functional effect, and results from previous epidemiologic studies (Millikan *et al*., 2005). The *XRCC3* ^{241}Met allele has in fact been associated with an increased number of micronuclei in peripheral lymphocytes of humans exposed to ionizing radiation (Angelini *et al*., 2005). No differences in homology-directed repair of DSB have, however, been found between the wild-type and the variant XRCC3 protein (Araujo *et al*., 2002), and DSB repair *in vitro* and acute normal tissue reaction after radiotherapy of breast cancer patients were not correlated (El Awady *et al*., 2005). Thus, the polymorphisms that have been investigated, although apparently functional, may not cover entirely the variability in gene function. In the future, a more detailed haplotype analysis of the genes and a comprehensive analysis, including variants in genes of both DSB repair pathways (homologous recombination and NHEJ), and consideration of late effects of radiotherapy will be necessary.

Polymorphisms in *TP53* and *p21* Genes and the Risk of Acute Side Effects After Radiotherapy

Our data indicated that *TP53* ^{72}Pro carriers had a decreased risk of developing acute skin toxicity after RT in normal weight patients. Functional analysis of the two variants showed that the ^{72}Arg form of *TP53* induced apoptosis more efficiently than the ^{72}Pro form; in contrast, the ^{72}Pro form appeared to induce a higher level of G1 arrest than the 72Arg form (Pim and Banks, 2004). Data about an effect of the *TP53* Arg^{72}Pro polymorphism on therapy outcome are rare but it has been shown that patients receiving chemo-radiotherapy for advanced head and neck cancer had higher response rates and survival when their tumors expressed the pro-apoptotic ^{72}Arg allele (Sullivan *et al*., 2004). Komarova *et al*. (2004) showed that growth arrest mediated by p53 plays an important role in inhibiting mitotic cell death in epithelia of the small intestine of mice and, thus, could reduce radiation

toxicity in these animals. Our results might be explained by ^{72}Pro inducing a high level of G1 arrest to permit more time to repair damaged DNA, thus reducing the risk of adverse reactions. However, this protective effect should be further studied *in vivo* and *in vitro*. In addition, apoptosis and cell death by mitotic catastrophe have been recognized as an important response to radiation in many cells (Komarova *et al*., 2004). If radiation-induced loss of cells is too extensive, the repopulating activity of the normal tissue during therapy might be insufficient resulting in acute side effects in the normal tissue (Hopewell *et al*., 2003). Therefore, the lower apoptosis rates induced by ^{72}Pro allele combined with the increased repair activity during G1 arrest might decrease the risk of acute skin toxicity after RT in individuals carrying the ^{72}Pro allele. Although it is unclear from our results which of the functional differences between the codon 72 polymorphic alleles are more important, both of them could account for the decreased risk of developing clinical radiation toxicity observed in Pro allele carriers.

We did not observe an independent effect of *p53*PIN3 polymorphism on the risk of clinical radiation toxicity to normal tissue after RT. This polymorphism was shown to be associated with reduced *TP53* mRNA expression levels in lymphoblastoid cell lines. As a possible mechanism, a reduced stability of the pre-mRNA carrying the 16 bp insertion was suggested (Gemignani *et al*., 2004). It is, however, difficult to assess the effects of the polymorphism on mRNA levels with the development of clinical radiation toxicity.

p21 plays a direct role in mediating p53-induced G1 arrest, with p53 being the transcription factor in this process. Thus, a possible combined effect of the polymorphisms in the two genes was suggested. We did not observe a significant effect of *p21* Ser^{31}Arg polymorphism associated with the risk of developing acute skin toxicity after RT. However, our data suggested that there may be combined effects of the *TP53* and *p21* polymorphisms since there was a difference in the occurrence of acute toxicity found in normal weight compared to overweight patients. Unfortunately, we did not have adequate power to detect the interaction effects at the predetermined significance level. Matsuzoe *et al*. (1999) reported that loss of p53 function decreased radiation sensitivity in non-small cell lung carcinoma; however, no significant association between the *p21* polymorphism and response to radiation was found. An interaction between *p21* Ser31Arg and *TP53* Arg72Pro was also not observed for the risk of breast cancer (Keshava *et al*., 2002). These findings are compatible to our results that the effects of *p21* Ser^{31}Arg polymorphism on normal tissue response to radiation may be negligible. However, due to the low variant allele frequency of *p21* ^{31}Arg (0.07 for our study population), larger clinical studies are needed to clearly confirm or exclude combined effects of *TP53* and *p21* polymorphism on acute response to radiation.

Joint Effects of the Polymorphisms in DNA Repair Genes and *TP53*

In our study, there was clear evidence that a greater decrease in the risk of acute skin toxicity after RT was associated with a larger number of variant alleles from polymorphisms of DNA repair and cell cycle control genes. Both DNA repair and cell cycle control play an important role

in the process of reproductive cell death caused by IR (Dahm-Daphi *et al*., 1998). Irradiated cells either die from failure to pass the next few mitosis (mitotic death), or are driven into apoptosis (apoptotic death). Otherwise, they pass through differentiation steps which will allow them to survive at the expense of reproductive capability. IR-induced DNA damages are repaired through several DNA maintenance mechanisms. If the repair is insufficient, a second prerequisite for expression of chromosomal damage at mitosis is the progress through the cell cycle. After irradiation, cells are normally blocked at the G1/S border which is regulated by a concert of proteins mainly ATM, p53, p21, retinoblastoma protein (RB), cyclin E and cdk2. Cells that successfully reenter the cell cycle will be further blocked at G2/M border from which they are regularly released into mitosis. It is possible that the statistical association between the number of the variant alleles from DNA repair and cell cycle control genes and the risk of developing acute skin toxicity after RT reflects the concerted action of base excision repair, nucleotide excision repair and DSBs repair, as well as apoptosis pathways.

Body Mass Index and Development of Acute Side Effects After Radiotherapy

In our population study, the risk of developing acute skin toxicity increased with increasing BMI (Twardella *et al*., 2003). This relationship is clinically recognized and is likely to be due to the association of a high BMI with a large breast size. RT of large breasts requires a special radiation protocol with tangential radiation fields, which often results in an increased maximum radiation dose at the surface. However, BMI could also be an indicator of as yet unknown individual factors that influence radiosensitivity. Thus, the strong association of high BMI with clinical radiation toxicity may override the effect of genotypes on the development of acute skin toxicity after RT in overweight patients. The significant interaction of DNA repair gene polymorphisms with BMI and their association with the development of acute skin toxicity after RT which is detectable only in normal weight patients seems consistent with this hypothesis. Further clinical data on the influence of BMI and radiation-induced complications are unfortunately sparse (Huang *et al*., 2000).

Epidemiological and Clinical Characteristics, Strengths and Limitations of the Study

We used a prospective study design to ensure standardized data collection on side effects at defined points during RT and the use of a predefined classification system comparable to other studies. In addition, the criteria for inclusion of patients into the study were defined *a priori* and allowed the recruitment of a study population rather homogenous for ethnic, therapeutic and clinical characteristics. Thus, our study includes only patients receiving primary RT of the breast after breast conserving surgery. Patients were never previously treated with chemotherapy to avoid confounding of side effect data by this treatment.

By choosing grade 2c (moist desquamation of the skin or interruption of RT because of side effects) as an indicator of clinical radiation toxicity, we selected an

indicator that is less prone to variability in classification than radiation-induced erythema (Twardella *et al*., 2003). However, we cannot exclude the possibility that there may still have been inter-observer variability in the evaluation of the degree of acute skin reaction. In addition, we tried to minimize sources of bias in design and analysis of our study by restriction on tumor type and type of side effects, and we accounted for confounding by treatment-related or patient-related characteristics in the data analysis. Using Cox regression to model the association between risk factors and the occurrence of acute skin toxicity after RT, we were able to account for differences in radiation dose received until the end of follow-up as well as the dose at first occurrence of severe acute skin reactions for each individual patient. Therefore, our results are not likely to be biased.

Power calculations were performed at the planning phase of the study to indicate the hazard ratios detectable for single gene polymorphisms with different genotype frequencies with 80% power ($\alpha = 0.05$) for a sample size of 443 patients, assuming a toxicity rate of 17%. This sample size is probably the largest among the relevant reported studies. However, when we conducted further analysis of genotype combinations with respect to their functions, the number of individuals with a specific genotype combination in our cohort became very small. The confidence intervals around the risk estimates are wide, especially when stratified by BMI, indicating a need to confirm our results by extending it to a much larger study population.

In conclusion, in our study, significant joint effects of *XRCC1* ^{399}Gln and the *APE1* ^{148}Glu variant alleles on the development of acute skin toxicity after RT were found in normal weight patients. Furthermore, the risk of developing acute skin toxicity after RT increased significantly with the number of variant alleles of three polymorphisms in DNA repair genes (*XRCC1* ^{399}Gln, *APE1* ^{148}Glu, *XPD* ^{751}Gln) and one polymorphisms in cell cycle control genes (*TP53* ^{72}Pro). Our data suggest that the genetic component of clinical radiation reactions should be considered as a polygenic trait. Genetic testing for only one or even a few genetic variants is unlikely to be of sufficient predictive power. Larger studies based on multiple genetic markers are necessary to generate genetic profiles to predict normal tissue responses after RT. Furthermore, genetic polymorphisms are likely to be associated with late reactions to RT and should be considered in future studies.

REFERENCES

Abdel-Rahman, S.Z., and El Zein, R.A. 2000. The 399Gln polymorphism in the DNA repair gene XRCC1 modulates the genotoxic response induced in human lymphocytes by the tobacco-specific nitrosamine NNK. *Cancer Lett. 159*: 63–71.

Andreassen, C.N., Alsner, J., and Overgaard, J. 2002. Does variability in normal tissue reactions after radiotherapy have a genetic basis–where and how to look for it? *Radiother. Oncol. 64*: 131–140.

Andreassen, C.N., Alsner, J., Overgaard, M., and Overgaard, J. 2003. Prediction of normal tissue radiosensitivity from polymorphisms in candidate genes. *Radiother. Oncol. 69*: 127–135.

Angelini, S., Kumar, R., Carbone, F., Maffei, F., Forti, G.C., Violante, F.S., Lodi, V., Curti, S., Hemminki, K., and Hrelia, P. 2005. Micronuclei in humans induced by exposure to low level of ionizing radiation: influence of polymorphisms in DNA repair genes. *Mutat. Res. 570*: 105–117.

Araujo, F.D., Pierce, A.J., Stark, J.M., and Jasin, M. 2002. Variant XRCC3 implicated in cancer is

functional in homology-directed repair of double-strand breaks. *Oncogene 21*: 4176–4180.

Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C.W., Chessa, L., Smorodinsky, N.I., Prives, C., Reiss, Y., Shiloh, Y., and Ziv, Y. 1998. Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science 281*: 1674–1677.

Bentzen, S.M., and Overgaard, M. 1991. Relationship between early and late normal-tissue injury after postmastectomy radiotherapy. *Radiother. Oncol. 20*: 159–165.

Bentzen, S.M., and Overgaard, J. 1994. Patient-to-Patient Variability in the Expression of Radiation-Induced Normal Tissue Injury. *Semin. Radiat. Oncol. 4*: 68–80.

Brock, W.A., Tucker, S.L., Geara, F.B., Turesson, I., Wike, J., Nyman, J., and Peters, L.J. 1995. Fibroblast radiosensitivity versus acute and late normal skin responses in patients treated for breast cancer. *Int. J. Radiat. Oncol. Biol. Phys. 32*: 1371–1379.

Chang-Claude, J., Popanda, O., Tan, X.L., Kropp, S., Helmbold, I., von Fournier, D., Haase, W., Sautter-Bihl, M.L., Wenz, F., Schmezer, P., and Ambrosone, C.B. 2005. Association between polymorphisms in the DNA repair genes, *XRCC1*, *APE1*, and *XPD* and acute side effects of radiotherapy in breast cancer patients. *Clin. Cancer Res. 11*: 4802–4809.

Dahm-Daphi, J., Dikomey, E., and Brammer, I. 1998. DNA-repair, cell killing and normal tissue damage. *Strahlenther Onkol. 174*: 8–11.

De Ruyck, K., Van Eijkeren, M., Claes, K., Morthier, R., De Paepe, A., Vral, A., De Ridder, L., and Thierens, H. 2005. Radiation-induced damage to normal tissues after radiotherapy in patients treated or gynecologic tumors: association with single nucleotide polymorphisms in *XRCC1*, *XRCC3*, and *OGG1* genes and *in vitro* chromosomal radiosensitivity in lymphocytes. *Int. J. Radiat. Oncol. Biol. Phys. 62*: 1140–1149.

Duell, E.J., Wiencke, J.K., Cheng, T.J., Varkonyi, A., Zuo, Z.F., Ashok, T.D., Mark, E.J., Wain, J.C., Christiani, D.C., and Kelsey, K.T. 2000. Polymorphisms in the DNA repair genes XRCC1 and ERCC2 and biomarkers of DNA damage in human blood mononuclear cells. *Carcinogenesis 21*: 965–971.

El Awady, R.A., Mahmoud, M., Saleh, E.M., El-Baky, H.A., Lotayef, M., Dahm-Daphi, J., and Dikomey, E. 2005. No correlation between radiosensitivity or double-strand break repair capacity of normal fibroblasts and acute normal tissue reaction after radiotherapy of breast cancer patients. *Int. J. Radiat. Biol. 81*: 501–508.

el Deiry, W.S., Harper, J.W., O'Connor, P.M., Velculescu, V.E., Canman, C.E., Jackman, J., Pietenpol, J.A., Burrell, M., Hill, D.E., and Wang, Y. 1994. WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res. 54*: 1169–1174.

Gemignani, F., Moreno, V., Landi, S., Moullan, N., Chabrier, A., Gutierrez-Enriquez, S., Hall, J., Guino, E., Peinado, M.A., Capella, G., and Canzian, F. 2004. A TP53 polymorphism is associated with increased risk of colorectal cancer and with reduced levels of *TP53* mRNA. *Oncogene 23*: 1954–1956.

Hoeijmakers, J.H. 2001. Genome maintenance mechanisms for preventing cancer. *Nature 411*: 366–374.

Hopewell, J.W., Nyman, J., and Turesson, I. 2003. Time factor for acute tissue reactions following fractionated irradiation: a balance between repopulation and enhanced radiosensitivity. *Int. J. Radiat. Biol. 79*: 513–524.

Hu, J.J., Smith, T.R., Miller, M.S., Mohrenweiser, H.W., Golden, A., and Case, L.D. 2001. Amino acid substitution variants of *APE1* and *XRCC1* genes associated with ionizing radiation sensitivity. *Carcinogenesis 22*: 917–922.

Huang, E.Y., Wang, C.J., Chen, H.C., Sun, L.M., Fang, F.M., Yeh, S.A., Hsu, H.C., Hsiung, C.Y., and Wu, J.M. 2000. Multivariate analysis of pulmonary fibrosis after electron beam irradiation for postmastectomy chest wall and regional lymphatics: evidence for non-dosimetric factors. *Radiother. Oncol. 57*: 91–96.

Hung, R.J., Hall, J., Brennan, P., and Boffetta, P. 2005. Genetic polymorphisms in the base excision repair pathway and cancer risk: a HuGE review. *Am. J. Epidemiol. 162*: 925–942.

Karran, P. 2000. DNA double strand break repair in mammalian cells. *Curr. Opin. Genet. Dev. 10*: 144–150.

Keshava, C., Frye, B.L., Wolff, M.S., McCanlies, E.C., and Weston, A. 2002. Waf-1 (p21) and p53 polymorphisms in breast cancer. *Cancer Epidemiol. Biomarkers Prev. 11*: 127–130.

Komarova, E.A., Kondratov, R.V., Wang, K., Christov, K., Golovkina, T.V., Goldblum, J.R.,

and Gudkov, A.V. 2004. Dual effect of p53 on radiation sensitivity in vivo: p53 promotes hematopoietic injury, but protects from gastro-intestinal syndrome in mice. *Oncogene 23*: 3265–3271.

Lunn, R.M., Langlois, R.G., Hsieh, L.L., Thompson, C.L., and Bell, D.A. 1999. XRCC1 polymorphisms: effects on aflatoxin B1-DNA adducts and glycophorin A variant frequency. *Cancer Res. 59*: 2557–2561.

Lunn, R.M., Helzlsouer, K.J., Parshad, R., Umbach, D.M., Harris, E.L., Sanford, K.K., and Bell, D.A. 2000. XPD polymorphisms: effects on DNA repair proficiency. *Carcinogenesis 21*: 551–555.

Maity, A., McKenna, W.G., and Muschel, R.J. 1994. The molecular basis for cell cycle delays following ionizing radiation: a review. *Radiother. Oncol. 31*: 1–13.

Matsuzoe, D., Hideshima, T., Kimura, A., Inada, K., Watanabe, K., Akita, Y., Kawahara, K., and Shirakusa, T. 1999. p53 mutations predict non-small cell lung carcinoma response to radiotherapy. *Cancer Lett. 135*: 189–194.

Millikan, R.C., Player, J.S., Decotret, A.R., Tse, C.K., and Keku, T. 2005. Polymorphisms in DNA repair genes, medical exposure to ionizing radiation, and breast cancer risk. *Cancer Epidemiol. Biomarkers Prev. 14*: 2326–2334.

Moullan, N., Cox, D.G., Angele, S., Romestaing, P., Gerard, J.P., and Hall, J. 2003. Polymorphisms in the DNA repair gene XRCC1, breast cancer risk, and response to radiotherapy. *Cancer Epidemiol. Biomarkers Prev. 12*: 1168–1174.

Parshad, R., Tarone, R.E., Price, F.M., and Sanford, K.K. 1993. Cytogenetic evidence for differences in DNA incision activity in xeroderma pigmentosum group A, C and D cells after X-irradiation during G2 phase. *Mutat. Res. 294*: 149–155.

Paulovich, A.G., Toczyski, D.P., and Hartwell, L.H. 1997. When checkpoints fail. *Cell 88*: 315–321.

Perez, C.A. and Brady, L.W. 1992. Principles and practice of radiation oncology. *JB Lippincott, Philadelphia, PA*.

Pim, D., and Banks, L. 2004. p53 polymorphic variants at codon 72 exert different effects on cell cycle progression. *Int. J. Cancer 108*: 196–199.

Popanda, O., Schattenberg, T., Phong, C.T., Butkiewicz, D., Risch, A., Edler, L., Kayser, K., Dienemann, H., Schulz, V., Drings, P., Bartsch, H., and Schmezer, P. 2004. Specific combinations of DNA repair gene variants and increased risk for non-small cell lung cancer. *Carcinogenesis 25*: 2433–2441.

Popanda, O., Tan, X.L., Ambrosone, C.B., Kropp, S., Helmbold, I., von Fournier, D., Haase, W., Sautter-Bihl, M.L., Wenz, F., Schmezer, P., and Chang-Claude, J. 2006. Genetic polymorphisms in the DNA double-strand break repair genes *XRCC3*, *XRCC2*, and *NBS1* are not associated with acute side effects of radiotherapy in breast cancer patients. *Cancer Epidemiol. Biomarkers Prev. 15*: 1048–1050.

Rafii, S., O'Regan, P., Xinarianos, G., Azmy, I., Stephenson, T., Reed, M., Meuth, M., Thacker, J., and Cox, A. 2002. A potential role for the *XRCC2* $R^{188}H$ polymorphic site in DNA-damage repair and breast cancer. *Hum. Mol. Genet. 11*: 1433–1438.

Rogers, P.B., Plowman, P.N., Harris, S.J., and Arlett, C.F. 2000. Four radiation hypersensitivity cases and their implications for clinical radiotherapy. *Radiother. Oncol. 57*: 143–154.

Seedhouse, C., Bainton, R., Lewis, M., Harding, A., Russell, N., and Das-Gupta, E. 2002. The genotype distribution of the *XRCC1* gene indicates a role for base excision repair in the development of therapy-related acute myeloblastic leukemia. *Blood 100*: 3761–3766.

Shen, M.R., Jones, I.M., and Mohrenweiser, H. 1998. Nonconservative amino acid substitution variants exist at polymorphic frequency in DNA repair genes in healthy humans. *Cancer Res. 58*: 604–608.

Spitz, M.R., Wu, X., Wang, Y., Wang, L.E., Shete, S., Amos, C.I., Guo, Z., Lei, L., Mohrenweiser, H., and Wei, Q. 2001. Modulation of nucleotide excision repair capacity by XPD polymorphisms in lung cancer patients. *Cancer Res. 61*: 1354–1357.

Stephens, M. and Donnelly, P. 2003. A comparison of bayesian methods for haplotype reconstruction from population genotype data. *Am. J. Hum. Genet. 73*: 1162–1169.

Sullivan, A., Syed, N., Gasco, M., Bergamaschi, D., Trigiante, G., Attard, M., Hiller, L., Farrell, P.J., Smith, P., Lu, X., and Crook, T. 2004. Polymorphism in wild-type p53 modulates response to chemotherapy in vitro and in vivo. *Oncogene 23*: 3328–3337.

Tan, X.L., Popanda, O., Ambrosone, C.B., Kropp, S., Helmbold, I., von Fournier, D., Haase, W.,

Sautter-Bihl, M.L., Wenz, F., Schmezer, P., and Chang-Claude, J. 2006. Association between *TP53* and *p21* genetic polymorphisms and acute side effects of radiotherapy in breast cancer patients. *Breast Cancer Res. Treat. 97*: 255–262.

Terwilliger, J., and Ott, J. 1994. Handbook of human genetic linkage. *Johns Hopkins University Press, Baltimore*, MD.

Twardella, D., and Chang-Claude, J. 2002. Studies on radiosensitivity from an epidemiological point of view - overview of methods and results. *Radiother. Oncol. 62*: 249–260.

Twardella, D., Popanda, O., Helmbold, I., Ebbeler, R., Benner, A., von Fournier, D., Haase, W., Sautter-Bihl, M.L., Wenz, F., Schmezer, P., and Chang-Claude, J. 2003. Personal characteristics, therapy modalities and individual DNA repair capacity as predictive factors of acute skin toxicity in an unselected cohort of breast cancer patients receiving radiotherapy. *Radiother. Oncol. 69*: 145–153.

Wang-Gohrke, S., Rebbeck, T.R., Besenfelder, W., Kreienberg, R., and Runnebaum, I.B. 1998. p53 germline polymorphisms are associated with an increased risk for breast cancer in German women. *Anticancer Res. 18*: 2095–2099.

Wang, Y., Spitz, M.R., Zhu, Y., Dong, Q., Shete, S., and Wu, X. 2003. From genotype to phenotype: correlating XRCC1 polymorphisms with mutagen sensitivity. *DNA Repair (Amst) 2*: 901–908.

36
^{18}F-Fluorodeoxyglucose/Positron Emission Tomography in Primary Breast Cancer: Factors Responsible for False-Negative Results

Rakesh Kumar and Neena Lal

INTRODUCTION

Positron emission tomography (PET) is a molecular imaging technique that uses radio- labeled molecules to image interactions of biological processes at the molecular level *in vivo*. Molecular imaging with PET is sensitive to these biological processes and this is exhibited without the evidence of anatomic changes on the conventional imaging. ^{18}F-fluoro-2-deoxy-D-glucose (FDG) is the most commonly used radiotracer in PET imaging. FDG is an analogue of glucose and the uptake is directly proportional to the glucose metabolism. Malignant tumors with high glucose metabolism show preferential uptake of FDG as compared to surrounding normal cells. After transport into tumor cell, FDG is phosphorylated by hexokinase into FDG-6-phosphate. However, ^{18}F-FDG-6-phosphate cannot continue through glycolysis because it is not a substrate for enzyme glucose-6-phosphate isomerase. As a result, ^{18}F-FDG-6-phosphate is biochemically trapped within the cell. This trapped molecule representing the metabolically active tissue like the cancer cell can be measured *in vivo* noninvasively using PET as "hot spots". This physiological process helps to differentiate normal cells from the abnormal cells on the PET imaging. ^{18}F-FDG-PET is now an established standard in the initial staging, monitoring the response to the therapy, and restaging after treatment of patients with various cancers.

Breast cancer is the second most common cause of cancer death affecting women after lung cancer. Approximately one in nine women will have breast cancer during her lifetime. The treatment of breast cancer has changed during the last decade progressing to less radical, together with primary chemotherapy. A comprehensive imaging modality, which detects the tumor in early stages, defines the extent of disease, monitors the treatment response, and

predicts tumor behavior in patients with breast cancer, will be very useful. During the past decade, the application of PET has remarkably improved the management of cancer patients. PET or PET/CT is currently the single most useful diagnostic modality in breast cancer patients, especially those with recurrent or metastatic disease. PET is also highly useful in predicting tumor response to chemotherapy and can differentiate between responder and non-responder early in the course of treatment. Despite many advantages of PET, extensive research has brought forward the shortcomings of PET as an imaging tool of primary breast cancer. A variable sensitivity (range 63–96%) and specificity (range 75–100%) of PET in evaluating primary breast lesions has been reported in the literature. Most of the initial PET studies done in smaller number of patients and with larger primary breast tumors demonstrated high sensitivity and high specificity of FDG-PET. However, recent studies done in breast tumors with smaller size demonstrated relatively lower diagnostic accuracy of PET. Major work has been done to find the relationship between FDG uptake and breast tumor size, grade, tumor type, estrogen and progesterone receptor status, proliferative index, glucose levels and axillary lymph node status. In this chapter, we will discuss various factors in detail, which can lead to false-negative results of PET and PET/CT when used for the diagnosis of primary breast cancer.

TUMOR SIZE

Size of the breast tumor is one of the most important factors determining the sensitivity of FDG-PET in primary breast cancer detection. There is a direct relationship between detection of the tumor by PET and size of the breast tumor. Most of the breast tumors of < 10 mm of size are usually missed by PET, mainly because only ~ 10 mm resolution obtained with PET. That is the reason why most of the PET studies which recruited patients with larger primary breast tumor demonstrated higher diagnostic accuracy as compared to PET studies done in patients with smaller primary breast tumors. Various studies have shown that as the lesion size decreases, false-negative rate of PET increases. However, recent incorporation of CT with PET led to significant improvement of resolution. The current PET/CT machines have resolution of ~ 4 mm.

Earlier landmark studies by Wahl *et al.* (1991) and Adler *et al.* (1993) found high sensitivity of PET mainly because larger tumors were imaged. Similarly, Avril *et al.* (2001) and Buck *et al.* (2002) found no significant correlation between tumor size and FDG uptake as mean diameter of tumors was 24 ± 15 and 28.3 ± 19 mm, respectively. But, recent studies have shown that standardized uptake value (SUV) decreases as the size of the lesion decreases, which can contribute to higher false-negative rates. Avril *et al.* (1997) in their phantom study, demonstrated a lower detection rate of 28% in lesions < 10 mm. In agreement with above study, Kumar *et al.* (2006a) also showed a sensitivity of 23% in primary breast cancer in patients with smaller lesions. The authors detected only 7 of 30 tumors with PET, which were ≤ 10 mm in size (Figure 36.1). In this study, the mean tumor size in false-negative lesions and true positive was 12.14 ± 13.26 and 20.94

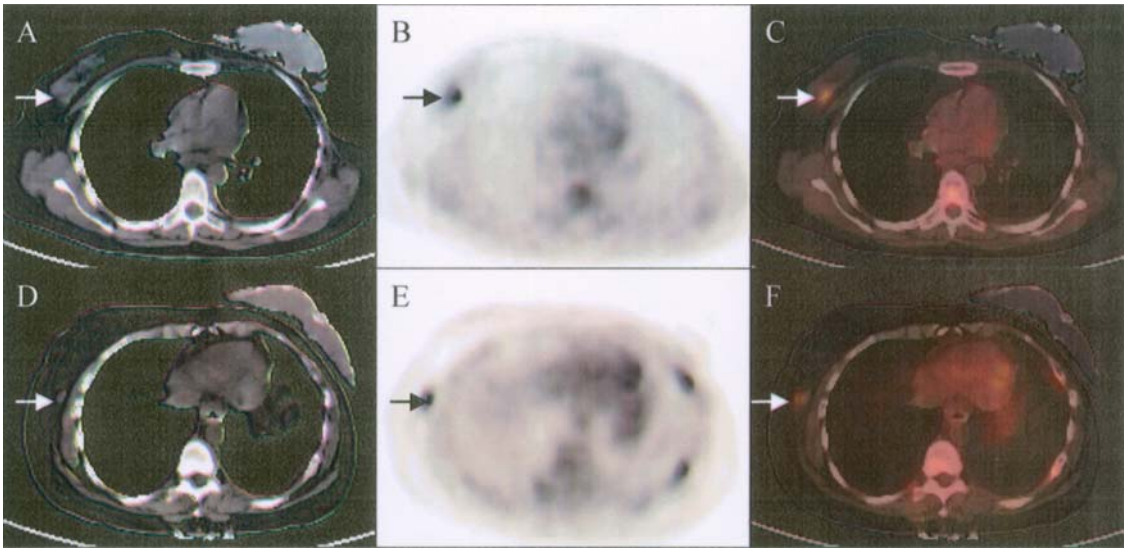


FIGURE 36.1. A 45-year old female post left mastectomy and chemotherapy for left breast cancer with prothesis on the same side. Whole body PET-CT scan was done for restaging. NCCT chest demonstrates dense right breast (A) and an intramammary lymphnode (D). PET scan shows two focal areas (B, E) of mildly increased FDG uptake (SUV 1.8, 1.5) in right breast region. PET-CT confirmed the two focal areas of increased FDG uptake are in breast (C) and intramammry lymphnode (F), respectively. Note the SUVs of two lesions are below the cut off threshold of 2.5 but both lesion were found to be malignant on histopathology

± 11.80 mm, respectively. This difference in tumor sizes of false-negative and true-positive was found to be statistically significant ($p = 0.003$). They also demonstrated that the odds of having FN results is 8.09 times (CI 3.38, 31.52) higher in smaller (≤ 10 mm) tumors than when compared with larger (> 10 mm) tumor ($p = 0.001$).

The potential explanation is that lower SUVs are greatly affected by partial volume effects in smaller tumor due to spread of counts over larger area. Another theory is that an increase in metabolic activity may occur with tumor growth. By correcting for partial volume effect, sensitivity improved from 75% to 92% while specificity decreased from 100% to 97%. In addition, another source of inaccuracy is the observation that breast tumors have lower phosphorylation of FDG as compared to lung cancer as demonstrated by Torizuka *et al.* (1998), which might lead to overall lower SUVs.

HISTOLOGICAL TUMOR TYPE

FDG-PET has been demonstrated to have higher diagnostic value in patients who have invasive ductal carcinoma (IDC) than those of invasive lobular carcinoma (ILC) of breast. Although, ILC accounts for only 8–14% of all breast cancers, its incidence is increasing, especially among postmenopausal women for unknown reasons as reported by Li *et al.* (2000). The FDG uptake of lobular carcinomas of breast is much below the threshold for cancer

detection, which is usually a SUV of > 2.5, leading to higher false-negative PET results. It was found by Buck *et al*. (2002) that FDG uptake in ductal breast cancer was significantly higher than that in lobular cancer (mean TBR 17.3 vs. 6.5, respectively, $p < 0.05$). They attributed this to more Ki-67 positive nuclei present in the ductal subtype as compared with lobular breast cancer ($p < 0.05$). Similar results were also reported by Avril *et al*. (2001) and Crippa *et al*. (1998). In another interesting study by Schirrmeister *et al*. (2001), it was found that lobular or tubular subtype of breast cancer contributed to almost 50% of false-negative results. In our study Kumar *et al*. (2006a) were unable to find any correlation between tumor types and false-negative PET results. Although we could not find any statistically significant results, there were more lobular carcinomas and carcinoma *in situ* in false-negative group as compared to true-positive group.

In various studies false-negative results are reported in slowly growing and well-differentiated histological subtypes of tumors such as tubular carcinoma, carcinoma *in situ*, and lobular carcinomas. Lower tumor cell density and diffuse surrounding tissue infiltration found in lobular cancers leading to lower SUV's can explain this. Slowly growing and well-differentiated breast tumors have lower glucose metabolism as compared to IDC and poorly differentiated tumors, and therefore can be missed by PET.

TUMOR GRADE

Cellular differentiation helps in determining the prognosis of the tumor with high-grade tumors having poor prognosis and vice versa. It was found by Adler *et al*. (1991) and Schulte *et al*. (1999) that FDG uptake was significantly related to pathological grade in bone and soft-tissue sarcoma. Similarly, Crippa *et al*. (1998) found that grade 3 breast tumors exhibited higher SUVs of 6.2 than grade 1 and 2 breast tumors that had mean SUV of 4.9. This difference in SUVs of different grades of breast tumors was found to be statistically significant. Similarly, Kumar *et al*. (2006a) studied 111 patients with breast tumors and reported that patients with true positive PET results had more high-grade tumors, while patients with false-negative PET results had more low-grade tumors. The odds of having false-negative result was 16.76 and 6.26 times higher with low-grade and moderate-grade tumors, respectively, than with high-grade tumor ($p = 0.002$ and $p = 0.005$, respectively). In contrast, Avril *et al*. (2001) and Buck *et al*. (2002) observed a tendency towards lower FDG uptake in differentiated cancers. However, this finding was not statistically significant. It has been very well established that low grade and well-differentiated tumors have lower glucose metabolism, and therefore leads to lower SUVs and false-negative PET results. Similarly, higher SUVs in higher histological tumor grades were mainly attributed to increased glucose metabolism in less differentiated tumors.

TUMOR GROWTH PATTERNS

Tumors cells can grow diffusely in the surrounding tissue or remain confined to small tumor area. Avril *et al*. (2001) demonstrated significantly lower SUVs in

diffusely growing tumors as compared to clearly defined tumors. A possible explanation given was that non-nodular tumors are more influenced by partial volume effects predominantly in the border area of the tumor. Also, it is interesting to note that, four of five tumors with a diffuse growth pattern were observed to be lobular carcinomas histopathologically. Invasive lobular carcinomas had significantly lower FDG uptake compared with invasive ductal carcinomas.

PROLIFERATIVE ACTIVITY

Usually tumors with high proliferative activity demonstrate higher FDG uptake and vice versa. However, different studies performed in breast cancer provide controversial results regarding proliferative activity and FDG uptake. Buck *et al*. (2002) noted that the relation between FDG uptake and Ki-67 expression in ductal breast cancer was significant, but a low correlation coefficient was found ($r = 0.63$). In lobular cancer, no significant relation was observed between FDG uptake and Ki-67 expression. Similarly, Avril *et al*. (2001) reported a significant relationship between the SUV and Ki-67 labeling index of malignant ductal breast tumors and not with lobular cancer. In contrast, Crippa *et al*. (1998) found that SUVs in patients with breast cancers were not related to the thymidine labeling index (TLI) of the tumors. It is interesting to note that Barnard *et al*. (1987) established that Ki-67, which is a monoclonal antibody, helps in determining the proliferative index of breast cancer cells, and its antigen is expressed in G1, S, G2 and M phases of the cell cycle but not in the G0 phase. However, TLI represents only the S-phase fraction. This might explain the above discrepancy.

TUMOR BLOOD VESSEL DENSITY

Avril *et al*. (2001) found a weak inverse relation between blood vessels densities and FDG uptake. Visual gradation of the entire tissue for blood vessel density was done and found lower FDG uptake with higher microvessel density. This could be explained by the fact that under hypoxic conditions, anaerobic metabolism of glucose converts it to lactate substrate that results in higher influx of glucose for energy production. Clavo and Wahl (1996) studied the effects of hypoxia on cultured cancer cells and found significant increase in FDG uptake with moderate hypoxia. It was reported by Helmlinger *et al*. (1997) that in solid tumors, hypoxia is present 100–200 μm beyond the blood vessel supply. In contrast, Oshida *et al*. (1998) using factor VIII-related polyclonal antibody, selected areas with highest vascular quantification and found positive relationship between FDG uptake and microvessel density. More research in this region is required to answer vital questions regarding the affect of perfusion on tissue FDG uptake.

TISSUE HETEROGENICITY

Tumor tissue has a heterogeneous cellularity, consisting of variable proportions of viable tumor cells, inflammatory cells like macrophages, granulation tissue, and necrotic areas. Therefore, FDG uptake by

these cells can affect the overall results obtained during imaging by PET. Malignant cells contribute from a few to up to > 90% of the tumor. One of the drawbacks of PET imaging is the limited spatial resolution; thus, FDG uptake in the tumor lesion represents the average of all uptake. Kubota *et al*. (1992, 1994) showed higher FDG uptake in inflammatory cells and granulation tissue than in malignant tumor cells using autoradiographic studies of tumors. However, non-metabolically active components, i.e., necrotic tissue, fibrotic scar or mucine may reduce FDG uptake in tumors. On the contrary, Brown *et al*. (1995) using syngeneic rat mammary cancers showed that highest FDG uptake was in areas of highest tumor cell density (80% of the total uptake and 72% of the section area), and lower levels were noted in granulation tissue, necrotic areas, fibrotic regions, and inflammatory cell infiltrates (15% of the total uptake and 20% of the section area). Similarly, Kumar *et al*. (2005) found higher FDG uptake in primary breast tumors than postsurgery/excisional biopsy inflammation of breast parenchyma. In this study we demonstrated that a positive change of 3.75% or more in SUV over the time has high sensitivity and specificity in differentiating inflammatory and malignant lesions. This is because the uptake of FDG continues to rise in tumors for several hours after FDG injection. In contrast, such prolonged period of FDG uptake is rare in inflammatory lesions or normal tissues.

UNIFOCAL VS MULTIFOCAL BREAST LESIONS

Breast cancer is often detected as multiple lesions clinically and/or histopathologically. Identification of unifocal or multifocal nature of breast cancer can help in determining the appropriate therapeutic strategy. Schirrmeister *et al*. (2001) found that the sensitivity and specificity of FDG-PET in detecting malignant breast lesions was 93% and 75%, respectively. They further found that the sensitivity, specificity, and accuracy, of positive predictive value and negative predictive value of FDG-PET in differentiating multifocal from unifocal cancer were 63%, 96%, and 86%, respectively. It is interesting to note that all the multifocal lesions that were missed were lobular cancers with maximum lesion size of 30 mm. The high false-negative rate in the detection of multifocal lesions can be explained by the histology and size of the tumor.

ESTROGEN AND PROGESTERONE RECEPTOR

Estrogen receptor (ER) and progesterone receptor (PR) status play an important role in the management of breast cancer. It was evaluated by Buck *et al*. (2002) that there was no positive correlation between FDG uptake and estrogen ($p = 0.79$) or progesterone receptor ($p = 0.34$) immunohistochemistry. Similarly, Kumar *et al*. (2005) did not find any correlation between FDG uptake and ER/PR status. Recently introduced ER/PR targeted breast imaging is more specific and found to have lower false-negative rates than FDG PET imaging.

BREAST DENSITY

According to the ACR Lexicon criteria, mammographic breast image can be

characterized into dense (grade III or IV) and nondense breast (grade I or II). In a retrospective study by Vranjesevic *et al*. (2003), the affect of normal breast density was evaluated in 45 women who had undergone whole-body FDG-PET for indications other than breast cancer. It was found that the FDG uptake was significantly higher in dense breast as compared to nondense fatty breast. In agreement with the above results Kumar *et al*. (2006c) found that dense breasts had significantly higher FDG uptake than nondense breasts. This can potentially be explained by the higher fibroglandular content of dense breast tissue compared to higher fatty content in nondense breasts. The maximum SUVs for all normal breast tissues were below 1.8 for breast parenchyma and 1.4 for nipple. Thus, despite higher FDG uptake in normal dense breast tissue, it is unlikely to result in false reports. However, higher background uptake can considerably affect the visual interpretation of PET and potentially contribute to false-negative reports (Figure 36.2). Under such circumstances quantification analysis like SUVs are not going to hamper the accuracy of PET as an imaging tool.

AXILLARY LYMPH NODE SPREAD

Axillary lymph node metastasis is the most important factor determining breast cancer prognosis. As of now, histological lymph node evaluation is considered gold standard in detecting lymph node spread, which can drastically change the staging and management. Kumar *et al*. (2006b) and Avril *et al*. (2001) found no relationship between primary breast tumor and axillary lymph node status ($p = 0.37$ and $p = 0.29$, respectively). Similarly, Buck *et al*. (2002) found that FDG uptake did not differ significantly in the primary breast tumor in those patients with node positive status ($n = 28$) from node negative status ($n = 48$).

Several studies have been performed to investigate the possibility of PET scanning as an independent imaging modality for detecting axillary lymph node status.

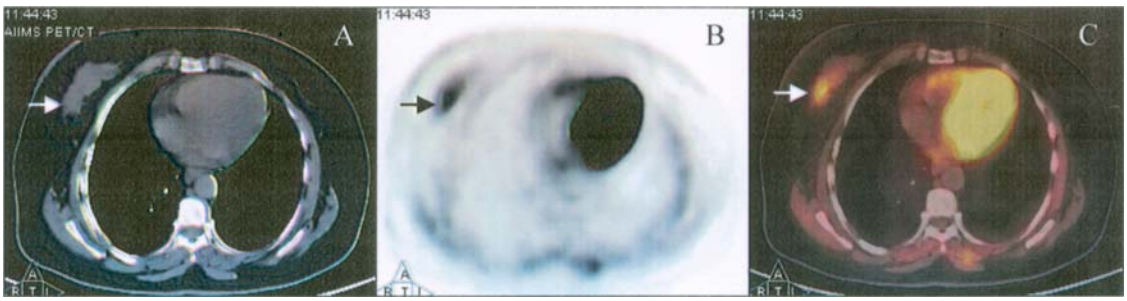


FIGURE 36.2. A 54-year old female with known right breast cancer, underwent whole body PET-CT scan for staging. NCCT chest demonstrates dense right breast (A). PET scan shows increased FDG uptake (SUV 1.7) in the entire right breast with a focal area of relatively increased uptake (SUV 2.7) (B). PET-CT confirmed increased FDG uptake in dense breast with a focal area of increased uptake in outer quadrant of right breast (C). In this patient increased FDG uptake in dense breast can mask lesion visually. However, SUV was above the cut off threshold of 2.5

Kumar *et al*. (2006b) found the sensitivity and specificity of FDG-PET for axillary lymph node detection as 44% and 95%, respectively. It was seen that the smallest lymph node detected by PET was 8 mm in size and that all the micrometastases with size range of 0.5–2 mm were missed. Among those with macrometastases and false-negative result it was seen that size ranged from 5 to 20 mm. They also found that malignancies with moderate and lower histological grade, lower SUV and fewer involved lymph nodes resulted in false-negative results. However, no association was found between size of primary tumor and false-negativity for lymph node spread. It was reported by Schirrmeister *et al*. (2001) that the sensitivity and specificity of FDG-PET in detecting axillary lymph node metastases was 79% and 92% ($p < 0.05$). A false-negative rate of 20% was found and the lesions which were missed were either small in size or were masked by intensive, symmetrical, nonspecific uptake. Those with prior history of chemotherapy were 7 and 10 mm is size, whereas those without prior chemotherapy were 4 mm or smaller in size and were missed. In agreement with above, Avril *et al*. (1996) found a sensitivity of 79% in detecting axillary lymph node spread. On the contrary, Utech *et al*. (1996) found a sensitivity of 100%. Also, Kumar *et al*. (2006b) and Buck *et al*. (2002) could not find any significant correlation between FDG uptake and axillary lymph node status. Due to the above controversy, FDG-PET cannot replace histopathological evaluation of lymph node status.

DISTANT SPREAD

Breast tumor metastases conform poor prognosis. However, the status of distant spread does not affect the primary tumor uptake. Kumar *et al*. (2006b) found that FDG uptake was not significantly affected by distant spread ($p = 0.165$). Whole-body PET scan can be used to assess distant metastases in a single sitting. It was noted by Schirrmeister *et al*. (2001) that none of the distant visceral metastases was missed by FDG-PET. However, previous reports by Cook *et al*. (1998) have suggested that FDG-PET is less sensitive in detecting osteoblastic metastases than conventional bone scans. In addition, Griffeth *et al*. (1993) showed that FDG-PET has limited application in detecting brain metastases due to high uptake by normal cerebral gray matter. Also, PET may not pick up smaller lesions.

BLOOD GLUCOSE LEVELS

Rapidly growing tumors are usually poorly differentiated and tend to have higher metabolic rates, thus leading to high glucose uptake. Due to the competition between the transport of endogenous glucose and FDG molecules into the cell, FDG uptake in cancer is sensitive to variation in blood glucose levels. This altered glucose metabolism has been studied noninvasively *in vivo* using FDG-PET. However, many *in vitro* animal studies have shown that FDG uptake by cancer cells tend to decline as blood glucose and insulin levels rise. Thus, FDG-PET imaging in uncontrolled diabetic patients may lead to false-negative results. The reliability of these studies under high plasma glucose levels is particularly doubtful. It is also interesting to note that cardiac and skeletal muscles show enhanced uptake with elevated blood glucose levels as shown by Knuuti *et al*. (1992). Langen *et al*. (1993) reported

markedly decreased FDG uptake (41.8 ± 15%) after glucose infusion in lung cancer patients. Wahl *et al*. (1992) also found that FDG uptake in breast cancer in rats was significantly decreased in hyperglycemia created by continuous glucose infusion. In another study by Wahl *et al*. (1991), they demonstrated that *in vitro* increasing levels of glucose in the medium lead to inhibition of FDG uptake in breast cancer cells which showed greater decline than the increase in glucose levels. This could be explained by saturability of the tumor uptake by excess glucose.

GLUCOSE TRANSPORTERS

The uptake of FDG in different tissues is dependant on the level of expression of Glut-1 facilitative glucose transporters by various cells and hexokinase enzyme levels. Brown and Wahl (1993) observed that glucose transporter 1 (Glut-1) is particularly overexpressed by cancer cells (upto 60%) in breast carcinomas. On the contrary, Avril *et al*. (2001) did not find any significant correlation between FDG uptake and Glut-1 expression. Only 30% of tumor cells were immunoreactive for Glut-1 receptors. More work is required in this area to clearly define the association between Glut-1 levels and FDG uptake.

DATA ACQUISITION AND DATA ANALYSIS

Physiological patient movement during the PET scanning process is another reason, which can lead to erroneous results. In supine position, the chest wall including the mammary glands move with the patients respiratory efforts leading to blurring of lesion on imaging. Therefore, Avril *et al*. (1997) recommended that patients be scanned in a prone position on a comfortable foam rubber support, with a hole in the foam that ensured that breasts could hang freely. This procedure reduced the motion artifacts enabling better target localization and tracer quantification *via* SUVs. Estimation of tracer uptake by tissue should take into account the attenuation of the emitted annihilation gamma rays, partial volume effect, and placements of region of interest (ROI) during the PET scanning procedure. Hoffman *et al*. (1979) observed that the restricted sampling and resolution lead to partial volume effects which contributed to underestimation of local tracer uptake, primarily in tumors smaller than twice the resolution of the imaging system. Thus, correction coefficients are very useful in such scenario, which is mostly obtained from phantom studies.

Different body compartments do not have the same FDG uptake. It has been observed that fat has a lower FDG uptake than other tissues. Therefore, FDG uptake in tumors will be overestimated in heavy patients. Thus, lean body mass as proposed by Zasadny and Wahl (1993) and body surface area as suggested by Kim *et al*. (1994) may prove to be helpful. Avril *et al*. (1997) did not find any improvement in diagnostic accuracy by applying this correction indicating that these may prove superior in patients who are significantly under- or overweight. Scintigraphic information can be analyzed either by visual interpretation or by quantitative regional tracer uptake. Visual analysis is operator-dependant and experience over time can identify the different regional tracer distribution. In comparison, quantitative parameters are more superior as they

are objective and observer-independent analysis methods.

RECENT DEVELOPMENTS

Lately, PET has undergone tremendous evolution. PET imaging alone lacks the anatomical landmarks of the conventional imaging like computed tomography (CT) and magnetic resonance imaging (MRI); hence, the addition of CT to PET improves detection efficiency and localization of lesions. This results in better specificity and sensitivity than either PET or CT alone. PET mammography is a dedicated tool, which has added benefits of both anatomic and functional imaging of breast. Targeted PET imaging would prove to be less costly and avoid soft-tissue attenuation, which would increase its sensitivity over PET alone. Application of this novel modality for radionucleide-guided biopsies is underway. Raylman *et al*. (2000) reported in their phantom studies that in the absence of breast background FDG uptake, PET mammography could detect lesions as small as 5 mm. However, in breasts with higher background uptake like metabolically active and thick breasts, PET mammography had decreased sensitivity, and lesions < 12 mm were missed.

In a study conducted by Stadnik *et al*. (2006), it was observed that MRI after ultra-small super paramagnetic iron oxide (USPIO) injection had a sensitivity of 100% and specificity of 80% for preoperative axillary lymph node staging in patients with breast cancer, and PET alone had a sensitivity of 80% and specificity of 100%. A combination of PET/MRI had a 100% sensitivity and specificity. Thus, PET/MRI has the potential added advantage of both structural and metabolic delineation of a breast lesion. Moy *et al*. (2007) investigated 23 patients with suspected recurrent or new breast cancer using whole-body PET, PET breast and MRI of breast as imaging tools. They found that MRI alone had a sensitivity of 92%, specificity of 52%, positive predictive value of 69%, and negative predictive value of 85%, whereas fusion of MRI and PET had a sensitivity of 63%, specificity of 95%, positive predictive value of 94%, and negative predictive value of 69%. These data show that there is considerable limitation of PET/MRI in breast pathology detection. Similarly, PET/CT has been used for characterization and localization of breast lesions. Tatsumi *et al*. (2006) retrospectively compared the accuracy of FDG-PET-CT with PET and CT alone in 75 patients with breast cancer. PET/CT established a significantly better accuracy than CT. PET/CT added incremental diagnostic confidence to PET in more than 50% of patients and regions with increased FDG uptake.

The role of dual-time-point imaging to further improve breast cancer diagnosis is also being utilized. Various studies have shown that FDG uptake do not reach maximum until after several hours. In a study by Kumar *et al*. (2005) 54 patients with breast cancer underwent two sequential PET scans 1-h apart. Of the lesions with invasive carcinoma 85% showed an increase and 15% showed no change or decrease in SUVs over time. The percentage change in SUVs was +12.6% ± 11.4% ($p = 0.003$). Of the inflammatory lesions 17% showed an increase and 83% showed no change or decrease in SUVs. The percentage change was −10.2% ± 16.5% ($p = 0.03$). Of the

normal breasts 3.5% showed an increase and 96.5% showed either no change or decreased uptake. Thus, it was seen that a percentage change of +3.75 or more in SUVs over time is highly sensitive and specific in differentiating inflammatory from malignant lesions. Targeted breast imaging using estrogen receptor radioligand is one of the newer scintigraphic techniques, which has an extensive scope in breast cancer detection and treatment monitoring. It is a noninvasive diagnostic tool for determining the functional receptors compared to *in vitro* biopsy or tumor resection and receptor assay. Encouraging results were obtained by Linden *et al*. (2006) using radio labelled estrogen analog, 16alpha-[F-18] fluoroestradiol, which is most widely used. The journey of these receptor specific ligands and PET up to date was reviewed by Kumar (2007). However, more research is required in this field to allow evidence-based clinical practice.

REFERENCES

Adler, L.P., Blair, H.F., Makley, J.T., Williams, R.P., Joyce, M.J., Leisure, G., al-Kaisi, N., and Miraldi, F. 1991. Noninvasive grading of musculoskeletal tumors using PET. *J. Nucl. Med. 32*: 1508–1512.

Adler, L.P., Crowe, J.P., al-Kaisi, N.K., and Sunshine, J.L. 1993. Evaluation of breast masses and axillary lymph nodes with [F-18] 2-deoxy-2-fluoro-D-glucose PET. *Radiology 187*: 743–750.

Avril, N., Dose, J., Janicke, F.,Bense, S., Ziegler, S., Laubenbacher, C., Romer, W., Pache, H., Herz, M., Allgayer, B., Nathrath, W., Graeff, H., and Schwaiger, M. 1996. Metabolic characterization of breast tumors with positron emission tomography using F-18 fluorodeoxyglucose. *J. Clin. Oncol. 14*:1848–1857.

Avril, N., Bense, S., Ziegler, S.I., Dose, J., Weber, W., Laubenbacher, C., Romer, W., Janicke, F., and Schwaiger, M. 1997. Breast imaging with fluorine- 18-FDG PET: quantitative image analysis. *J. Nucl. Med. 38*: 1186–1191.

Avril, N., Menzel, M., Dose, J., Schelling, M., Weber, W., Janicke, F., Nathrath, W., and Schwaiger, M. 2001. Glucose metabolism of breast cancer assessed by 18F-FDG PET: histologic and immunohistochemical tissue analysis. *J. Nucl. Med. 42*: 9–16.

Barnard, N.J., Hall, P.A., Lemoine, N.R., and Kadar, N. 1987.Proliferative index in breast carcinoma determined in situ by Ki67 immunostaining and its relationship to clinical and pathological variables. *J. Pathol. 152*: 287–295.

Brown, R.S., and Wahl, R.L. 1993. Overexpression of Glut-1 glucose transporter in human breast cancer: an immunohistochemical study. *Cancer 72*: 2979–2985.

Brown, R.S., Leung, J.Y., Fisher, S.J., Frey, K.A., Ethier, S.P., and Wahl, R.L. 1995. Intratumoral distribution of tritiated fluorodeoxyglucose in breast carcinoma. I. Are inflammatory cells important? *J. Nucl. Med. 36*: 1854–1861.

Buck, A., Schirrmeister, H., Kuhn, T., Shen, C., Kalker, T., Kotzerke, J., Dankerl, A., Glatting, G., Reske, S., and Mattfeldt, T. 2002. FDG uptake in breast cancer: correlation with biological and clinical prognostic parameters. *Eur. J. Nucl. Med. Mol. Imaging 29*: 1317–1323.

Clavo, A.C., and Wahl, R.L. 1996. Effects of hypoxia on the uptake of tritiated thymidine,L-leucine, L-methionine and FDG in cultured cancer cells. *J. Nucl. Med. 37*: 502–506.

Cook, G.J., Houston, S., Rubens, R. Maisey, M.N., and Fogelman, I. 1998. Detection of bone metastases in breast cancer by 18-FDG PET: differing metabolic activity in osteoblastic and osteolytic lesions. *J. Clin. Oncol. 16*: 3375–3379.

Crippa, F., Seregni, E., Agresti, R., Chiesa, C., Pascali, C., Bogni, A., Decise, D., De Sanctis, V., Greco, M., Daidone, M.G., and Bombardieri, E. 1998. Association between F-18 fluorodeoxyglucose uptake and postoperative histopathology, hormone receptor status, thymidine labelling index and p53 in primary breast cancer: a preliminary observation. *Eur. J. Nucl. Med. 25*: 1429–1434.

Griffeth, L.K., Rich, K.M., Dehdashti, F., Simpson, J.R., Fusselman, M.J., McGuire, A.H., and Siegel, B.A. 1993. Brain metastases from non-central nervous system tumors: evaluation with PET. *Radiology 186* : 37–44.

Helmlinger, G., Yuan, F., Dellian, M., and Jain, R.K. 1997. Interstitial pH and pO2 gradients in solid tumors in vivo: high-resolution measurements reveal a lack of correlation. *Nat. Med. 3*: 177–182.

Hoffmann, E.J., Huang, S., and Phelps, M.E. 1979. Quantitation in positron emission computed tomography: effect of object size. *J. Compai. Assisi. Tomogr. 3*: 299–308.

Kim, C.K., Gupta, N.C., Chandramouli, B., and Alavi, A. 1994. Standardized uptake values of FDG: body surface area correction is preferable to body weight correction. *J. Nucl. Med. 35*: 164–167.

Knuuti, J., Nuutila, P., Ruotsalainen, U., Saraste, M., Harkonen, R., Ahonen, A., Teras, M., Haaparanta, M., Wegelius, U., Haapanen, A., Hartiala, J., and Voipio-Pulkki, L.M. 1992. Euglycemic hyperinsulinemic clamp and oral glucose load in stimulating myocardial glucose utilization during positron emission tomography. *J. Nucl. Med. 33*: 1255–1262.

Kubota, R., Yamada, S., Kubota, K., Ishiwata, K., Tamahashi, N., and Ido, T. 1992. Intratumoral distribution of fluorine-18- fluorodeoxyglucose in vivo: high accumulation in macrophages and granulation tissue studied by microautoradiography. *J. Nucl. Med. 33*: 1972–1980.

Kubota, R., Kubota, K., Yamada, S., Tada, M., Ido, T., and Tamahashi, N. 1994. Active and passive mechanisms of [fluorine-18] fluorodeoxyglucose uptake by proliferating and prenecrotic cancer cells *in vivo*: a microautoradiographic study. *J. Nucl. Med*. *35*:1067–75.

Kumar, R. 2007. Targeted Functional Imaging in Breast Cancer. *Eur. J. Nucl. Med. Mol. Imaging 34*: 346–353.

Kumar, R., Loving, V.A., Chauhan, A., Zhuang, H., Mitchell, S., and Alavi, A. 2005. Potential of dual-time-point imaging to improve breast cancer diagnosis with (18) F-FDG PET. *J. Nucl. Med. 46*: 1819–1824.

Kumar, R., Chauhan, A., Zhuang, H., Chandra, P., Schnall, M., and Alavi, A. 2006a. Clinicopathologic factors associated with false negative FDG–PET in primary breast cancer. *Breast Cancer Res. Treat. 98*: 267–274.

Kumar, R., Zhuang, H., Schnall, M., Conant, E., Damia, S., Weinstein, S., Chandra, P., Czerniecki, B., and Alavi, A. 2006b. FDG PET positive lymph nodes are highly predictive of metastasis in breast cancer. *Nucl. Med. Commun. 27*: 231–236.

Kumar, R., Chauhan, A., Zhuang, H., Chandra, P., and Alavi, A. 2006c. Stanadarized upatke value in normal breast: effect of age, density and menopausal status. *Mol. Imaging Biol. 8*: 355–362.

Langen, K.J., Braun, U., Kops, E.R., Herzog, H., Kuwert, T., Nebeling, B., and Feinendegen, L.E. 1993. The influence of plasma glucose levels on fluorine-18-fluorodeoxyglucose uptake in bronchial carcinomas. *J. Nucl. Med. 34*: 355–359.

Li, C.I., Anderson, B.O., Porter, P., Holt, S.K., Daling, J.R., and Moe, R.E. 2000. Changing incidence rate of invasive lobular breast carcinoma among older women. *Cancer 88*: 2561–2569.

Linden, H.M., Stekhova, S.A., Link, J.M., Gralow, J.R., Livingston, R.B., Ellis, G.K., Petra, P.H., Peterson, L.M., Schubert, E.K., Dunnwald, L.K., Krohn, K.A., and Mankoff, D.A. 2006. Quantitative fluoroestradiol positron emission tomography imaging predicts response to endocrine treatment in breast cancer. *J. Clin. Oncol. 24*: 2793–2799.

Moy, L., Ponzo, F., Noz, M.E., Maguire, G.Q., Jr, Murphy-Walcott, A.D., Deans, A.E., Kitazono, M.T., Travascio, L., and Kramer, E.L. 2007. Improving specificity of breast MRI using prone PET and fused MRI and PET 3D volume datasets. *J. Nucl. Med. 48*: 528–537.

Oshida, M., Uno, K., Suzuki, M., Nagashima, T., Hashimoto, H., Yagata, H., Shishikura, T., Imazeki, K., and Nakajima, N. 1998. Predicting the prognoses of breast carcinoma patients with positron emission tomography using 2-deoxy-2-fluoro[18F]-D-glucose. *Cancer 82*: 2227–2234.

Raylman, R.R., Majewski, S., Wojcik, R., Weisenberger, A.G., Kross, B., Popov, V., and Bishop, H.A. 2000. The potential role of positron emission mammography for detection of breast cancer. A phantom study. *Med. Phys. 27*: 1535–1543.

Schirrmeister, H., Kuhn, T., Guhlmann, A., Santjohanser, C., Horster, T., Nussle, K., Koretz, K., Glatting, G., Rieber, A., Kreienberg, R., Buck, A.C., and Reske, S.N. 2001. Fluorine-18 2-deoxy-2-fluoro-D-glucose PET in the preoperative staging of breast cancer: comparison with the standard staging procedures. *Eur. J. Nucl. Med. 28*: 351–358.

Schulte, M., Brecht-Krauss, D., Heymer, B., Guhlmann, A., Hartwig, E., Sarkar, M.R.,

Diederichs, C.G., Schultheiss, M., Kotzerke, J., and Reske, S.N. 1999. Fluorodeoxyglucose positron emission tomography of soft tissue tumours: is a non-invasive determination of biological activity possible? *Eur. J. Nucl. Med. 26*: 599–605.

Stadnik, T.W., Everaert, H., Makkat, S., Sacre, R., Lamote, J., and Bourgain, C. 2006. Breast imaging. Preoperative breast cancer staging: comparison of USPIO-enhanced MR imaging and 18F-fluorodeoxyglucose (FDC) positron emission tomography (PET) imaging for axillary lymph node staging–initial findings. *Eur. Radiol. 16*: 2153–2160.

Tatsumi, M., Cohade, C., Mourtzikos, K.A., Fishman, E.K., and Wahl, R.L. 2006. Initial experience with FDG-PET/CT in the evaluation of breast cancer. *Eur. J. Nucl. Med. Mol. Imaging 33*: 254–262.

Torizuka, T., Zasadny, K.R., Recker, B., and Wahl, R.L. 1998. Untreated primary lung and breast cancers: correlation between F-18 FDG kinetic rate constants and findings of in vitro studies. *Radiology 207*: 767–774.

Utech, C.I., Young, C.S., and Winter, P.F. 1996. Prospective evaluation of fluorine-18 fluorodeoxyglucose positron emission tomography in breast cancer for staging of the axilla related to surgery and immunocytochemistry. *Eur. J. Nucl. Med. 23*:1588–1593.

Vranjesevic, D., Schiepers, C., Silverman, D.H., Quon, A., Villalpando, J., Dahlbom, M., Phelps, M.E., and Czernin, J. 2003. Relationship between 18F-FDG uptake and breast density in women with normal breast tissue. *J. Nucl. Med. 44*: 1238–1242.

Wahl, R.L., Cody, R.L., Hutchins, G.D., and Mudgett, E.E. 1991. Primary and metastatic breast carcinoma: initial clinical evaluation with PET with the radiolabeled glucose analogue 2-[F-18]-fluoro-2-deoxy-D-glucose. *Radiology 179*: 765–770.

Wahl, R.L., Henry, C.A., and Ethier, S.P. 1992. Serum glucose: effects on tumor and normal tissue accumulation of 2-[F-l8]-fluoro-2-deoxy-D-glucose FDG in rodents with mammary carcinoma. *Radiology 183*: 643–647.

Zasadny, K.R., and Wahl, R.L. 1993. Standardized uptake values of normal tissues at PET with 2-[fluorine-18]-fluoro-2-deoxy-D-glucose: variations with body weight and a method for correction. *Radiology 189*: 847–850.

37
Sentinel Lymph Node Surgery During Prophylactic Mastectomy (Methodology)

Judy C. Boughey and Isabelle Bedrosian

INTRODUCTION

Prophylactic mastectomy is an increasingly sought after procedure in the current era of health awareness, screening mammography, and genetic counseling (Tuttle *et al*., 2007). Although historically applied as a risk reducing procedure in women with a strong family history of breast cancer (Hartmann *et al*., 1999), increasingly women with a personal history of breast cancer are also seeking risk reduction in the contralateral breast through this procedure. Therefore, currently there are two main groups of patients who are undergoing counseling for prophylactic mastectomy: those without a personal history of breast carcinoma but with a high risk family pedigree and those with a personal diagnosis of breast cancer.

Patients with a history of breast cancer are known to have a risk of 0.5–1% per year of developing a contralateral breast cancer (Hill-Kayser *et al*., 2006). This risk estimate is most representative of women without a strong family history of the disease, and additionally does not reflect potential reductions in risk that could be achieved through chemoprevention strategies. Nonetheless, despite considerable controversy among surgeons regarding the role of contralateral prophylactic mastectomy, an increasing number of anxious patients are requesting this procedure. Removal of the normal breast acts as risk reduction and provides potential psychological benefit to the patient who wishes to avoid ever having to face breast cancer again. Contralateral prophylactic mastectomy has been shown to decrease the risk of development of contralateral breast cancer by 90–95% (van Sprundel *et al*., 2005; Herrinton *et al*., 2005; Goldflam *et al*., 2004; McDonnell *et al*., 2001). Contralateral prophylactic mastectomy hypothetically offers increased life expectancy to young women with BRCA mutations (Schrag *et al*., 2000). Currently there are no data to support that contralateral prophylactic mastectomy will impact overall survival in the majority of patients.

Patients who have not been diagnosed with breast cancer but are at high risk of

developing this cancer, either because of a strong family history of breast cancer, documented mutations in *BRCA1* or *BRCA2* genes or personal diagnosis of high-risk pathology such as lobular carcinoma *in situ,* atypical lobular hyperplasia, or atypical ductal hyperplasia, may consider bilateral prophylactic mastectomy (BPM) for risk reduction. As awareness increases regarding the familial predisposition to the development of breast cancer, more patients are pursuing genetic testing, and the identification of *BRCA1* or *BRCA2* gene mutations has increased the number of patients considering bilateral prophylactic mastectomy. Bilateral prophylactic mastectomy is associated with a substantial reduction in the incidence of subsequent breast cancer in women at high risk of this cancer on the basis of a family history of breast cancer and patients known to be *BRCA 1* or *BRCA 2* mutation carriers (Hartmann *et al*., 1999, 2001; Meijers-Heijboer *et al*., 2001; Rebbeck *et al*., 2004) by 90–100%. Long term survival outcomes for patients undergoing bilateral prophylactic mastectomy are also poorly defined, although in these instances, there is no competing risk of death from an existing carcinoma, thereby theoretically such patients also stand to gain in terms of overall survival.

The Society of Surgical Oncology has issued a position statement on prophylactic mastectomy (http://www.surgonc.org/default.aspx?id=179). Clinicopathological diagnoses identifying patients at increased risk of breast cancer and therefore with indications for bilateral prophylactic mastectomy include: atypical lobular or ductal hyperplasia; lobular carcinoma *in situ*; known *BRCA* mutation or strong family history (multiple first degree relatives and/or multiple successive generations, or multiple family members with premenopausal or bilateral breast cancer or male breast cancer). Prophylactic mastectomy of the contralateral breast may be strongly considered for: diffuse microcalcifications, breasts where subsequent surveillance will be difficult, lobular carcinoma *in situ*, breasts of disproportionately large size, and risk reduction for patients at high risk of contralateral breast cancer (as listed for bilateral prophylactic mastectomy).

Across the multiple studies evaluating prophylactic mastectomy in both contralateral prophylactic mastectomy and bilateral prophylactic mastectomy specimens, there is a 5% incidence that the prophylactic mastectomy specimen may harbor an occult breast cancer (Goldflam *et al*., 2004; King *et al*., 2004; McDonnell *et al*., 2001; Boughey *et al*., 2006b; Gershenwald *et al*., 1998). Upon evaluating this more closely, however, the majority of these are in fact ductal carcinoma in situ (DCIS) and the incidence of an invasive breast cancer in the prophylactic mastectomy specimen is 1.8% for patients undergoing contralateral prophylactic mastectomy and 1.9% for those patients undergoing bilateral prophylactic mastectomy (Boughey *et al*., 2006b).

The sentinel lymph node is the first lymph node or group of lymph nodes encountered in the lymphatic drainage of the breast, generally identified by lymphatic mapping (Lyman *et al*., 2005). This technique was first described in breast cancer by Giuliano *et al*. (1994),

and subsequently validated in a multicenter study (Krag *et al*., 1998). Sentinel lymph node surgery has been shown to be reliable for axillary staging of early stage invasive breast cancer in patients with clinically negative axillae, with a sentinel lymph node identification rate of 97%, false-negative rate of 9.7%, and negative predictive value of 96% (Julian *et al*., 2004). Sentinel lymph node surgery is widely used in breast surgery for the axillary staging of patients with early stage breast cancer (T1 and T2 tumors) with clinically negative nodes. Its use has also expanded to patients with larger tumors (Bedrosian *et al*., 2000), multicentric tumors (Lyman *et al*., 2005), DCIS with mastectomy, male breast cancer (Boughey *et al*., 2006a), patients with a prior excisional breast biopsy (Czerniecki *et al*., 1999; Miner *et al*., 1999) and patients who have received neoadjuvant chemotherapy (Breslin *et al*., 2000; Xing *et al*., 2006).

Prior to the era of sentinel lymph node surgery, nodal staging was not routinely performed at the time of risk reduction surgery. However, currently there is an ongoing debate regarding the routine use of sentinel lymph node surgery during prophylactic mastectomy. Discovery of an occult invasive cancer in patients undergoing prophylactic mastectomy would require an axillary lymph node dissection for axillary staging of this invasive cancer if it were diagnosed after the time of mastectomy. An alternative approach advocated by some centers is to perform routine sentinel lymph node surgery on all patients undergoing prophylactic mastectomy to allow staging of all patients in the event of finding an occult cancer (Dupont *et al*., 2000).

PREOPERATIVE EVALUATION PRIOR TO SENTINEL LYMPH NODE SURGERY AND PROPHYLACTIC MASTECTOMY

Prior to undergoing prophylactic mastectomy the patient should have a clinical breast examination performed by the breast surgeon and at least a two view diagnostic mammogram to evaluate for any evidence of malignancy in the breast. Additional imaging such as breast ultrasound can be performed to evaluate any palpable abnormalities or abnormalities detected on mammography. Magnetic resonance imaging is increasingly being used to evaluate patients for breast cancer diagnosis, and for patients at increased risk of breast cancer. Bilateral breast magnetic resonance imaging in these patients will sometimes detect otherwise occult malignancy not detected on clinical breast examination and mammography. In women with a recent breast cancer diagnosis, 3% will have an otherwise occult invasive breast cancer detected in the opposite breast by magnetic resonance imaging alone (Lehman *et al*., 2007). Of these, only 60% will be invasive cancer. Therefore, magnetic resonance imaging detects contralateral invasive breast cancer in 1.8% of patients with an index breast tumor.

Black (2006) evaluated the efficacy and cost of magnetic resonance imaging and sentinel lymph node surgery in detecting occult cancer in prophylactic mastectomy specimens. Magnetic resonance imaging detected only one of the four occult malignancies (one of the two invasive breast cancers). The strategy of preoperative

magnetic resonance imaging with selective sentinel lymph node added $1,207 per patient, and the strategy of routine sentinel lymph node added $644 per patient. They concluded that preoperative magnetic resonance imaging adds significant cost and misses most occult cancer in prophylactic mastectomy specimens. Routine use of magnetic resonance imaging prior to prophylactic mastectomy for the detection of occult malignancy to direct nodal staging has not demonstrated benefit for these patients. However, in the event of an abnormality suspicious for malignancy on mammogram, ultrasound, or magnetic resonance imaging, then sentinel lymph node surgery for nodal staging at the time of mastectomy would be recommended.

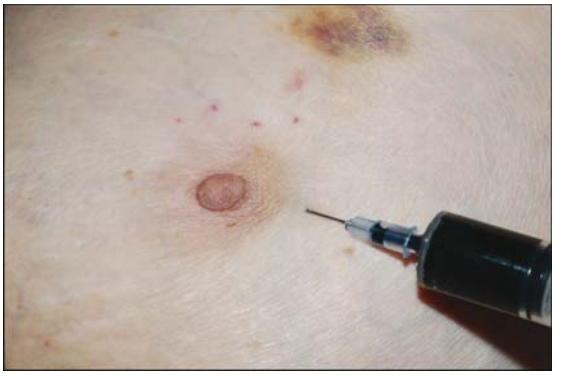


FIGURE 37.1. Injection of blue dye into the subareolar location

METHODOLOGY OF SENTINEL LYMPH NODE SURGERY AT THE TIME OF PROPHYLACTIC MASTECTOMY

Choice of Mapping Agent

Sentinel lymph node surgery can be performed using either radioactive tracer or vital blue dye or both (Figure 37.1). The American Society of Breast Surgeons consensus statement recommends the use of dual tracers for the accurate identification of the sentinel lymph node (http://www.breastsurgeons.org/officialstmts/sentinel.shtml). The sentinel lymph node identification rate is increased and the false-negative rate is decreased with use of dual tracers (Kim *et al.*, 2006). The most commonly used radioactive tracer in the United States is technetium 99 m-labeled (Tc^{99m}) sulfur colloid. The colloid may be filtered or unfiltered. Average size of unfiltered sulfur colloid particles is 50–1,000 nm. Use of a 0.22 μm filter keeps the average particle size below 200 nm, which results in more rapid transit to the sentinel lymph node. Overall the size of the particles affects the rate of drainage from the breast along the lymphatics to the sentinel lymph nodes. Several other radiolabeled colloids (not approved for use in the United States) are used outside the United States for lymphatic mapping including, Tc^{99m} antimony colloid, Tc^{99m} tin colloid, and Tc^{99m} albumin colloid. In Europe, Tc^{99m} colloidal albumin is most commonly utilized. Radiolabeled albumin colloid has a smaller particle size, and, therefore, more rapid lymphatic migration. This allows for injection after induction of general anesthesia, which can reduce pain associated with the colloid injection.

The dose of radioactive tracer injected per patient ranges between 0.1 to 1 mCi (3.7–37 MBq). This is 4% of that administered for a conventional bone scan (Waddington *et al.*, 2000) and no isolation, precautions or special radiation monitoring is required. The authors use 0.5 mCi of

^{99m}Tc-labeled sulfur colloid injected on the morning of surgery, at least 30 min prior to start of operation or 2.5 mCi on the day prior to surgery (Solorzano *et al*., 2001).

The most commonly used vital blue dye in the United States is isosulfan blue (Lymphazurin, United States Surgical Corporation, Norwalk, CT). Usual dose is 5 ml of 1% isosulfan blue dye. Alternatives include methylene blue dye and patent V blue dye, which is used in the United Kingdom. Use of lymphazurin is associated with the potential risk of anaphylaxis with cardiovascular collapse and hypotension in 0.1–2% of cases (Cimmino *et al*., 2001; Montgomery *et al*., 2002; Cox *et al*., 2000; Wilke *et al*., 2006) and methylene blue dye can be associated with skin necrosis (Stradling *et al*., 2002). Preoperative prophylaxis with intravenous glucocorticoid, diphenhydramine, and famotidine was found to reduce the severity, but not the overall incidence of adverse reactions to isosulfan blue dye (Raut *et al*., 2005). Use of methylene blue dye has associated risk of skin necrosis; however, dilution of 0.5 ml of methylene blue (10 mg/ml) with 3.5 ml of normal saline decreases the risk of skin necrosis, while still providing reliable sentinel lymph node mapping (Zakaria *et al*., 2007). Patent blue V dye is the most commonly used blue dye in Europe, with a usual dose of 1–2 ml of 2.5% patent blue dye V (Guerbet, Roissy-Charles-de-Gaulle, France). Whichever blue dye is utilized, it is injected at the start of the operation under sterile conditions and followed by massage for 3–5 min prior to skin incision.

In the case of a patient undergoing prophylactic surgery, it is important to minimize any side-effects or potential complications. Therefore, the authors avoid the use of vital blue dyes for sentinel lymph node surgery in prophylactic mastectomy, and use Tc^{99m} sulfur colloid as the sole tracer, although some authors still use dual tracers in this situation (Table 37.1). A caveat to this is that if the patient is undergoing concomitant mastectomy for invasive disease and would be receiving the blue dye for lymphatic mapping of the index tumor, then the patient is exposed to lymphazurin for the index tumor and the argument may be made to use lymphazurin on both sides.

Site of Injection of Mapping Agents

The site of injection of mapping agent in a prophylactic mastectomy, which has no index tumor, was extrapolated from our clinical experience with sentinel lymph node mapping in patients with invasive breast cancer. In prophylactic mastectomy, the tracer is most commonly injected in the subareolar location or as reported by some authors, in the upper outer quadrant of the breast (King *et al*., 2004). Other authors report the use of six equally spaced periareolar injections to subtend all four quadrants and the subareolar plexus (Dupont *et al*., 2000). Overall, in patients with an invasive tumor, studies have found that the sentinel lymph node can be identified by the use of intraparenchymal, subdermal, intradermal, or subareolar injections, and these staged the axilla with comparable accuracy. The breast is developed embryologically from the centrally located nipple breast bud, and, therefore, the mammary lymphatics drain through the subareolar plexus of Sappey to the axillary nodal basin. Because it is felt that the entire breast and its overlying skin may function as a single lymphatic unit in most patients,

TABLE 37.1. Sentinel lymph node surgery in prophylactic mastectomy patients.

Paper	Number of PMs	Tracer used	Injection site	Incision for SLN surgery	Average number of SLN	Number with occult cancer in PM	Number with occult invasive cancer in PM	Number positive SLN with invasive cancer in PM	Number positive SLN with no invasive cancer in PM
Boughey *et al*. (2006)	436	Tc^{99m} +/– isosulfan blue	Subareolar	Mastectomy or separate axillary incision	n/a	22 (5%)	8 (1.8%)	0	2/106 (1.9%)
King *et al*. (2004)	163	0.1 mCi (3.7 MBq) unfiltered Tc^{99m} and 4 ml isosulfan blue	Intradermal Tc^{99m}, intraparenchymal blue dye in upper outer quadrant	Mastectomy or separate axillary incision	2.3	13 (8.0%)	4 (2.5%)	2	1/163 (0.6%)
Dupont *et al*. (2000)	57	0.45 mCi Tc^{99m} and isosulfan blue	Four quadrant periareolar injection (6 injections)	Mastectomy incision	2.6	2 (3.5%)	2	0	2/57 (3.5%)
Soran *et al*. (2006)	155	0.45–0.495 mCi filtered Tc^{99m} and isosulfan blue dye	Not given	Mastectomy incision	2.6	5 (3.2%)	2 (1.3%)	0	2/80 (2.5%)

there has been widespread acceptance of these multiple injection sites for sentinel lymph node surgery in patients with an invasive tumor, and, therefore, it follows that the use of a subareolar or a periareolar intradermal injection site should reliably stage the axilla in patients undergoing sentinel lymph node surgery for prophylactic mastectomy. Intradermal, subareolar and intraparenchymal injections show similar drainage patterns to the axilla; however, intradermal and subareolar injections are less likely to demonstrate extra-axillary sites of drainage such as internal mammary nodes. The vital blue dye, if utilized, is similarly injected in the subareolar or periareolar location.

Timing of Injection of Mapping Agent

Isosulfan blue dye is rapidly absorbed by the lymphatics, so it must be injected within 5–10 min of the procedure, and the sentinel lymph node surgery should be performed as the first part of the operation. Radioactive colloid particles travel more slowly. After intraparenchymal injection, at least 30–60 min are required for the colloid to localize in the sentinel lymph node. Absorption is faster after intradermal and subareolar injection. Injection of the radioactive colloid the day prior to surgery allows more flexibility in the operating schedule, and an increased dose can be administered (Solorzano *et al*., 2001; McCarter *et al*., 2001).

Lymphoscintigraphy

A lymphoscintigram is obtained in some institutions after the radioactive tracer injection in patients with breast cancer. Lymphoscintigraphy can show some unexpected patterns of lymphatic drainage to the supraclavicular, internal mammary, contralateral axilla or Rotter's nodes in breast cancer patients (Jansen *et al*., 2000). The clinical relevance of this finding is uncertain, and the role of routine preoperative lymphoscintigraphy in breast cancer patients continues to be debated. In patients undergoing a prophylactic mastectomy, the role of preoperative lymphoscintigraphy is minimal and the majority of surgeons would forego preoperative lymphoscintigraphy in these patients. In the event that preoperative lymphoscintigraphy showed extra-axillary drainage, the authors would not recommend evaluating these additional nodal basins in the absence of an invasive tumor in the breast, because this can add significant morbidity to the prophylactic operation, without any known clinical benefit.

Surgical Technique

The sentinel lymph node dissection can be performed prior to or after completion of the mastectomy dissection. When blue dye is utilized for lymphatic mapping the kinetics of blue dye drainage favor evaluation of the sentinel lymph node at the start of the operation, prior to the mastectomy. Otherwise, sentinel lymph node dissection is usually performed after completion of the mastectomy. As dissection is performed along the lateral edge of the breast tissue, close to the axilla, attention should be paid to identify any blue lymphatic channels or nodes (if blue dye was utilized) and any palpable nodes or radioactive nodes, as detected with the gamma probe (Figures 37.2 and 37.3). When the mastectomy specimen has been resected and prior to submission to pathology, the axillary tail of the breast is

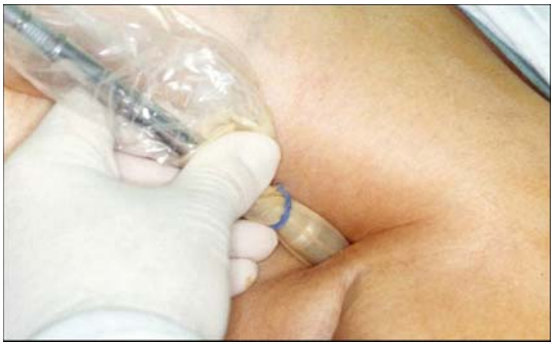


FIGURE 37.2. Locating the sentinel lymph node through the skin using the gamma probe

evaluated using the gamma probe to ensure that none of the sentinel lymph nodes are included in the mastectomy specimen. If an area of increased radioactivity or a blue lymph node is identified within the mastectomy specimen, the sentinel lymph node is dissected out from the mastectomy specimen and submitted to pathology separately as a sentinel lymph node.

Attention is then turned to the axilla, where the sentinel lymph node is identified using the gamma probe and/or the identification of blue lymphatics. If a simple mastectomy without reconstruction is performed, the axilla can be accessed through the incision utilized for the mastectomy to allow focused dissection in the axilla to identify the sentinel lymph nodes in the standard fashion. If a skin-sparing mastectomy with immediate reconstruction is being performed, then dependent on the size of the incision and access to the axilla, the sentinel lymph node surgery can be performed either through the mastectomy incision or with a small counter incision in the axilla. In our experience, in patients with larger breasts, it is easy to manipulate the overlying skin to allow adequate access to the axilla as opposed to patients with a smaller breast size where access to the axilla may be limited through a skin-sparing mastectomy incision. When a counter incision in the axilla is required, a gamma probe is used to identify the area in the axilla with the highest radioactivity counts, and the skin incision is placed in this location. Care must be taken to avoid placing the incision through the hair bearing tissue and sweat glands of the axilla.

The sentinel lymph node is defined as any lymph node in the axilla that is radioactive, blue or palpably abnormal. Any node with a radioactivity count that is 10% or greater of the most radioactive node excised should similarly be excised. Any node that is blue (if blue dye is used), is excised and any palpably abnormal lymph nodes should also be removed, even if it does not contain any radioactivity or blue dye staining. Occasionally, lymph nodes that are completely replaced by tumor may present in this manner. In the majority, most authors report a mean of two to three sentinel lymph nodes removed per patient (Solorzano *et al*., 2001; Povoski *et al*., 2007; Chagpar *et al*., 2006). It is uncommon to remove more than four lymph nodes and in the setting of a prophylactic operation, as in any sentinel lymph node surgery, attention should be paid to minimize disruption of lymphatics and damage to intercostobrachial nerves. Dissection should be focused and directed to minimize the morbidity associated with sentinel lymph node surgery.

An alternative and more selective method for sentinel lymph node evaluation in patients undergoing prophylactic mastectomy is to limit the sentinel lymph node surgery to those patients whose intraoperative breast findings are worrisome for carcinoma. In this approach, all patients undergoing prophylactic mastectomy have

radioactive colloid injected preoperatively. At the time of surgery when the prophylactic mastectomy has been performed, immediate intraoperative pathologic evaluation of the breast specimen is obtained. The prophylactic mastectomy specimen is sliced into thin slices of 1–2 cm width, each slice palpated separately to evaluate for any areas of thickening or abnormality, and then frozen sections of any abnormal areas are obtained. This allows the intraoperative diagnosis of any occult cancer at the time of prophylactic mastectomy. Additionally, sections from each quadrant and the nipple are submitted for histological analysis. If breast cancer is identified, then sentinel lymph node dissection and evaluation follows. Otherwise, no lymph nodes are harvested from the axilla; thus, limiting the potential complications of sentinel lymph node dissection to those patients that require nodal evaluation.

If the sentinel lymph node is unable to be identified at the time of prophylactic mastectomy, axillary lymph node dissection for staging of the axilla is not recommended. Final pathological analysis of the mastectomy specimen should be awaited, and then in the event that an occult invasive malignancy is found, the patient would need to return to the operating room for axillary lymph node dissection for accurate axillary staging of the occult malignancy.

Pathological Analysis of Sentinel Lymph Node

Intraoperative analysis of the sentinel lymph node can allow identification of lymph node metastasis if present and if identified at the time of surgery, enables the surgeon to proceed to an immediate completion axillary lymph node dissection. This spares the patient a potential reoperation. Intraoperative analysis of the sentinel lymph node can be performed either by immediate frozen section analysis or intraoperative touch preparation techniques. These methodologies have been shown to be limited in their ability to detect micrometastasis, which tend to be associated with smaller invasive cancers. The sensitivity of frozen section increases as the size of the primary tumor increases and the benefit of frozen section ranges from 4% for Tla tumors to 38% for T2 tumors (Weiser *et al.*, 2000). Therefore, some surgeons / institutions use intraoperative evaluation only for larger invasive tumors and not for small volume disease or prophylactic mastectomy, as occult disease in a prophylactic mastectomy specimen is most commonly T1 tumor. Pathological analysis of the sentinel lymph nodes involves embedding the sentinel lymph node in paraffin and examination with serial sectioning with hematoxylin and eosin staining and immunohistochemical staining with anticytokeratin antibodies (Yared *et al.*, 2002). In the event that no invasive cancer is found in the prophylactic mastectomy specimen, the use of

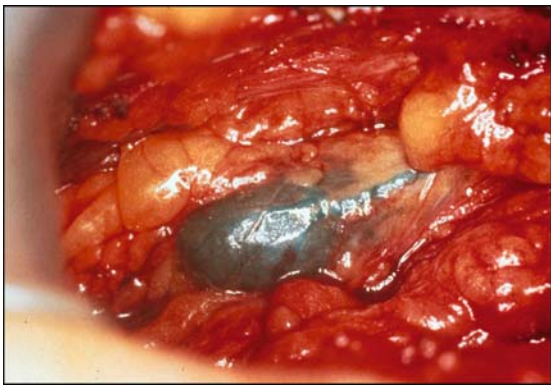


FIGURE 37.3. Intraoperative sentinel lymph node identification

immunohistochemical staining is questionable, because the findings may not be clinically relevant (Lyman *et al*., 2005).

INDICATIONS FOR PERFORMING SENTINEL LYMPH NODE SURGERY AT THE TIME OF PROPHYLACTIC MASTECTOMY

There have been few studies evaluating the use of sentinel lymph node surgery at the time of prophylactic mastectomy and although sentinel lymph node surgery is an accurate and minimally invasive method of evaluating the lymphatic basin, the indication for this at the time of prophylactic mastectomy is currently debated. The complication rates of sentinel lymph node surgery are not negligible and include axillary wound infection 1.0%, axillary seroma 7.1%, and axillary hematoma 1.4%. Of significance are the reported long-term complications of axillary paresthesias (9–22%), lymphedema (7%), and decreased range of motion in the shoulder (0–4%) (Wilke *et al*., 2006; Lucci *et al*., 2007; Fleissig *et al*., 2006; Veronesi *et al*., 2003; Schrenk *et al*., 2000). Unfortunately, the majority of studies evaluating the complications of sentinel lymph node surgery are in patients undergoing wide local excision and, therefore, the true morbidity related to the addition of sentinel lymph node surgery in a patient undergoing a prophylactic mastectomy is not well documented. Overall, the incidence of finding an occult cancer at the time of prophylactic mastectomy is 5%; however, the majority of these are DCIS which does not require nodal staging. Therefore, the patients that benefit from sentinel lymph node surgery at the time of prophylactic mastectomy are the 2% (range 0.1–3.5%) (Dupont *et al*., 2000; King *et al*., 2004; Goldflam *et al*., 2004; McDonnell *et al*., 2001) of patients with invasive cancer found within a prophylactic mastectomy specimen. We performed a decision analysis model at our institution evaluating the complications associated with routine sentinel lymph node surgery on every patient undergoing prophylactic mastectomy in comparison to performing a subsequent axillary lymph node dissection only on the individuals found to have an occult invasive cancer. This analysis revealed that routine use of sentinel lymph node surgery is not warranted during prophylactic mastectomy. In fact, the probability of complications was nine-fold higher with routine sentinel lymph node surgery compared with directed axillary lymph node dissection. The complication rates for the two strategies become equivalent in the model scenario when the prevalence of occult invasive cancer in the prophylactic mastectomy is projected to be 28%. Although no group of patients has been shown to harbor such a high risk of occult invasive malignancy, there are patients who are at > 1.8% risk of finding carcinoma in the prophylactic mastectomy breast. These include postmenopausal women and women with a history of invasive lobular carcinoma or lobular carcinoma in situ in the index breast (Boughey *et al*., 2006b) where the risk of invasive cancer in the prophylactic mastectomy specimen may be as high as 10%. In these patients, the risk to benefit ratio is more favorable, and therefore consideration can be given to routine sentinel lymph node biopsy at the time of prophylactic mastectomy.

One additional consequence of performing sentinel lymph node surgery in all patients undergoing prophylactic mastectomy is the identification of patients without any occult invasive cancer in their prophylactic mastectomy specimen who are nonetheless found to have cancer cells in the sentinel lymph node. Some have argued that these 'metastatic' deposits may represent benign transport of epithelial cells (Bleiweiss *et al*., 2006). However, it is difficult to distinguish such benign epithelial transport from true metastatic cells draining an ipsilateral undetected primary breast cancer. Therefore, the true clinical relevance of finding cancer cells in the sentinel lymph node without a corresponding carcinoma in the breast is unknown and the treatment of this finding is also debated. In some studies, patients have undergone an axillary lymph node dissection, and in other studies patients have not been subjected to further surgical or medical treatment (Dupont *et al*., 2000; King *et al*., 2004).

In cases when a patient has a locally advanced index cancer and where a positive sentinel lymph node is found in the contralateral prophylactic mastectomy, the positive sentinel lymph node may be due to cross-axillary metastasis from the index breast cancer. In a series of 52 patients undergoing sentinel lymph node and prophylactic mastectomy at Massachusetts General Hospital, only two positive sentinel lymph nodes were found, which were both metastases from the known cancers (Black, 2006). Similarly, at Moffit Cancer Center, 421 patients undergoing sentinel lymph node and contralateral prophylactic mastectomy, nine patients were found to have a positive sentinel lymph node (2%). All of these were metastases from the known locally advanced cancer in the index breast (Alvarado *et al*., 2006). Therefore, it has been suggested that sentinel lymph node surgery may be considered in contralateral prophylactic mastectomy in patients with locally advanced primary breast cancer, for staging of the index breast tumor, rather than staging of the prophylactic breast (Alvardo *et al*., 2006). In such patients, when bilateral mastectomy is being planned where one is for invasive breast cancer and the other is for risk reduction, an argument can also be made for preoperative lymphoscintigraphy of the index tumor side in order to identify potential cross axillary drainage, and thus assist with interpretation of pathology findings in the sentinel lymph node on the prophylactic mastectomy side.

In conclusion, patients with abnormalities on imaging of the prophylactic mastectomy breast or patients at increased risk of occult malignancy should be considered for sentinel lymph node surgery at the time of prophylactic mastectomy. Because the overall incidence of invasive cancer in the prophylactic mastectomy specimen is low, the routine use of sentinel lymph node surgery in all patients undergoing prophylactic mastectomy remains controversial. The morbidity of adding sentinel lymph node surgery to a prophylactic procedure should be minimized by choice of mapping agents and minimal disruption of axillary lymphatics. Lymphoscintigraphy is not generally required.

REFERENCES

Alvarado, M.D., Cox, C.E., Carter, W.B., Hoover, S., and Laronga, L.C.H, 2006. Moffitt Cancer Center, Tampa, FL. Use of sentinel lymph node biopsy in a prophylactic mastectomy setting. Breast Cancer Research and Treatment, *29th*

Annual San Antonio Breast Cancer Symposium. 100:S82.

Bedrosian, I., Reynolds, C., Mick, R., Callans, L.S., Grant, C.S., Donohue, J.H., Farley, D.R., Heller, R., Conant, E., Orel, S.G., Lawton, T., Fraker, D.L., and Czerniecki, B.J., 2000. Accuracy of sentinel lymph node biopsy in patients with large primary breast tumors. *Cancer 88*: 2540–2545.

Black, D.M., 2006. Detecting Occult Breast Cancer in Prophylactic Mastectomies: Sentinel Node Biopsy vs. Pre-Operative MRI. *Society of Surgical Oncology 59th Annual Cancer Symposium. Ann. Surg. Oncol.13: 9*.

Bleiweiss, I.J., Nagi, C.S., and Jaffer, S., 2006. Axillary sentinel lymph nodes can be falsely positive due to iatrogenic displacement and transport of benign epithelial cells in patients with breast carcinoma. *J. Clin. Oncol. 24: 2013–2018*.

Boughey, J.C., Bedrosian, I., Meric-Bernstam, F., Ross, M.I., Kuerer, H.M., Akins, J.S., Giordono, S.H., Babiera, G.V., Ames, F.C., and Hunt, K.K., 2006a. Comparative analysis of sentinel lymph node operation in male and female breast cancer patients. *J. Am. Coll. Surg. 203: 475–480*.

Boughey, J.C., Khakpour, N., Meric-Bernstam, F., Ross, M.I., Kuerer, H.M., Singletary, S.E., Babiera, G.V., Arun, B., Hunt, K.K., and Bedrosian, I., 2006b. Selective use of sentinel lymph node surgery during prophylactic mastectomy. *Cancer 107: 1440–1447*.

Breslin, T.M., Cohen, L., Sahin, A., Fleming, J.B., Kuerer, H.M., Newman, L.A., Delpassand, E.S., House, R., Ames, F.C., Feig, B.W., Ross, M.I., Singletary, S.E., Buzdar, A.U., Hortobagyi, G.N., and Hunt, K.K., 2000. Sentinel lymph node biopsy is accurate after neoadjuvant chemotherapy for breast cancer. *J. Clin. Oncol. 18:* 3480–3486.

Chagpar, A.B., Scoggins, C.R., Martin, R.C., 2nd, Carlson, D.J., Laidley, A.L., E1-Eid, S.E., McGlothin, T.O., and McMasters, K.M., 2006. University of Louisville Breast Sentinel Lymph Node Study. Prediction of sentinel lymph node-only disease in women with invasive breast cancer. *Am. J. Surg. 192*: 882–887.

Cimmino, V.M., Brown, A.C., Szocik, J.F., Pass, H.A., Moline, S., De, S.K., and Domino, E.F., 2001. Allergic reactions to isosulfan blue during sentinel node biopsy–a common event. *Surgery 130*: 439–442.

Cox, C.E., Salud, C.J., and Harrinton, M.A., 2000. The role of selective sentinel lymph node dissection in breast cancer. *Surg. Clin. North Am. 80*: 1759–1777.

Czerniecki, B.J., Scheff, A.M., Callans, L.S., Spitz, F.R., Bedrosian, I., Conant, E.F., Orel, S.G., Berlin, J., Helsabeck, C., Fraker, D.L., and Reynolds, C., 1999. Immunohistochemistry with pancytokeratins improves the sensitivity of sentinel lymph node biopsy in patients with breast carcinoma. *Cancer 85*: 1098–1103.

Dupont, E.L., Kuhn, M.A., McCann, C., Salud, C., Spanton, J.L., and Cox, C.E., 2000. The role of sentinel lymph node biopsy in women undergoing prophylactic mastectomy. *Am. J. Surg. 180*: 274–277.

Fleissig, A., Fallowfield, L.J., Langridge, C.I., Johnson, L., Newcombe, R.G., Dixon, J.M., Kissin, M., and Mansel, R.E., 2006. Post-operative arm morbidity and quality of life. Results of the ALMANAC randomized trial comparing sentinel node biopsy with standard axillary treatment in the management of patients with early breast cancer. *Breast Cancer Res. Treat. 95*: 279–293.

Gershenwald, J.E., Hunt, K.K., Kroll, S.S., Ross, M.I., Baldwin, B.J., Feig, B.W., Ames, F.C., Schusterman, M.A., and Singletary, S.E., 1998. Synchronous elective contralateral mastectomy and immediate bilateral breast reconstruction in women with early-stage breast cancer. *Ann. Surg. Oncol. 5*: 529–538.

Giuliano, A.E., Kirgan, D.M., Guenther, J.M., and Morton, D.L., 1994. Lymphatic mapping and sentinel lymphadenectomy for breast cancer. *Ann. Surg. 220*: 391–398; discussion 398–401.

Goldflam, K., Hunt, K.K., Gershenwald, J.E., Singletary, S.E., Mirza, N., Kuerer, H.M., Babiera, G.V., Ames, F.C., Ross, M.I., Feig, B.W., Sahin, A.A., Arun, B., and Meric-Bernstam, F., 2004. Contralateral prophylactic mastectomy. Predictors of significant histologic findings. *Cancer 101*: 1977–1986.

Hartmann, L.C., Schaid, D.J., Woods, J.E., Crotty, T.P., Myers, J.L., Arnold, P.G., Petty, P.M., Sellers, T.A., Johnson, J.L., McDonnell, S.K., Frost, M.H., and Jenkins, R.B., 1999. Efficacy of bilateral prophylactic mastectomy in women with a family history of breast cancer. *N. Engl. J. Med. 340*: 77–84.

Hartmann, L.C., Sellers, T.A., Schaid, D.J., Frank, T.S., Soderberg, C.L., Sitta, D.L., Frost, M.H., Grant, C.S., Donohue, J.H., Woods, J.E.,

McDonnell, S.K., Vockley, C.W., Deffenbaugh, A., Couch, F.J., and Jenkins, R.B., 2001. Efficacy of bilateral prophylactic mastectomy in BRCA1 and BRCA2 gene mutation carriers. *J. Natl. Cancer. Inst. 93*: 1633–1637.

Herrinton, L.J., Barlow, W.E., Yu, O., Geiger, A.M., Elmore, J.G., Barton, M.B., Harris, E.L., Rolnick, S., Pardee, R., Husson, G., Macedo, A., and Fletcher, S.W., 2005. Efficacy of prophylactic mastectomy in women with unilateral breast cancer: a cancer research network project. *J. Clin. Oncol. 23*: 4275–4286.

Hill-Kayser, C.E., Harris, E.E., Hwang, W.T., and Solin, L.J., 2006. Twenty-year incidence and patterns of contralateral breast cancer after breast conservation treatment with radiation. *Int. J. Radiat. Oncol. Biol. Phys. 66*: 1313–1319.

Jansen, L., Doting, M.H., Rutgers, E.J., de Vries, J., Olmos, R.A., and Nieweg, O.E., 2000. Clinical relevance of sentinel lymph nodes outside the axilla in patients with breast cancer. *Br. J. Surg. 87*: 920–925.

Julian, T., and Krag, D., 2004. A randomized phase III clinical trial to compare sentinel node resection to conventional axillary dissection in clinically node-negative breast cancer patients. *Breast Cancer Res. Treat.*: abstract 140, 2004

Kim, T., Giuliano, A.E., and Lyman, G.H., 2006. Lymphatic mapping and sentinel lymph node biopsy in early-stage breast carcinoma: a metaanalysis. *Cancer 106*: 4–16.

King, T.A., Ganaraj, A., Fey, J.V., Tank, L.K., Hudis, C., Norton, L., Cody, H.S., 3rd, and Borgen, P.I., 2004. Cytokeratin-positive cells in sentinel lymph nodes in breast cancer are not random events: experience in patients undergoing prophylactic mastectomy. *Cancer 101*: 926–933.

Krag, D., Weaver, D., Ashikaga, T., Moffat, F., Klimberg, V.S., Shriver, C., Feldman, S., Kusminsky, R., Gadd, M., Kuhn, J., Harlow, S., and Beitsch, P., 1998. The sentinel node in breast cancer–a multicenter validation study. *N. Engl. J. Med. 339*: 941–946.

Lehman, C.D., Gatsonis, C., Kuhl, C.K., Hendrick, R.E., Pisano, E.D., Hanna, L., Peacock, S., Smazal, S.F., Maki, D.D., Julian, T.B., DePeri, E.R., Bluemke, D.A., and Schnall, M.D., 2007. ACRIN Trial 6667 Investigators Group. MRI evaluation of the contralateral breast in women with recently diagnosed breast cancer. *N. Engl. J. Med. 356*: 1295–1303.

Lucci, A., McCall, L.M., Beitsch, P.D., Whitworth, P.W., Reintgen, D.S., Blumencranz, P.W., Leitch, A.M., Saha, S., Hunt, K.K., and Giuliano, A.E. 2007. Surgical complications associated with sentinel lymph node dissection (SLND) plus axillary lymph node dissection compared with SLND alone in the American College of Surgeons Oncology Group Trial Z0011. *J. Clin. Oncol. 25*: 3657–3663.

Lyman, G.H., Giuliano, A.E., Somerfield, M.R., Benson, A.B., 3rd, Bodurka, D.C., Burstein, H.J., Cochran, A.J., Cody, H.S., 3rd, Edge, S.B., Galper, S., Hayman, J.A., Kim, T.Y., Perkins, C.L., Podoloff, D.A., Sivasubramaniam, V.H., Turner, R.R., Wahl, R., Weaver, D.L., Wolff, A.C., and Winer, E.P., 2005. American Society of Clinical Oncology. American Society of Clinical Oncology guideline recommendations for sentinel lymph node biopsy in early-stage breast cancer. *J. Clin. Oncol. 23*: 7703–7720.

McCarter, M.D., Yeung, H., Yeh, S., Fey, J., Borgen, P.I., and Cody, H.S., 3rd., 2001. Localization of the sentinel node in breast cancer: identical results with same-day and day-before isotope injection. *Ann. Surg. Oncol. 8*: 682–686.

McDonnell, S.K., Schaid, D.J., Myers, J.L., Grant, C.S., Donohue, J.H., Woods, J.E., Frost, M.H., Johnson, J.L., Sitta, D.L., Slezak, J.M., Crotty, T.B., Jenkins, R.B., Sellers, T.A., and Hartmann, L.C., 2001. Efficacy of contralateral prophylactic mastectomy in women with a personal and family history of breast cancer. *J. Clin. Oncol. 19*: 3938–3943.

Meijers-Heijboer, H., van Geel, B., van Putten, W.L., Henzen-Logmans, S.C., Seynaeye, C., Menke-Pluymers, M.B., Bartels, C.C., Verhoog, L.C., van den Ouweland, A.M., Niermeijer, M.F., Brekelmans, C.T., and Kliin, J.G., 2001. Breast cancer after prophylactic bilateral mastectomy in women with a BRCA1 or BRCA2 mutation. *N. Engl. J. Med. 345*: 159–164.

Miner, T.J., Shriver, C.D., Jaques, D.P., Maniscalco-Theberge, M.E., and Krag, D.N., 1999. Sentinel lymph node biopsy for breast cancer: the role of previous biopsy on patient eligibility. *Am. Surg. 65*: 493–498; discussion 498–499.

Montgomery, L.L., Thorne, A.C., Van Zee, K.J., Fey, J., Heerdt, A.S., Gemignani, M., Port, E., Petrek, J., Cody, H.S., 3rd, and Borgen, P.I., 2002. Isosulfan blue dye reactions during sentinel lymph node mapping for breast cancer. *Anesth. Analg. 95*: 385–388.

Povoski, S.P., Young, D.C., Walker, M.J., Carson, W.E., Yee, L.D., Agnese, D.M., and Farrar, W.B., 2007. Re-emphasizing the concept of adequacy of intraoperative assessment of the axillary sentinel lymph nodes for identifying nodal positivity during breast cancer surgery. *World. J. Surg. Oncol. 5*: 18.

Raut, C.P., Hunt, K.K., Akins, J.S., Daley, M.D., Ross, M.I., Singletary, S.E., Marshall, G.D., Jr., Meric-Bernstam, F., Babiera, G., Feig, B.W., Ames, F.C., and Kuerer, H.M., 2005. Incidence of anaphylactoid reactions to isosulfan blue dye during breast carcinoma lymphatic mapping in patients treated with preoperative prophylaxis: results of a surgical prospective clinical practice protocol. *Cancer 104*: 692–699.

Rebbeck, T.R., Friebel, T., Lynch, H.T., Neuhausen, S.L., Van't Veer, L., Garber, J.E., Evans, G.R., Narod, S.A., Isaacs, C., Matloff, E., Daly, M.B., Olopade, O.I., and Weber, B.L., 2004. Bilateral prophylactic mastectomy reduces breast cancer risk in BRCA1 and BRCA2 mutation carriers: the PROSE Study Group. *J. Clin. Oncol. 22*: 1055–1062.

Schrag, D., Kuntz, K.M., Garber, J.E., and Weeks, J.C., 2000. Life expectancy gains from cancer prevention strategies for women with breast cancer and BRCA1 or BRCA2 mutations. *JAMA 283*: 617–624.

Schrenk, P., Rieger, R., Shamiyeh, A., and Wayand, W., 2000. Morbidity following sentinel lymph node biopsy versus axillary lymph node dissection for patients with breast carcinoma. *Cancer 88*: 608–614.

Solorzano, C.C., Ross, M.I., Delpassand, E., Mirza, N., Akins, J.S., Kuerer, H.M., Meric, F., Ames, F.C., Newman, L., Feig, B., Singletary, S.E., and Hunt, K.K., 2001. Utility of breast sentinel lymph node biopsy using day-before-surgery injection of high-dose 99mTc-labeled sulfur colloid. *Ann. Surg. Oncol. 8*: 821–827.

Soran, A., Falk, J., Bonaventura, M., Keenan, D., Ahrendt, G., and Johnson, R., 2006. Is routine sentinel lymph node biopsy indicated in women undergoing contralateral prophylactic mastectomy? Magee-Womens Hospital Experience. *Ann. Surg. Oncol. 14*: 646–651.

Stradling, B., Aranha, G., and Gabram, S., 2002. Adverse skin lesions after methylene blue injections for sentinel lymph node localization. *Am. J. Surg. 184*: 350–352.

Tuttle, T.M., Habermann, E., Grund, E., Morris, T., and Virnig, B., 2007. Increasing use of contralateral prophylactic mastectomy among breast cancer patients: a trend toward more aggressive surgical treatment. *Ann. Surg. Oncol. 14*: 7.

van Sprundel, T.C., Schmidt, M.K., Rookus, M.A., Brohet, R., van Asperen, C.J., Rutgers, E.J., Van't Veer, L.J., and Tollenaar, R.A., 2005. Risk reduction of contralateral breast cancer and survival after contralateral prophylactic mastectomy in BRCA1 or BRCA2 mutation carriers. *Br. J. Cancer. 93*: 287–292.

Veronesi, U., Paganelli, G., Viale, G., Luini, A., Zurrida, S., Galimberti, C., Intra, M., Veronesi, P., Robertson, C., Maisonneuve, P., Renne, G., De Cicco, C., De Lucia, F., and Gennari, R., 2003. A randomized comparison of sentinel-node biopsy with routine axillary dissection in breast cancer. *N. Engl. J. Med. 349*: 546–553.

Waddington, W.A., Keshtgar, M.R., Taylor, I., Lakhani, S.R., Short, M.D., and Ell, P.J. 2000. Radiation safety of the sentinel lymph node technique in breast cancer. *Eur. J. Nucl. Med. 27*: 377–391.

Weiser, M.R., Montgomery, L.L., Susnik, B., Tan, L.K., Borgen, P.I., and Cody, H.S., 2000. Is routine intraoperative frozen-section examination of sentinel lymph nodes in breast cancer worthwhile? *Ann. Surg. Oncol. 7*: 651–655.

Wilke, L.G., McCall, L.M., Posther, K.E., Whitworth, P.W., Reintgen, D.S., Leitch, A.M., Gabram, S.G., Lucci, A., Cox, C.E., Hunt, K.K., Herndon, J.E., 2nd, and Giuliano, A.E., 2006. Surgical complications associated with sentinel lymph node biopsy: results from a prospective international cooperative group trial. *Ann. Surg. Oncol. 13(4)*: 491–500.

Xing, Y., Foy, M., Cox, D.D., Kuerer, H.M., Hunt, K.K., and Cormier, J.N., 2006. Meta-analysis of sentinel lymph node biopsy after preoperative chemotherapy in patients with breast cancer. *Br. J. Surg. 93*: 539–546.

Yared, M.A., Middleton, L.P., Smith, T.L., Kim, H.W., Ross, M.I., Hunt, K.K., and Sahin, A.A., 2002. Recommendations for sentinel lymph node processing in breast cancer. *Am. J. Surg. Pathol. 26*: 377–382.

Zakaria, S., Hoskin, T., and Degnim, A.C., 2007. Safety of methylene blue dye for lymphatic mapping in breast cancer. *Ann. Surg. Oncol 14*: 90.

38
Breast Conservation Surgery: Methods

Lloyd A. Mack, Robert L. Lindsay, and Walley J. Temple

INTRODUCTION

Breast conservation therapy (BCT) has become the preferred treatment for early stage breast cancer in most women. Breast conserving therapy is defined as the complete removal of a breast tumor (invasive breast cancer or duct carcinoma in situ) with a surrounding margin of normal tissue performed in a cosmetically acceptable manner followed by irradiation therapy (Schwartz *et al*., 2006). Well-designed prospective randomized trials (level one evidence) and a meta-analysis of these trials confirm no difference in disease-free or overall survival comparing total mastectomy with BCT (Fisher *et al*., 2002; Veronesi *et al*., 2002; Early Breast Trialists' Colloborative Group 1995). Long-term follow-up of eighteen or more years in both North American and European trials confirm no difference in overall survival rates (Fisher *et al*., 2002; Veronesi *et al*., 2002; Poggi *et al*., 2003). Inclusion criteria varied somewhat between trials for tumor size (strictly ≤ 2 cm in the Milan trial vs. ≤ 5 cm in the NCI trial), excision type (quadrantectomy vs. wide local excision), and margin status (microscopically negative in the NSABP-06 and Milan trials vs. clear gross margins for the NCI trial) (Fisher *et al*., 2002; Veronesi *et al*., 2002; Poggi *et al*., 2003).

There appears to be minor differences in local recurrence rates among these trials and patient subtypes, although no direct comparison studies have been performed. The goal of breast conservation therapy should be a local recurrence rate of 5–10% at 10 years of follow-up or less than 1%/year (Schwartz *et al*., 2006). Quadrantectomy appears to have a lower local recurrence rate of 8.8% at 20 years median follow-up compared to 14.3% for wide local excision at 20 years of median follow-up, although no trial has compared these procedures directly (Veronesi *et al*., 2002; Fisher *et al*., 2002). Further, the European quadrantectomy trial had a strict tumor size criteria of ≤ 2 cm compared to wide local excision trials accepting tumors up to 5 cm in size. Margin status is important with a local recurrence rate of 8.8 and 14.3% in the EORTC and NSABP-06 trials, respectively, at 20 years follow-up with negative microscopic margins (Veronesi *et al*., 2002; Fisher *et al*., 2006). This compares to a 22% local recurrence rate at a median follow-up of 18 years in the NCI trial where negative margins were

not required to enter the trial (Poggi *et al*., 2003). Of 121 patients entering the breast conservation arm of the trial, microscopic margin status was not assessed (Poggi *et al*., 2003). Furthermore, some data note a higher local recurrence rate among young women less than 45 years (Veronesi *et al*., 2002). Specifically, Veronesi *et al*. (2002) describe the highest local recurrence rate among this patient group with 1.05 recurrences per 100 woman-years of observation compared to 0.63 per 100 woman-years of observation overall. However, this is not felt to be a contraindication to BCT as the local recurrence rate is higher after either BCT or total mastectomy in this age group, and the majority of local recurrences are salvageable in the BCT group (Early Breast Trialists' Colloborative Group, 1995). Clearly, whole breast irradiation is required to minimize local recurrences with a consistent decrease in this problem by two-thirds (Early Breast Trialists' Colloborative Group, 2000). It is also important to reiterate that the metaanalysis comparing local recurrences between BCT and total mastectomy do not show significant differences (Early Breast Trialists' Colloborative Group, 1995).

Indications and contraindications for breast conservation therapy are generally agreed upon, although many prior absolute contraindications are becoming more relative (Schwartz *et al*., 2006). Most women with breast cancers ≤ 3 cm are candidates for BCT, and many women with larger tumors are also candidates if good cosmetic results can be achieved (Schwartz *et al*., 2006). Oncoplastic surgical techniques are extending the boundaries of when BCT is possible with good oncologic and cosmetic outcomes for those with large tumors relative to the size of the breast. Moreover, large tumors may be down-staged with neoadjuvant chemotherapy and still undergo breast conservation surgery (Singletary, 2001). Diffuse calcifications or multicentric tumors are contraindications to BCT as the goal of complete removal of the breast tumor cannot be achieved (Schwartz *et al*., 2006; Singletary, 2001). The inability to obtain clear margins is a contraindication to breast conservation therapy (Schwartz *et al*., 2006). Tumors located close to or beneath the areola may require removal of the nipple-areolar complex (Singletary, 2001). This is not a contraindication to breast conservation surgery and techniques are evolving to successfully address such defects in selected individuals (McMulley *et al*., 2006).

Prior irradiation such as mantle field radiation for Hodgkin disease is a contraindication (Schwartz *et al*., 2006; Singletary, 2001). Furthermore, most radiation oncologists feel that a personal history of scleroderma, systemic or discoid lupus or dermatomyositis are relative contraindications to BCT (Schwartz *et al*., 2006). Axillary nodal status, tumor histology, and family history of breast cancer are not contraindications to BCT if tumor-free margins are achieved as there is no associated difference in local recurrence rates (Singletary, 2001; Kurtz *et al*., 1989; Haas *et al*., 1989; Chabner *et al*., 1998). A systematic review of the management of BRCA 1 and BRCA 2 associated breast cancer notes conflicting reports on local recurrence risk in this subgroup (Liebens *et al*., 2007). Most studies find no difference in local recurrence compared to sporadic breast cancer patients, but some found a higher risk in this patient group. There are not sufficient data to preclude BCT in a

well-informed patient with BRCA 1 or BRCA 2 associated breast cancer. The presence of extensive duct carcinoma in situ should not be a contraindication to breast conservation therapy as long as final margins are negative (Gage *et al*., 1996). Finally, pregnancy is no longer considered a contraindication to BCT. There is no concern regarding surgery during pregnancy other than increased vascularity which makes adequate hemostasis important. Irradiation therapy is contraindicated during pregnancy and should be reserved until delivery as exposure to the fetus can be significant (Schwartz *et al*., 2006; Petrek, 1994). Overall, ~ 90% and 72% of women with stage I and II breast cancer, respectively, are candidates for breast conservation therapy (Morrow *et al*., 1998)

Most importantly, both retrospective and prospective data confirm the majority of women prefer breast conserving surgery and treatment and have improved long-term psychological outcomes correlating with better overall quality of life. In a prospective trial of women felt to be candidates for BCT, ~ 70% anticipated having breast conserving surgery preoperatively. Important factors which influenced their decision making included their surgeon's advice and the possibility of breast cancer cure; stressing the importance of a knowledgeable surgeon and the accumulated evidence regarding breast cancer surgery. (Temple *et al*., 2006) A large 5-year prospective study of quality of life following BCT or mastectomy without reconstruction indicates significantly better body image role, and sexual function scores in the BCT group (Engel *et al*., 2004) Furthermore, those in the mastectomy group felt more insecure, avoided contact with people, and their daily habits were more affected. During the longer term, quality of life improved significantly following treatment in the BCT group but not in the mastectomy group. Despite some inconsistencies in the literature, most large, prospective, long-term trials with validated instruments confirm that, where possible, breast conservation therapy is the treatment of choice for women diagnosed with breast cancer (Fallowfield, 1995; Moyer *et al*., 1997; Temple *et al*., 2006; Engel *et al*., 2004). This chapter discusses the surgical techniques or methods employed in breast conservation surgery. It does not discuss the techniques of mastectomy or axillary node assessment which are discussed in other chapters of this volume.

SURGICAL METHODS

The goals of breast conserving surgery include complete removal of the breast tumor with good margins, acceptable cosmesis, and minimal long-term local recurrence. Wide local excision (lumpectomy) and quadrantectomy are two operations performed in breast conservation surgery, although wide local excision is performed more frequently (Veronesi *et al*., 1983; Fisher, 1979). The two operations differ in magnitude and biologic concept and are compared in the following paragraphs.

Skin Incisions

The placement of the skin incision is important for adequate exposure, the quality of the cosmetic outcome, and its ability to be incorporated into a mastectomy, if

necessary. The most common skin incisions used in wide local excision are short, curvilinear, circumareolar or transverse incisions placed over the underlying tumor (Figure 38.1). These incisions are placed in the line of skin cleavage and generally lead to an acceptable scar on the surface, but other factors including the volume of tissue removed and tumor location influence symmetry and distortion. They allow flexibility in the placement of future incisions if mastectomy or breast reconstruction is needed. In addition, these incisions are used to facilitate the removal of wire-localized breast tumors. Transverse incisions high on the breast may be visible and may be difficult to incorporate into a mastectomy incision and should be placed lower on the breast, if possible (i.e., beneath the top of the bra line) (Schrotia, 2001). Incisions in the lower half of the breast or close to the inframammary crease can cause exaggerated ptosis of the breast and misalignment of the nipples. A vertical incision in this location will generally prevent this deformity but can be difficult to plan mastectomy incisions, if necessary (Schrotia, 2001).

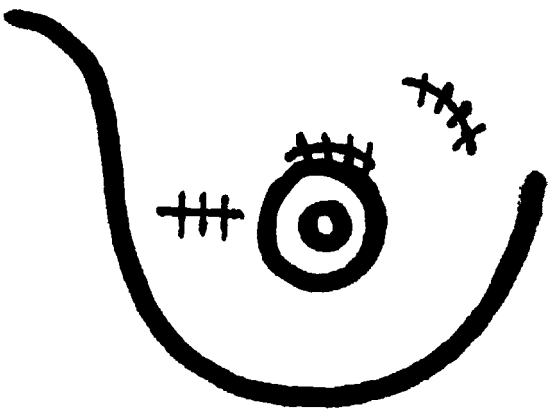


FIGURE 38.1. Standard and alternate skin incisions for wide local excision.

The above incisions are in contrast to the classic incision of a quadrantectomy, where relatively long radial incisions are made (Veronesi *et al*., 1983). Other options for skin incisions include those in the inframammary fold, along the lateral border of pectoralis major, or via the same incision as sentinel lymph node biopsy or axillary node dissection. Incisions in the inframammary fold are made with upward and medial retraction of the breast. Ideally, adequate margins are obtained at the first operation as reoperation or incorporation into a mastectomy is difficult, if needed. Access to the breast tumor can be difficult with incisions along the lateral border of the pectoralis major or via an axillary approach depending on tumor location, but can be adequate and cosmetically sound when oncoplastic techniques are used. Oncoplastic skin incisions, such as fishtailing of a quadrantectomy scar or reduction mammoplasty incisions, are useful to prevent nipple areolar distortion or for cancers in large ptotic breasts (Figure 38.2) (Schrotia, 2001) Oncoplastic incisions can allow wide exposure of the breast tissue involved with tumor, may follow the pattern of standard reduction mammoplasty techniques or may be adapted in cases where the tumor is in a location that is not normally removed in the standard reduction procedures. The specific techniques of oncoplastic surgery are discussed later.

Tumor Removal

In a wide local excision, the tumor is removed with ~ 1 cm of normal breast tissue and does not usually involve removal of skin or pectoralis fascia or muscle

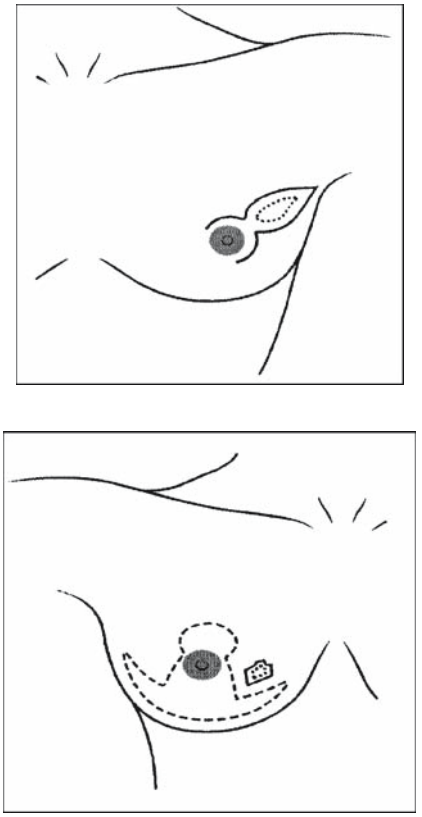


FIGURE 38.2. Oncoplastic mammaplasty incisions. Figure adapted from Schrotia A. Techniques for improving the cosmetic outcome of Breast-conserving surgery. EJSO 2001;27:109–112. Copyright Elsevier. Reproduced with permission.

unless within that 1 cm. If the tumor is on chest wall fascia, pectoralis muscle or close to skin then an appropriate margin of 1 cm is also taken of these tissues. In a quadrantectomy, at least a 2–3 cm cuff of normal breast tissue is removed and the entire quadrant was removed in early descriptions of the procedure. Furthermore, overlying skin and underlying pectoralis fascia are removed in continuity (Veronesi *et al*., 1983). Typically, the tumor and surrounding normal breast tissue is removed with an electrocautery device to ensure adequate hemostasis. The surgeons place their fingers between the palpable breast mass and the line of dissection to ensure a clear gross margin. Adequate retraction and tension are required. Care must be taken to avoid injuring the skin with the electrocautery or transmission of heat injury through the retractor.

Regardless of the method of tumor removal, once the specimen is removed, proper orientation with sutures by the surgeon is necessary for the pathologist's interpretation of margins. Further excision of inadequate margins cannot be achieved without it. Specimen radiograph is also required to ensure removal of nonpalpable lesions. Either wire-guided localization as commonly used in North America or the technique of radioguided lesion localization with a radioactive tracer may be used to remove nonpalpable breast tumors (Nadeem *et al*., 2005). Many surgeons recommend placing clips to demarcate the surgical bed to assist in delivering adequate irradiation therapy. Both boost volume as well as delineating the borders of the tangential field can be improved via this relatively simple process. Placement of balloon brachytherapy or the MammoSite radiation system may be considered for partial breast brachytherapy, although data comparing this technique to external beam radiation is quite preliminary (Graham and Fourquet, 2006). A balloon catheter is inserted into the lumpectomy cavity either via the main incision or tunneled from a small counter-incision and inflated with 30–70 mL of saline to conform to the cavity. Following brachytherapy given during 5–7 days, the balloon is deflated and withdrawn.

Although there is no debate on the need to achieve clear microscopic margins circumferentially around the tumor,

there is considerable debate regarding the ideal width of clear margin required. Most experts agree that a wider margin implies a lower likelihood of local recurrence, although there is a paucity of evidenced-based data to support a specific tumor margin width (Schwartz *et al*., 2006). Most of the randomized trials of BCT required microscopically free margins, although some only required grossly free margins at the time of surgery (Fisher *et al*., 2002; Veronesi *et al*., 2002; Poggi *et al*., 2003). Dillon *et al*. (2006) describe 612 patients undergoing breast conservation surgery with 211 (34%) having radial margins < 5 mm at the time of pathologic assessment. One hundred sixty-one patients had a reoperation with 87 having residual disease. Residual disease was present in 58%, 56%, and 45% of those with tumor-margin distances of < 1, ≥ 1 and < 2 mm, and ≥ 2 and < 5 mm, respectively. A retrospective analysis of 341 women with stage I or II breast cancer noted a lower local recurrence rate of 1.8% in those with ≥ 2 mm margins compared to 8.9% in those with clear but < 2 mm margins at median follow-up of 56 months (Kunos *et al*., 2006) In tumors where there is difficulty obtaining clear, wide margins, this may be reflective of the biology of the disease.

Intraoperative margin assessment appears prudent to avoid the possibility of a second procedure. Either the entire specimen margin should be inked or individual cavity margin shavings (~ 0.5 cm) can be submitted separately for pathologic assessment (Barthlmes *et al*., 2003). Intraoperative frozen section assessment was accurate in 84% of the cases among 97 patients in one study (Cendan *et al*., 2005). Furthermore, 19 patients (24%) avoided a second operation as positive margins were reexcised at the time of initial surgery. Similarly, Barros *et al*. (2003) noted 38 (37.3%) of 102 patients had additional surgery for inadequate margins at time of frozen section analysis. Sensitivity and specificity of frozen section margin assessment ranges from 77% to 90% and 97–100%, respectively (Cendan *et al*., 2005; Barros *et al*., 2003; Weber *et al*., 1997). An alternative is to take the gross specimen, properly oriented, to the pathologist for intraoperative gross sectioning and frozen section of the closest gross margin. If clear, generally all margins will be clear. Interestingly, residual disease is detected in ~ 60% of positive margin specimens if resected immediately at the first operation compared to ~ 33% if at a delayed operation (Nasir and Rainsbury, 2003). Nasir and Rainsbury (2003) conclude that there is a more accurate assessment of residual disease at the margins at the first operation compared to a later operation once local tissue destruction and repair mechanisms have been initiated.

Closure of Defect/Skin Closure

Most surgeons close the skin/dermis with an absorbable suture without attempting to close the underlying cavity or defect following wide local excision. The seroma cavity should be allowed to accumulate to retain the most natural contour of the breast (Choi *et al*., 2006). In 266 patients from the Danish randomized trial for breast conservation surgery, 73% felt their cosmetic outcome with this technique was good or excellent (Johansen *et al*., 2002). Breast fibrosis, skin telangiectasia and breast retraction were associated with a less satisfactory result. Fibrosis and telangiectasia may be more related to irradiation than

surgery. Six percent of women underwent further plastic surgical reconstruction and another 15% wished to. This occurred more frequently in younger women, women with larger tumors, and larger breasts. Significant treatment related factors influencing an inferior cosmetic outcome following multivariate analysis included a direct anterior electron field and adjuvant systemic therapy. Skin incision length was assessed by multivariate analysis but was not significant. Skin incision orientation or placement was not assessed.

Oncoplastics

The boundaries of breast conservation surgery may be extended with the relatively new area of oncoplastics. Moreover, the boundaries between breast and plastic surgery are breaking down and newer techniques combining oncologic and cosmetic outcomes are being developed. Oncoplastics is no longer solely confined to post-mastectomy reconstruction. Reconstruction after a breast-sparing operation is a relatively new oncoplastic approach to facilitate immediate reconstruction of larger resection defects. These techniques are useful if it is anticipated that by excising a volume of > 10% or excision in a sensitive site (medial or inferior), that the outcome will be unsatisfactory in appearance; significant asymmetry, nipple ptosis, or retraction (Cochrane *et al*., 2003). These techniques are divided into volume replacement procedures and volume displacement procedures.

Volume replacement procedures combine resection with immediate reconstruction of the defect or cavity. Examples include the use of breast implants or latissimus dorsi mini-flaps to reconstruct deficits in the breast tissue. The goal is to restore symmetry following the loss of a significant amount of breast tissue. Insertion of tissue expanders/implants carry some risks. In a series by Nano *et al*. (2005), 10 of 124 implants were removed due to infection (4/124) or extracapsular rupture (6/124). Quality of life (Functional Assessment of Cancer Therapy-Breast) was also assessed by this group and no differences were found among different reconstruction methods including implant insertion, latissimus dorsi mini-flaps for partial mastectomy, or transverse rectus abdominis myocutaneous (TRAM) flaps following mastectomy. Latissimus dorsi mini- flaps are harvested via an axillary incision with the muscle being inserted into the defect left after excision of the tumor mass (Figure 38.3) (Shrotia, 2001; Rainsbury, 2002). This technique appears most useful for upper or central defects.

Volume displacement techniques combine resection with breast reduction techniques and frequently involve a contralateral breast reduction to ensure symmetry (Anderson *et al*., 2005). In its simplest form, a local flap of adjacent breast tissue is rotated, transposed or advanced to fill the defect following tumor excision (Bold *et al*., 1997). This is performed with extension of the incision and dissection of breast tissue off of the pectoralis fascia. A more common oncoplastic technique is the use of reduction mammaplasty. One of the standard breast reduction incisional patterns may be used that facilitates good exposure for tumor excision with good margins (Figure 38.4). Modifications may be used if the tumor is located in an area that is not normally removed in the standard reduction techniques (Figure 38.5). The pedicle chosen for nipple areola pres-

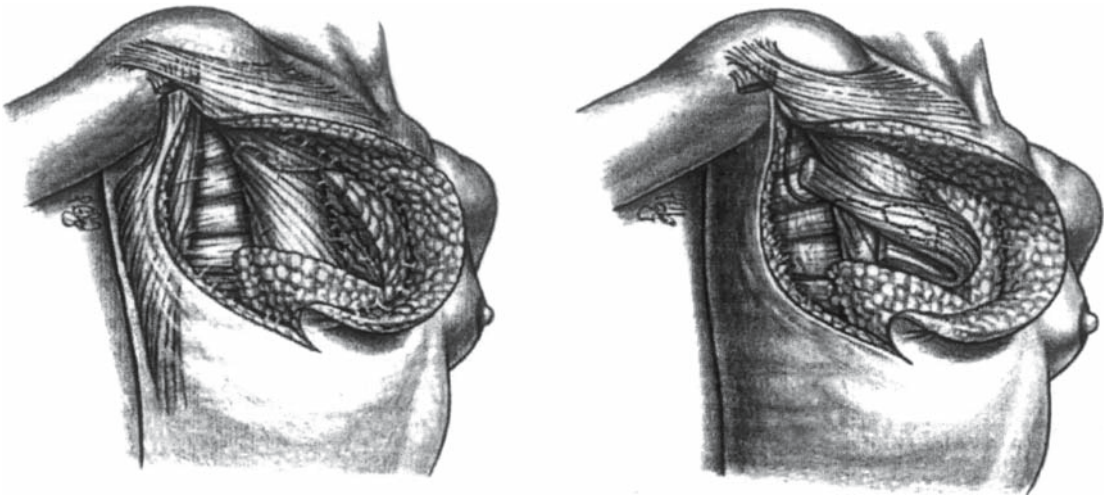


FIGURE 38.3. Oncoplastic latissimus dorsi mini-flap reconstruction.
Figure adapted from Raja et al (1997). Extending the role of breast conserving surgery by immediate volume replacement.

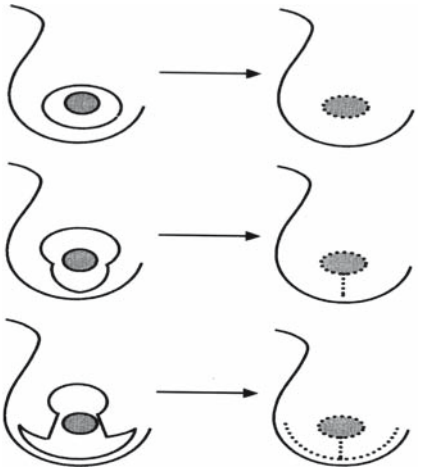


FIGURE 38.4. Reduction mammaplasty. Figure adapted from McCulley and Macmillan (2005) Planning and use of therapeutic mammoplasty. Copyright Elsevier. Reproduced with permission.

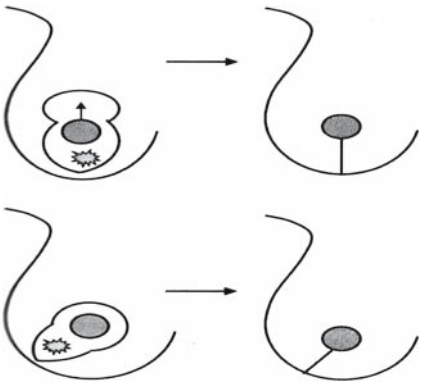


FIGURE 38.5. Modified reduction mammaplasty incisions. Figure adapted from McMulley and Macmillan (2005). Planning and use of therapeutic mammoplasty. Copyright Elsevier. Reproduced with permission.

ervation may be altered according to the location of planned resection (McMulley *et al*., 2006). Additional oncoplastic volume displacement techniques include the round block technique for reconstruction of defects of central resections and reconstruction of the nipple/areolar complex when these structures are involved by tumor and resected (Benelli, 1990; Schoeller and Huemer, 2006; McMulley *et al*., 2006). While a complete review of all oncoplastic techniques is beyond the scope of this

chapter, references by Masetti *et al*. (2006), Anderson *et al*. (2005), and McCulley *et al*. (2006) are quite comprehensive.

Regardless of the oncoplastic technique employed, it is imperative to meet the oncologic objectives of complete tumor resection with clear margins and long-term local control. Extensive internal remodeling of the breast can increase the complexity of a subsequent reexcision if a negative margin is not achieved. Generally, these patients require mastectomy but this most likely would have been the treatment alternative if oncoplastic techniques had not been used. A few small studies in expert centers are reassuring. Kaur *et al*. (2005) prospectively compared 30 patients undergoing quandrantectomy to 30 undergoing an oncoplastic reduction mammaplasty type resection. The mean volume of tissue resected was significantly larger in the oncoplastic group and margins were more often negative. Similarly, Giacolone *et al*. (2007) prospectively compared 41 patients with quadrandectomy and 31 with oncoplastic resections. Again, median tissue volume was larger in the oncoplastic group and the nearest margins were larger. However, there was no difference in re-operation rates for involved margins. Although reassuring, neither study is randomized or adequately powered to detect differences in local control. A review by Asgeirsson *et al*. (2005) indicates a general lack of oncological outcomes or long-term results in most studies of oncoplastic techniques. With intermediate follow-up of up to 4.5 years, local recurrence rates vary from 0% to 1.8% per year. Further, cosmetic failures still occur varying from 0% to 18%. All studies in this review are small with generally < 50 patients and follow-up times all < 5 years. However, a recently published, larger trial of 148 consecutive patients with median follow-up of 74 months is encouraging (Rietjens *et al*., in press). These authors note a 91% complete excision rate with 5% of patients having focally involved margins and 3% with close (< 2 mm) margins. Furthermore, the local recurrence rate was 3% with > 5 year follow-up. While oncoplastic techniques are appealing and likely oncologically sound, additional sufficiently large, prospective cohort studies with adequate follow-up are required.

Local tissue complications including delayed wound healing, areas of fat necrosis which may require biopsy, or nipple areola tissue loss or sensation loss may occur in up to 17% of patients (Munhoz *et al*., 2006) Complications may also occur with a contralateral reduction mammaplasty. Finally, oncoplastic techniques take longer than a wide local excision but require less time than mastectomy and reconstruction.

Minimally Invasive/Ablative Techniques

Minimally invasive techniques are being developed in the treatment of invasive breast cancer. Tumor ablation approaches include cryotherapy, radiofrequency ablation, as well as less studied techniques such as ethanol ablation, laser therapy, microwave techniques or focused ultrasound (Singletary, 2001; Vlastos and Verkooijen, 2007). The two most promising techniques appear to be cryotherapy and radiofrequency ablation, both with percutaneous ultrasound guided applications. In cryotherapy, a hypoechoic iceball is formed with resulting cellular damage. In a small study of 21 patients, all tumors < 1 cm were completely ablated (Sabel *et al*., 2004). Larger tumors were not completely destroyed. In radiofrequency

ablation, frictional heat is generated by intracellular ions moving in response to an alternating current. A pilot study of 20 patients confirmed successful ablation of tumor cells within the zone of ablation for lesions < 2 cm in size. However, residual tumor beyond the ablated area was found in one patient and was not apparent on preoperative imaging. While appealing, these techniques are still experimental with standard tumor resection being performed to assess for viable cells and final margins.

REFERENCES

Anderson, B.O., Masetti, R., and Silverstein, M.J. 2005. Oncoplastic approaches to partial mastectomy: an overview of volume-displacement techniques. *Lancet 6*: 145–157.

Asgeirsson, K.S., Rasheed, T., McCulley, S.J., and Macmillan R.D. 2005. Oncological and cosmetic outcomes of oncoplastic breast conserving surgery. *Eur. J. Surg. Oncol. 31*: 817–823.

Barros, A., Pinotti M., Ricci, M.D., Nisida, A.C., and Pinotti J.A. 2003. Immediate effects of intraoperative evaluation of surgical margins over the treatment of early infiltrating breast carcinoma. *Tumori 89*: 42–45.

Barthlmes, L.B., Awa, A.A., and Crawford D.J. 2003. Effect of cavity margin shavings to ensure completeness of excision on local recurrence rates following breast conserving surgery. *Eur. J. Surg. Oncol. 29*: 644–648.

Benelli, L. 1990. A new periareaolar mammoplasty: round block technique. *Aesthetic Plast. Surg. 14*: 99–106.

Bold, R.J., Kroll, S.S., Baldwin, B.J., Ross, M.I., and Singletary, S.E. 1997. Local rotational flaps for breast conservation therapy as an alternative to mastectomy. *Ann. Surg. Oncol. 4*: 540–544.

Cendan, J.C., Coco, D., and Copeland III, E.M. 2005. Accuracy of intraoperative frozen-section analysis of breast cancer lumpectomy-bed margins. *J. Am. Coll. Surg. 201*: 194–198.

Chabner, E., Nixon, A., Gelman, R., Hetelekidis, S., Recht, A., Bornstein, B., Connolly, J., Schnitt, S., Silver, B., Manola, J., Harris, J., and Garber, J. 1998. Family history and treatment outcome in young women after breast-conserving surgery and radiation therapy for early-stage breast cancer. *J. Clin. Oncol. 16*: 2045–2051.

Choi, J.Y., Alderman, A.K., and Newman, L.A. 2006. Aesthetic and reconstruction considerations in oncologic breast surgery. *J. Am. Coll. Surg. 202*: 943–952.

Cochrane, R.A., Valasiadou, P., Wilson, A.R., Al-Ghazal, S.K., and Macmillan, R.D. 2003. Cosmesis and satisfaction after breast-conserving surgery correlates with the percentage of breast volume excised. *Br. J. Surg. 90*: 1505–1509.

Dillon, M.F., Hill, A.D.K., Quinn, C.M., McDermott, E.W., and O'Higgins, N.A. 2006. Pathologic assessment of adequate margin status in breast-conserving surgery. *Ann. Surg. Oncol. 3*: 333–339.

Early Breast Cancer Trialists' Collaborative Group. 1995. Effects of radiotherapy and surgery in early breast cancer: an overview of the randomized trials. *N. Engl. J. Med. 333*: 1444–1455.

Early Breast Cancer Trialists' Collaborative Group. 2000. Favourable and unfavourable effects on long-term survival of radiotherapy for early breast cancer: an overview of the randomised trials. *Lancet 355*: 1757–1770.

Engel, J., Kerr, J., Schlesinger-Raab, A., Sauer, H., and Holzel, D. 2004. Quality of life following breast-conserving therapy or mastectomy: results of a 5-year prospective study. *Breast J. 10*: 223–231.

Fallowfield, L.J. 1995. Assessment of quality of life in breast cancer. *Acta Oncol. 34*: 689–694.

Fisher, B. 1979. Sounding board. Breast-cancer management: alternatives to radical mastectomy. *N. Engl. J. Med. 301*: 326–328.

Fisher, B., Anderson, S., Bryant, J., Margolese, R.G., Deutsch, M., Fisher, E.R., Anderson, S., Bryant, J., Margolese, R.G., Deutsch, M., Fisher, E.R., Jeong, J-H., and Wolmark, N. 2002. Twenty-year follow-up of a randomized trial comparing total mastectomy, lumpectomy, and lumpectomy plus irradiation for the treatment of invasive breast cancer. *N. Engl. J. Med. 347*: 1233–1241.

Gage, I., Schnitt, S.J., Nixon, A.J., Silver, B., Recht, A., Troyan, S.L., Eberlein, T., Love, S.M., Gelman, R., Harris, J.R., and Connolly, J.L. 1996. Pathologic margin involvement and the

risk of recurrence in patients treated with breast-conserving therapy. *Cancer 78*: 1921–1918.

Giacalone, P-L., Roger, P., Dubon, O., El Gareh, N., Rihaoul, S., Taourel, P., and Daures, J.P. 2007. Comparative study of the accuracy of breast resection in oncoplastic surgery and quadrantectomy in breast cancer. *Ann. Surg. Oncol. 14*: 605–614.

Graham, P., and Fourquet, A. 2006. Placing the boost in breast-conservation radiotherapy: a review of the role, indications and techniques for breast-boost radiotherapy. *Clin. Oncol. 18*: 210–219.

Haas, J.A., Schultz, D.J., Peterson, M.E., and Solin, L.J. 1998. An analysis of age and family history on outcome after breast-conservation treatment: the University of Pennsylvania experience. *Cancer J. Sci. Am. 4*: 308–315.

Johansen, J., Overgaard, J., Rose, C., Engelholm, S.A., Gadeberg, C.C., Kjaer, M., Kamby, C., Juul-Christensen, J., Blichert-Toft, M., and Overgaard, M. 2002. Cosmetic outcome and breast morbidity in breast-conserving surgery. *Acta. Oncol. 41*: 369–380.

Kaur, N., Petit, J-Y., Rietjens, M., Maffini, F., Luini, A., Gatti, G., Rey, P.C., Urban, C., and De Lorenzi, F. 2005. Comparative study of surgical margins in oncoplastic surgery and quadrantectomy in breast cancer. *Ann. Surg. Oncol. 12*: 1–7.

Kunos, C., Latson, L., Overmoyer, B., Silverman, P., Shenk, R., Kinsella, T., and Lyons, J. 2006. Breast conservation surgery achieving > = 2 mm tumor-free margins results in decreased local-regional recurrence rates. *Breast J. 12*: 28–36.

Kurtz, J.M., Jacquemier, J., Torhosrst, J., Spitalier, J.M., Amalric, R., Hunig, R., Walther, E., Harder, F., Almendral, A., and Brandone, H. 1989. Conservation therapy for breast cancers other than infiltrating ductal carcinoma. *Cancer 63*: 1630–1635.

Liebens, F.P., Carly, B., Pastijn, A., and Rozenberg, S. 2007. Management of BRCA1/2 associated breast cancer: a systematic qualitative review of the state of knowledge in 2006. *Eur. J. Cancer 43*: 238–257.

Masetti, R., Leone, A.D., Franceschini, G., Magno, S., Terribile, D., Fabbri, M.C., and Chiesa, F. 2006. Oncoplastic techniques in the conservative surgical treatment of breast cancer: an overview. *Breast J. 12*(Suppl. 2): S174–S180.

McMulley, S.J., and Macmillan, R.D. 2005. Planning and use of therapeutic mammoplasty - Nottingham approach. *Br. J. Plast. Surg. 58*: 889–901.

McMulley, S.J., Durani, P., and Macmillan, R.D. 2006. Therapeutic mammaplasty for centrally located breast tumors. *Plast. Reconstr. Surg. 117*: 366–373.

Morrow, M., Bucci, C., and Rademaker, A. 1998. Medical contraindications are not a major factor in the underutilization of breast conserving therapy. *J. Am. Coll. Surg. 186*: 269–274.

Moyer, A. 1997. Psychosocial outcomes of breast-conserving surgery versus mastectomy: a meta-analytic review. *Health Psychology 16*: 284–298.

Munhoz, A.M., Montag, E., Arruda, E.G., Aldrighi, C., Gemperli, R., Aldrighi, J.M., and Ferreira, M.C. 2006. Critical analysis of reduction mammaplasty techniques in combination with conservative breast surgery for early breast cancer treatment. *Plast. Reconstr. Surg. 117*: 1091–1103.

Nadeem, R., Chagla, L.S., Harris, O., Desmond, S., Thind, R., Titterrell, C., and Audisio, R.A. 2005. Occult breast lesions: a comparison between radioguided occult lesion localisation (ROLL) vs. wire-guided lumpectomy (WGL). *Breast 14*: 283–289.

Nano, M.T., Gill, P.G., Kollias, J., Bochner, M.A., Carter, N., and Winefield, H.R. 2005. Qualitative assessment of breast reconstruction in a specialist breast unit. *Aust. N. Z. J. Surg. 75*: 445–453.

Nasir, N., and Rainsbury, R.M. 2003. The timing of surgery affects the detection of residual disease after wide local excision of breast carcinoma. *Eur. J. Surg. Oncol. 29*: 718–720.

Petrek, J.A. 1994. Breast cancer and pregnancy. *J. Natl. Cancer Inst. Monogr. 16*: 113–121.

Poggi, M.M., Danforth, D.N., Sciuto, L.C., Smith, S.L., Steinberg, S.M., Liewehr, D.J., Menard, C., Lippman, M.E., Lichter, A.S., and Altemus, R.M. 2003. Eighteen-year results in the treatment of early breast carcinoma with mastectomy versus breast conservation therapy. *Cancer 98*: 697–702.

Rainsbury, R.M. 2002. Breast-sparing reconstruction with latissimus dorsi miniflaps. *Eur. J. Surg. Oncol. 28*: 891–895.

Raja, M.A.K., Straker, V.F., and Rainsbury, R.M. 1997. Extending the role of breast-conserving

surgery by immediate volume replacement. *Br. J. Surg. 84*: 101–105.

Rietjens, M., Urban, C.A., Rey, P.C., Mazzarol, G., Maisonneuve, P., Garusi, C., Intra, M., Yamaguchi, S., Kaur, N., De Lorenzi, F., Matthes, A.G., Zurrida, S., and Petit, J.Y. 2008. Long-term oncological results of breast conservative treatment with oncoplastic surgery. *Breast 16*: 387–395.

Sabel, M.S., Kaufman, C.S., Whitworth, P., Chang, H., Stocks, L.H., Simmons, R., and Schultz, M. 2004. Cryoablation of early-stage breast cancer: work-in-progress report of a multi-institutional trial. *Ann. Surg. Oncol. 11*: 542–549.

Schoeller, T., and Huemer, G.M. 2006. Immediate reconstruction of the nipple/areola complex in oncoplastic surgery after central quandrantectomy. *Ann. Plast. Surg. 57*: 611–615.

Schwartz, G.F., Veronesi, U., Clough, K.B., Dixon, J.M., Fentiman, I.S., Heywang-Kobrunner, S.H., Holland, R., Hughes, K.S., Mansel, R.E., Margolese, R., Mendelson, E.B., Olivotto, I.A., Palazzo, J.P., Solin, L.J., and the Consensus Conference Committee 2006. Consensus on breast conservation. *J. Am. Coll. Surg. 203*: 198–207.

Shrotia, S. 2001. Techniques for improving the cosmetic outcome of breast conservation therapy. *Eur. J. Surg. Oncol. 27*: 109–112.

Singletary, S.E. 2001. New approaches to surgery for breast cancer. *Endocr. Relat. Cancer 8*: 265–286.

Temple, W.J., Russell, M.L., Parsons, L.L., Huber, S.M., Jones, C.A., Bankes, J., and Eliasziw, M. 2006. Conservation surgery for breast cancer as the preferred choice: a prospective analysis. *J. Clin. Oncol. 24*: 3367–3373.

Veronesi, U., Del Vecchio, M., Luini, A., Rasponi, A., and Zucali, R. 1983. The quadrantectomy, axillary dissection and radiotherapy (QU.A.RT) technique in early breast cancer. *Int. Adv. Surg. Oncol. 6*: 141–165.

Veronesi, U., Cascinelli, N., Mariani, L., Greco, M., Saccozzi, R., Luini, A., Aguilar, M., and Marubini, E. 2002. Twenty-year follow-up of a randomized study comparing breast-conserving surgery with radical mastectomy for early breast cancer. *N. Engl. J. Med. 347*: 1227–1232.

Vlastos, G., and Verkooijen, H.M. 2007. Minimally invasive approaches for diagnosis and treatment of early-stage breast cancer. *Oncologist 12*: 1–10.

Weber, S., Storm, F.K., Stitt, J., and Mahvi, D.M. 1997. The role of frozen section analysis of margins during breast conservation surgery. *Cancer J. Sci. Am. 3*: 273–277.

39
Lymph Node-Negative Breast Carcinoma: Assessment of HER-2/*neu* Gene Status as Prognostic Value

Gloria Peiró

INTRODUCTION

Patients with breast carcinoma and negative lymph-nodes are considered to have a good prognosis. Nevertheless, about one-third will develop metastases. Although useful prognostic information can be obtained from clinico-pathologic data, the identification of molecular genetic alterations for better selection of patients who will need additional or specific treatment has become relevant. Among them, HER-2/*neu* has been proved to have a role in the pathogenesis of breast carcinoma and in a significant number of other human tumors (Slamon *et al*., 1989; Peiró *et al*., 2004).

HER-2 gene (17q21) encodes a 185 kD transmembrane glycoprotein with tyrosine kinase (TK) activity that functions as a growth factor receptor. In normal cells, protein expression is primarily regulated by transcription activation and gene amplification. Oncogenic activation results in abnormally large amounts of the nonmutated receptor on the cell surface, which, in turn, may lead to autoactivation of the TK domain, activation of signal transduction pathways, and cellular transformation or proliferation. The extracellular domain of the receptor protein, also called p105, can be cleaved from the cell surface by matrix metalloproteases, and then, released into the blood.

In breast carcinoma, HER-2 overexpression and/or amplification have been documented between 10% to 30% of the tumors, and can occur early in the development of the disease. It is known that HER-2 overexpression occurs due to HER-2/*neu* gene amplification in > 90% of the cases (Slamon *et al*., 1989). Both, overexpression and gene amplification have important biological signification in breast carcinoma. As a prognostic marker, HER-2/*neu* status predicts the progression and the outcome of the disease (Andrulis *et al*., 1998). Regarding its role as a predictive marker, it is used to select the most appropriate adjuvant therapy, and to determine the therapeutic response to chemotherapy and/or endocrine therapy (Peiró *et al*., 2007a). In fact, it is considered as a

surrogate marker of anthracycline sensitivity, because it is associated with amplification of *topoisomerase 2-alpha gene* in about one-third of the cases (Tanner *et al*., 2006).

In node-negative breast carcinoma, the prognostic relevance of HER-2/*neu* has been controversial. Although some investigators have found that overexpression and/or amplification confer a worse prognosis, others have been unable to confirm these results (Dandachi *et al*., 2004; Peiró *et al*., 2007b).

Currently, the most important application of HER-2/*neu* testing is in the selection of patients for specific treatment with trastuzumab (Herceptin™). Trastuzumab is a humanized monoclonal antibody targeted against the extracellular domain of the HER-2/*neu* oncogene. It improves survival only in positive cases in both primary and disseminated diseases in adjuvant (Piccart-Gebhart *et al*., 2005; Romond *et al*., 2005), and more recently in neoadjuvant setting (Buzdar *et al*., 2005). Although this therapy is effective in selected patients, a favorable treatment response is not achieved in all cases.

Presently, analysis of HER-2/*neu* status has become a common practice in surgical pathology laboratories. Several technical approaches have been proved to be useful for the assessment, but some of them are beyond the scope of the majority of laboratories due to technical and economical reasons. On the other hand, consensus regarding the best method or reagents has not yet been reached (Wolff *et al*., 2007). Hence, there is an increasing demand for new methods to accurately assess HER-2/*neu* status in the routine clinical practice, which would provide the clinicians more relevant information than the standard tests.

Several assays are available in clinical laboratories for the measurements of protein overexpression (immunohistochemistry [IHC] and enzyme immunoassay [ELISA]; mRNA overexpression (quantitative RT-PCR [qPCR]; and for gene amplification (*in situ* hybridization -ISH- methods, and qPCR). Technically difficult and time-consuming methods to identify gene amplification and protein overexpression include Southern, Northern and Western blotting techniques (Giai *et al*., 1994). However, considering the patient's life expectancies, the cost of the different tests and that of the specific treatment itself, it is highly important to use the most sensitive and cost-effective diagnostic method. In clinical practice, the most commonly used methods are IHC for the protein expression and fluorescence *in situ* hybridization (FISH) for the evaluation of gene amplification, both approved by the Food and Drug Administration (FDA). However, there is no gold standard at present. No assay currently available is perfectly accurate to identify all patients who will benefit from trastuzumab.

PROTEIN OVEREXPRESSION

Enzyme Linked Immunosorbent Assay (ELISA)

It is a quantitative method that measures HER-2 protein in the tumor as well as circulating HER-2 extracellular domain in serum (s-HER-2). Serum ELISA has been FDA-approved for monitoring response of breast carcinoma to treatment. It can be used when primary tumor samples are unavailable and eliminates the need of a biopsy. It is rapid, cost-effective, and fully automated; thereby, offering a high degree

of standardization and reproducibility. Good agreements of the results between ELISA, IHC, and FISH have been demonstrated (Tse *et al*., 2005).

The extracellular domain of HER-2 is an ideal target for therapeutic approaches. Detection levels predict the response to hormonal therapy in estrogen receptor (ER)-positive tumors and to Trastuzumab (Kostler *et al*., 2004). Furthermore, it has been shown to be useful for assessing the prognosis in node-negative breast carcinoma. Dittadi *et al*. (1997), based on the results of HER-2 protein (p185) expression evaluated by ELISA, were able to classify the patients as "low risk" and "high risk", and showed a significant relationship with the disease free survival in multivariate analysis. More recent studies also showed that patient survival was unaffected by tamoxifen, with opposite results regarding adjuvant chemotherapy (Eppenberger-Castori *et al*., 2001).

The results of Olsen *et al*. (2007) have revealed that measuring HER-2 in tissue extracts by ELISA seems to be more sensitive than IHC and FISH, and support that this technique offers a clinically valuable alternative to semiquantitative IHC assessment of breast tumor HER-2 overexpression. Moreover, it affords the opportunity to evaluate HER-2 phosphorylation, which may represent an important predictive parameter of receptor functionality (Eppenberger-Castori *et al*., 2001).

Immunohistochemistry

It is a quantitative method that remains the optimal first-line testing gene expression method that is widely used in diagnostic clinical applications despite its several weaknesses. This technique is readily available cost-effective, and the scoring system (Dako) based on the assessment of the intensity and completeness of membrane staining has been approved by the Food and Drug Administration (FDA) for diagnostic use (Table 39.1). Two commercially available HER-2 IHC kits, HercepTest™ (Dako) and the Ventana Pathway™ are approved for determining eligibility for patients to receive trastuzumab.

The reliability of the IHC assay has been questioned due to the high rate of discordance among the studies, usually attributed to the wide variation in antibody (Ab) sensitivity and specificity from field to field, the effect of fixation and pretreatments, the lack of a universal scoring system, and interobserver variability. The main concern is the accuracy, especially in (2+) positive reactions (Yaziji *et al*., 2004). Studies have evidenced that standardization of the assay yields an excellent correlation with gene copy status (Perez *et al*., 2006). To overcome the mentioned inconveniences related with this technique, several guidelines have been given by the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) panel to define the cut off points for HER-2 scores, as well as requirements for test validation and tissue fixation, rejection criteria and the suggestion of participation in external proficiency testing (Wolff *et al*., 2007)). The four-tiered scoring system proposed by Dako has been slightly modified. A positive IHC assay should be considered if there is intense membranous staining on > 30% of invasive tumor cells. A negative assay (scores 0 or 1+) is defined when there is no immunostaining or incomplete membranous staining in any proportion of tumor cells. An equivocal assay is considered if there is (2+), non-uniform or

TABLE 39.1. Advantages and disadvantages of IHC, FISH, and CISH methods for the analysis of HER-2 in breast carcinoma.

	IHC	FISH	CISH
Advantages	• Available for routine use • Low cost • Rapid and easy performance and evaluation • Bright field microscope • Permanent staining	• Standardized cut off for positivity • Direct visualization of the gene copies • Internal control • Accurate • High concordance	• Lower cost • Suitable for routine use • Easier to perform • Correlation with histology • Permanent reaction • Standard light microscope • No additional equipment
Disadvantages	Wide variation related to: • Differences of material • Sensitivity and specificity of Abs • Detection method • Non-uniform scoring systems • Subjectiveness • Inaccuracy	• Morphology difficult due to dark-field visualization • Staining fades • Time consuming • Technically demanding • Expensive equipment • Experienced personal	• False negativity related with technical problems • Nonsimultaneous chromosome 17 probe

weak intensity with circumferential distribution in at least 10% of neoplastic cells, and/or (3+) but in < 30% cells (Wolff *et al*., 2007).

A close correlation between IHC and FISH has been demonstrated for negative and (3+) cases. Recent data from the National Surgical Adjuvant Breast Program (NSABP) (Paik *et al*., 2002) has shown that certified laboratories, defined as those performing high-volume HER-2 testing and demonstrating high concordance between IHC and FISH results, approach 98% interlaboratory concordance when tumors assessed as (3+) were reanalyzed by both methods at the NSABP laboratory. In addition, previous studies comparing the specificity of different antibodies concluded that immunohistochemical HER-2 testing should be performed in accredited, approved central laboratories to avoid inconclusive results. A central reference or large local laboratories are those with high number of cases (> 250 cases per year) or > 100 cases a month (NSABP), quality assurance controls (internal and external), automated IHC, and therefore with a high level of training (technique and interpretation). On the other hand, small laboratories (< 250 cases/year or < 100 cases/month) should not do it and send it to larger laboratories. However, a high test volume does not ensure an accurate test result. Of note, there are laboratories capable of performing quality testing with lower volume. Therefore, a test should be offered if an individual laboratory can properly validate an assay and perform acceptably in an external validation (Perez *et al*., 2006).

Among the requirements for testing validation are: (1) an assay must be tested on 25–100 cases with previously validated assay; (2) an external laboratory for validation must be used; (3) 95% concordance among the assays as well as the evaluators and the fixatives if other than formalin must be shown. Exclusion criteria for IHC are: (1) tissues fixed in other than formalin unless validated in another fixative; (2) severe tissue artifact, and (3) fixation of < 1 h for core needle biopsies or < 6 h or > 48 h for excisional biopsies (Wolff *et al*., 2007).

Nonetheless, it is known that HER-2 must be activated (phosphorylated) to exert its effect. Therefore, the most common tests

for the routine analysis do not demonstrate the functional activity. A study (Thor *et al*., 2000) in a large series of patients with invasive breast carcinoma has shown autophosphorylated HER-2 (pHER-2) in 12% of tumors detected by IHC. The results of HER-2 and pHER-2 expression provided the most significant prognostic value in node-positive cases by multivariate analyses. Moreover, there were too few pHER-2-positive cases and events in the node-negative patient group to allow statistical analysis in that subgroup.

Previous studies have recommended a two phase testing algorithm based on first line IHC evaluation and second line FISH or CISH (Peiró *et al*., 2007b) assessment of borderline (2+) or < 30% (3+) cases (Figure 39.1).

Survival correlated with IHC results in (3+) cases in some studies, but in others, protein expression did not have a negative effect (Joensuu *et al*., 2003). Finally, in patients who received adjuvant tamoxifen alone, a univariate analysis also indicated a subgroup with HER-2-positive and ER-negative to have the worst clinical outcome (Peiró *et al*., 2007a).

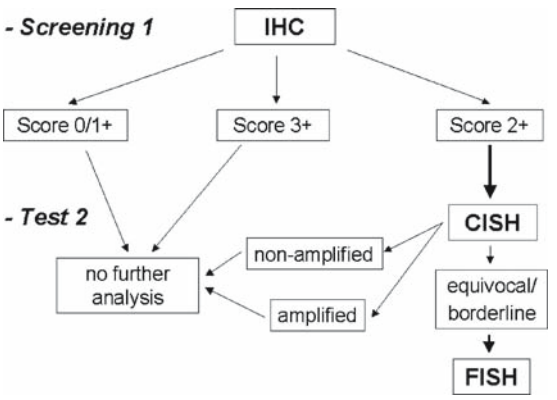


FIGURE 39.1. Testing algorithm for HER-2 determination. (Peiró *et al*., 2007.)

mRNA OVEREXPRESSION

Quantitative Real-Time Reverse Transcription-PCR (RT-PCR)

Real-time PCR is considered the "gold standard" for gene expression analysis and commonly used for validation of microarray results). This technique is not yet used for routine analysis. It evaluates quantitatively the levels of expression of HER-2 DNA and RNA requiring small amounts of tumor tissue. Measurement of mRNA is possible in fresh frozen tissue and in formalin-fixed paraffin-embedded tissue (FFPE). This method is fast and inexpensive, yielding quantitative results insensitive to interobserver variability and amenable to standardized scoring. Furthermore, it has several advantages over IHC with respect to quantitation and dynamic range (Vinatzer *et al*., 2005).

Paik *et al*. (2004) tested whether the results of a RT-PCR assay of 21 prospectively selected genes, including HER-2 in paraffin-embedded tumor tissue, would correlate with the likelihood of distant recurrence in patients with node-negative tamoxifen-treated breast cancer, who were enrolled in the NSABP clinical trial B-14. Based on the results, an algorithm was defined to calculate a recurrence score that allowed the classification of patients into risk groups. In a multivariate Cox model, the recurrence score provided significant predictive power that was independent of age and tumor size and, it was also predictive of overall survival. In contrast, Esteva *et al*. (2005) found no association between the recurrence score and the likelihood of recurrent disease in ER-positive patients with node-negative breast cancer who did not receive adjuvant tamoxifen or chemotherapy. However, this discrepancy could

be attributed in part to patient's cohort included in each study.

Quantitative RT-PCR appears to be clinically as useful in the assessment of HER-2 status as IHC and FISH, yielding comparable correlations of HER-2 status with the overall and disease-free survival. Hence, it is a promising complement or alternative to current methods for HER-2 testing (Vinatzer *et al*., 2005; Bergqvist *et al*., 2007). However, currently there is few data available.

More recently, Lamy *et al*. (2006) have proposed to assess the mRNA expression of HER-2 using a unique technique, a real-time nucleic acid sequence-based amplification (NASBA). HER-2 mRNA expression was compared to qPCR and ELISA and they demonstrated an excellent concordance. This approach is rapid, highly sensitive and a standardized method that could be complementary to the existing techniques, especially for small tumors. Therefore, NASBA is well suited for assessing HER-2 status in breast tumor samples.

cDNA Microarray-Based Method

Microarray technology is a powerful tool for measuring RNA expression for thousands of genes at the same time. This process, called transcriptional profiling, represents a technological breakthrough in the analysis of biological specimens. Analysis of gene expression patterns allows screening for individual genes that are differentially expressed between normal and diseased tissues in the hope of finding novel targets for drug development or finding new single-gene markers of clinical outcome. It may also be used as a classification tool to sort cancer into various clinically relevant subgroups, which is not currently possible with other methods. Currently, several microarray platforms are used for generating RNA expression profile (RNA-EP), but no gold standards exist. Moreover, this technique is not available for routine analysis because it is not cost-effective and time-consuming with low reproducibility. A recent comparison study has shown a relatively large difference in data obtained in laboratories using the same platform (Irizarry *et al*., 2005). Most RNA-EP techniques require intact full-length RNA. Therefore, the results among studies might not be comparable when FFPE material is used for expression array analysis, since RNA fragmentation occurs during storage in formalin (Bergqvist *et al*., 2007).

Van't Veer *et al*. (2002) in a series of primary breast tumors of 117 young patients and applying supervised classification, were able to identify a gene expression signature that was predictive of a short interval to distant metastases ("poor prognosis" signature) in patients with negative lymph nodes. Apparently, the findings might provide a strategy to select patients who would benefit from adjuvant therapy. HER-2 determination by this methodology has shown prognostic value with regard to disease-free survival and breast cancer-corrected survival, even at 10 years of follow-up. However, the lymph-node status was not specified (Bergqvist *et al*., 2007).

Nowadays, the available evidence does not support the use of RNA-EP as complementary or alternative methods to currently available HER-2 tests. In addition, the indication that HER-2-positive patients by RT-PCR and RNA-EP might have a worse long-term prognosis than those by standard methods, does not seem to be a strong

enough evidence to consider the new tests as an upcoming standard.

GENE AMPLIFICATION

The demand of HER-2/*neu* gene amplification testing has been recently strengthen by Kauraniemi *et al*. (2004), who reported that trastuzumab treatment induces a dose-dependent growth reduction in breast cancer cell lines with HER-2/*neu* amplification, whereas nonamplified cell lines are practically resistant.

Southern and Slot Blotting

They were the first developed methods for the study of HER-2/*neu* gene status in breast carcinoma. They can be performed in frozen, fresh or paraffin-embedded specimens. Despite some inconveniences related with the quality and quantity of the extracted tumor DNA, several investigators have shown that gene amplification predicted shorter DFS in node-negative breast cancer, independently of ER level and tumor size. Thus, this technology may serve to identify a subset of high-risk patients (Andrulis *et al*., 1998).

Fluorescence *In Situ* Hybridization (FISH)

FISH analysis is seen as the gold standard method for detecting HER-2/*neu* amplification, with 98% of sensitivity and 100% specificity when compared with other assays (Yaziji *et al*., 2004). Two FISH assays are FDA-approved: the Ventana Inform™ that detects only HER-2/*neu* gene copies and the Abbott-Vysis Pathvision™ which includes the chromosome 17 probe.

The advantages of this technique are the more objective scoring system and internal control from non-neoplastic cells. However, it is not practical for routine histopathologic laboratories because of the additional expensive equipment and its time consuming technique (Jacobs *et al*., 1999) (Table 39.1). A close correlation between IHC and FISH has been demonstrated for negative and (3+) cases, and confirmation of the results by FISH is suggested only in equivocal cases (2+) (Yaziji *et al*., 2004). However, the comparison of the data is difficult occasionally because of the different criteria to define amplification. More recently, a guideline has been developed to overcome this inconvenience (Wolff *et al*., 2007).

A positive FISH assay is defined if there are > 6 gene copies/nucleus (no chromosome 17 – Chr17-probe) or ratio of HER-2/*neu*/Chr17 > 2.2. An equivocal FISH assay is defined when HER-2/*neu*/Chr17 from 1.8 to 2.2 or when an average gene copy numbers between 4 to 6 (no Chr17) or those cases with ratios between 2.0–2.2 (previously called positive) are detected. A negative result is defined if HER-2/*neu*/Chr17 < 1.8 or an average of < 4 HER-2/*neu* gene copies per cell are seen (Wolff *et al*., 2007).

The exclusion criteria for FISH are: (1) fixatives other than formalin, unless validated in others; (2) fixation > 48 h; (3) small amounts of invasive tumor; and (4) non-uniform or < 75% of identifiable signals. For any laboratory performing HER-2/*neu* testing, an internal quality assurance is required. In addition, an accredited laboratory must participate in external proficiency testing twice a year and it must score at least 90% (Wolff *et al*., 2007).

Slamon *et al*. (1989) in a series of 181 lymph node-negative breast carcinoma patients found that HER-2/*neu* amplification was not associated with survival in uni- and multivariate analysis. A few studies indicate that HER-2/*neu* gene status provides better prognostic information and it might be associated with clinical response to specific therapy with trastuzumab (Tubbs *et al*., 2001). Recently, Elkin *et al*. (2004) estimated the cost-effectiveness of alternative HER-2/*neu* testing and trastuzumab treatment strategies. They concluded that it is more cost-effective to use FISH alone or as confirmation of all positive HercepTest™ results than using FISH to confirm only weakly positive results or using HercepTest™ alone.

The evaluation of the feasibility of automating the methodology and quantification of the FISH assay using whole sections or tissue microarrays of automated versus manual showed a good correlation. Furthermore, disease free survival was significantly shorter for patients with HER-2/*neu* amplified tumors (Tubbs *et al*., 2006).

Bright-Field *In Situ* Hybridization

Recently, novel second generation in situ hybridization detection methods based upon polymerized lg detection chemistry, autometallography or enzyme metallography have been developed.

Chromogenic In Situ Hybridization (CISH)

This new assay, based on the hybridization of a specific digoxigenin-labeled DNA probe and a peroxidase reaction, allows the assessment of the gene copy number in neoplastic cells using as internal hybridization control normal cells. It appears to be an attractive method for clinical HER-2/*neu* analysis owing to its specific targeting of neoplastic cells and retrospective potential. HER-2/*neu* gene signals are scored in histological tumor sections using a conventional microscope. Depending on the number of gene copies in the nuclei, tumors are classified as non amplified, those showing one to two brown intranuclear spots (diploid) or three to five spots (chromosomally polyploid), thus, defined as 1–5 copies; and amplified when ≥ 6 copies or large gene copy clusters are seen in at least 50% of nuclei (Tanner *et al*., 2000).

The results of CISH and two-colour FISH were concordant in 80–100% in several studies, including those performed on tissue microarrays. Occasionally, FISH was more sensitive than CISH in the subgroup of negative/non definitive cases detecting amplification (Peiró *et al*., 2007b). Studies on interobserver reproducibility have also shown a nearly perfect agreement (Isola *et al*., 2004).

The advantages of CISH over FISH are the lower costs, easier to perform, interpretation of the results by a standard light microscope allowing the correlation of morphological features with gene copy number, and the permanent reaction product (Table 38.1). However, minor difficulties related with technical problems such as occasional high background, excessive tissue digestion or low signal intensity could cause false negativity in some cases (Tanner *et al*., 2000). The inability of simultaneous identification of chromosome 17 is the main disadvantage of CISH technique. Although polysomy 17 is not considered a significant factor for HER-2 expression, it may result in an increased protein production to the IHC level of (2+) (Isola

et al., 2004), and exceptionally of (3+) (Lal *et al*., 2003). Isotopic in situ hybridization for HER-2 mRNA performed on cases with chromosome 17 polysomy showed no increased HER-2 mRNA expression, suggesting that in the absence of HER-2/*neu* amplification it does not have a significant biologic influence on HER-2 gene expression (Downs-Kelly *et al*., 2005b). To eliminate the polysomy variable, several authors recommend to apply a chromosome 17 centromeric probe in cases with more than two copies. About 21% of the cases would likely require confirmatory chromosome 17 (Isola *et al*., 2004).

The available data regarding the clinical value of CISH support that it is useful for selecting prognostic groups among breast carcinoma patients with negative lymph nodes (Joensuu *et al*., 2003; Peiró *et al*., 2007b).

Silver Enhanced In Situ Hybridization (SISH)

SISH is a newly developed technology that allows the quantification of the number of HER-2/*neu*-specific signals and centromeric chromosome 17 by using consecutive tumor sections. Both probes (against HER-2/*neu* DNA and chromosome 17) are labeled with dinitrophenol (DNP) and detected via peroxidase labeled multimer, followed by enzyme metallography with silver. It combines bright field microscopy with molecular analysis and full automation, with stable and discrete chromogenic reaction. Therefore, it appears to be particularly suited for routine application in surgical pathology (Downs-Kelly *et al*., 2005a).

Using a semi-quantitative score, cases are considered amplified if neoplastic nuclei contain six or more HER-2/*neu* signals, polysomic if > 30% of tumor cells contain 3–5 signals per nucleus and nonamplified if one or two signals per nuclei are detected. When the centromeric chromosome 17 probe is also determined, the scoring criteria according to the guidelines of the ASCO/CAP for FISH can be applied. The comparison of SISH and FISH has shown that SISH is equally reliable in determining HER-2/*neu* amplification as FISH (Dietel *et al*., 2007).

A recent modification of the methodology allows the simultaneous detection of HER-2/*neu* gene amplification and HER-2 protein expression (Downs-Kelly *et al*., 2005a). This assay detects HER-2/*neu* gene amplification via deposition of metallic silver by enzyme metallography combined with HER-2 protein detection by IHC using alkaline phosphatase and fast red K substrate visualization. This combination of gene and protein detection displayed a specificity of 100% and an accuracy of 92.6%, facilitated the recognition of gene/protein discordances and allowed for efficient interpretation of the slide by conventional light microscopy. Apparently, this new bright-field ISH assay can routinely and non-ambiguously detect endogenous HER-2/*neu* signals, essential for a reliable clinical HER-2 assay. In combination with HER-2 protein, it enables improved diagnosis in borderline cases. Currently, there are no data available regarding the clinical value of this new assay.

Quantitative Real-Time *RT-PCR (qPCR)*

This recent technique is an alternative method for determining HER-2/*neu* status, which enables the quantification of gene copy using DNA as template from freshly frozen or FFPE tissue, preferably containing

> 50% tumor cells. The continuous nature of the data along with the wholly automated quantitation process contributes to its high degree of accuracy and more cost-effective than FISH. The results are expressed as the ratio of HER-2/*neu* to reference gene copies in the sample, normalized against the ratio of HER-2/*neu* to reference gene copies in the calibrator DNA. A ratio of ≥ 2.0 has been considered positive (Tse *et al*., 2005). However, a clinically significant amplification ratio has not been established yet.

The results of qPCR correlate well with those obtained by IHC/C(F)ISH/RNA-EP. Thus, this technique has been proposed as an alternative to the conventional methods (Tse *et al*., 2005). Current data from Bergqvist *et al*. (2007) has evidenced statistically significant association regarding recurrence free survival and breast cancer-corrected survival after 5 and 10 years of follow-up.

In spite of the fact that there are advantages in using some of the methods detailed before, there is still some controversy on deciding the most efficient way to determine HER-2 status. In the not too far future, there will be an urgency to do in-depth testing on the outcome of the anti-HER-2 therapy and to correlate the methods which were used to spot HER-2 status with the accuracy of the therapy and end result.

REFERENCES

Andrulis, I. L., Bull, S. B., Blackstein, M. E., Sutherland, D., Mak, C., Sidlofsky, S., Pritzker, K. P., Hartwick, R. W., Hanna, W., Lickley, L., Wilkinson, R., Qizilbash, A., Ambus, U., Lipa, M., Weizel, H., Katz, A., Baida, M., Mariz, S., Stoik, G., Dacamara, P., Strongitharm, D., Geddie, W., and McCready, D. 1998. Neu/erbB-2 amplification identifies a poor-prognosis group of women with node-negative breast cancer. Toronto Breast Cancer Study Group. *J. Clin. Oncol. 16*: 1340–1349.

Bergqvist, J., Ohd, J., Smeds, J., Klaar, S., Isola, J., Nordgren, H., Elmberger, G., Hellborg, H., Bjohle, J., Borg, A. L., Skoog, L., and Bergh, J. 2007. Quantitative real-time PCR analysis and microarray-based RNA expression of HER2 in relation to outcome. *Ann. Oncol. 18*: 845–850.

Buzdar, A. U., Ibrahim, N. K., Francis, D., Booser, D. J., Thomas, E. S., Theriault, R. L., Pusztai, L., Green, M. C., Arun, B. K., Giordano, S. H., Cristofanilli, M., Frye, D. K., Smith, T. L., Hunt, K. K., Singletary, S. E., Sahin, A. A., Ewer, M. S., Buchholz, T. A., Berry, D., and Hortobagyi, G. N. 2005. Significantly higher pathologic complete remission rate after neoadjuvant therapy with trastuzumab, paclitaxel, and epirubicin chemotherapy: results of a randomized trial in human epidermal growth factor receptor 2-positive operable breast cancer. *J. Clin. Oncol. 23*: 3676–3685.

Dandachi, N., Dietze, O., and Hauser-Kronberger, C. 2004. Evaluation of the clinical significance of HER2 amplification by chromogenic in situ hybridisation in patients with primary breast cancer. *Anticancer Res. 24*: 2401–2406.

Dietel, M., Ellis, I. O., Hofler, H., Kreipe, H., Moch, H., Dankof, A., Kolble, K., and Kristiansen, G. 2007. Comparison of automated silver enhanced in situ hybridisation (SISH) and fluorescence ISH (FISH) for the validation of HER2 gene status in breast carcinoma according to the guidelines of the American Society of Clinical Oncology and the College of American Pathologists. *Virchows Arch. 451*: 19–45.

Dittadi, R., Brazzale, A., Pappagallo, G., Salbe, C., Nascimben, O., Rosabian, A., and Gion, M. 1997. ErbB2 assay in breast cancer: possibly improved clinical information using a quantitative method. *Anticancer Res. 17*: 1245–1247.

Downs-Kelly, E., Pettay, J., Hicks, D., Skacel, M., Yoder, B., Rybicki, L., Myles, J., Sreenan, J., Roche, P., Powell, R., Hainfeld, J., Grogan, T., and Tubbs, R. 2005a. Analytical validation and interobserver reproducibility of EnzMet GenePro: a second-generation bright-field metallography assay for concomitant detection of HER2 gene status and protein expression in invasive carcinoma of the breast. *Am. J. Surg. Pathol. 29*: 1505–1511.

Downs-Kelly, E., Yoder, B. J., Stoler, M., Tubbs, R., Skacel, M., Grogan, T., Roche, P., and Hicks, D. G. 2005b. The influence of polysomy 17 on HER2 gene and protein expression in adenocarcinoma of the breast: a fluorescent in situ hybridization, immunohistochemical, and isotopic mRNA in situ hybridization study. *Am. J. Surg. Pathol. 29*: 1221–1227.

Elkin, E. B., Weinstein, M. C. Winer, E. P., Kuntz, K. M., Schnitt, S. J., and Weeks, J. C. 2004. HER-2 testing and trastuzumab therapy for metastatic breast cancer: a cost-effectiveness analysis. *J. Clin. Oncol. 22*: 854–863.

Eppenberger-Castori, S., Kueng, W., Benz, C., Caduff, R., Varga, Z., Bannwart, F., Fink, D., Dieterich, H., Hohl, M., Muller, H., Paris, K., Schoumacher, F., and Eppenberger, U. 2001. Prognostic and predictive significance of ErbB-2 breast tumor levels measured by enzyme immunoassay. *J. Clin. Oncol. 19*: 645–656.

Esteva, F. J., Sahin, A. A., Cristofanilli, M., Coombes, K., Lee, S. J., Baker, J., Cronin, M., Walker, M., Watson, D., Shak, D., and Hortobagyi, G. N. 2005. Prognostic role of a multigene reverse transcriptase-PCR assay in patients with node-negative breast cancer not receiving adjuvant systemic therapy. *Clin. Cancer Res. 11*: 3315–3319.

Giai, M., Roagna, R., Ponzone, R., De Bortoli, M., Dati, C., and Sismondi, P. 1994. Prognostic and predictive relevance of c-erbB-2 and ras expression in node positive and negative breast cancer. *Anticancer Res. 14*: 1441–1450.

Irizarry, R. A., Warren, D., Spencer, F., Kim, I. F., Biswal, S., Frank, B. C., Gabrielson, E., Garcia, J. G., Geoghegan, J. Germino, G., Griffin, C., Hilmer, S.C., Hoffman, E., Jedlicka, A. E., Kawasaki, E., Martinez-Murillo, F., Morsberger, l., Lee, H., Petersen, D., Quackenbush, J., Scott, A., Wilson, M., Yang, I., Ye, S. Q., and Yu, W. 2005. Multiple-laboratory comparison of microarray platforms. *Nat. Methods 2*: 345–350.

Isola, J., Tanner, M., Forsyth, A., Cooke, T. G., Watters, A. D., and Bartlett, J. M. 2004. Interlaboratory comparison of HER-2 oncogene amplification as detected by chromogenic and fluorescence in situ hybridization. *Clin. Cancer Res. 10*: 4793–4798.

Jacobs, T. W., Gown, A. M., Yaziji, H., Barnes, M. J., and Schnitt, S. J. 1999. Comparison of fluorescence in situ hybridization and immunohistochemistry for the evaluation of HER-2/neu in breast cancer. *J. Clin. Oncol. 17*: 1974–1982.

Joensuu, H., Isola, J., Lundin, M., Salminen, T., Holli, H., Kataja, V., Pylkkanen, L., Turpeenniemi-Hujanen, T., von Smitten, K., and Lundin, J. 2003. Amplification of erbB2 and erbB2 expression are superior to estrogen receptor status as risk factors for distant recurrence in pT1N0M0 breast cancer: a nationwide population-based study. *Clin. Cancer Res. 9*: 923–930.

Kauraniemi, P., Hautaniemi, S., Autio, R., Astola, J., Monni, O., Elkahloun, A., and Kallioniemi, A. 2004. Effects of Herceptin treatment on global gene expression patterns in HER2-amplified and nonamplified breast cancer cell lines. *Oncogene 23*: 1010–1013.

Kostler, W. J., Steger, G. G., Soleiman, A., Schwab, B., Singer, C. F., Tomek, S., Brodowicz, T., Krainer, M., Wiltschke, C., Horvat, R., Jakesz, R., and Zielinski, C. C. 2004. Monitoring of serum Her-2/neu predicts histopathological response to neoadjuvant trastuzumab-based therapy for breast cancer. *Anticancer Res. 24*: 1127–1130.

Lal, P., Salazar, P. A., Ladanyi, M., and Chen, B. 2003. Impact of polysomy 17 on HER-2/neu immunohistochemistry in breast carcinomas without HER-2/neu gene amplification. *J. Mol. Diagn. 5*: 155–159.

Lamy, P. J., Verjat, T., Paye, M., Servanton, A. C., Grenier, J., Leissner, P., and Mougin, B. 2006. NASBA: a novel approach to assess hormonal receptors and ERBB2 status in breast cancer. *Clin. Chem. Lab. Med. 44*: 3–12.

Olsen, D. A., Ostergaard, B., Bokmand, S., Wamberg, P. A., Jakobsen, E. H., and Brandslund, I. 2007. HER-2 protein concentrations in breast cancer cells increase before immunohistochemical and fluorescence in situ hybridization analysis turn positive. *Clin. Chem. Lab. Med. 45*: 177–182.

Paik, S., Bryant, J., Tan-Chiu, E., Romond, E., Hiller, W., Park, K., Brown, A., Yothers, A., Anderson, S., Smith, R., Wickerham, D. L., and Wolmark, N. 2002. Real-world performance of HER2 testing–National Surgical Adjuvant Breast and Bowel Project experience. *J. Natl. Cancer Inst. 94*: 852–854.

Paik, S., Shak, S., Tang, G., Kim, C., Baker, J., Cronin, M., Baehner, F. L., Walker, M. G., Watson, D., Park, T., Hiller, W., Fisher, E. R., Wickerham, D. L., Bryant, J., and Wolmark, N. 2004. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. *N. Engl. J. Med. 351*: 2817–2826.

Peiro, G., Mayr, D., Hillemanns, P., Lohrs, U., and Diebold, J. 2004. Analysis of HER-2/neu amplification in endometrial carcinoma by chromogenic in situ hybridization. Correlation with fluorescence in situ hybridization, HER-2/neu, p53 and Ki-67 protein expression, and outcome. *Mod. Pathol. 17*: 227–237.

Peiro, G., Adrover, E., Aranda, F. I., Peiro, F. M., Niveiro, M., and Sanchez-Paya, J. 2007a. Prognostic implications of HER-2 status in steroid receptor-positive, lymph node-negative breast carcinoma. *Am. J. Clin. Pathol. 127*: 780–786.

Peiro, G., Aranda, F. I., Adrover, E., Niveiro, M., Alenda, C., Paya, A., and Segui, J. 2007b. Analysis of HER2 by chromogenic in situ hybridization and immunohistochemistry in lymph node-negative breast carcinoma: prognostic relevance. *Hum. Pathol. 38*: 26–34.

Perez, E. A., Suman, V. J., Davidson, N. E., Martino, S., Kaufman, P. A., Lingle, W. L., Flynn, P. J., Ingle, J. N., Visscher, D., and Jenkins, R. B. 2006. HER2 testing by local, central, and reference laboratories in specimens from the North Central Cancer Treatment Group N9831 intergroup adjuvant trial. *J. Clin. Oncol. 24*: 3032–3038.

Piccart-Gebhart, M. J., Procter, M., Leyland-Jones, B., Goldhirsch, A., Untch, M., Smith, I., Gianni, L., Baselga, J., Bell, R., Jackisch, C., Cameron, D., Dowsett, M., Barrios, C. H., Steger, G., Huang, C. S., Andersson, M., Inbar, M., Lichinitser, M., Lang, I., Nitz, U., Iwata, H., Thomssen, C., Lohrisch, C., Suter, T. M., Ruschoff, J., Suto, T., Greatorex, V., Ward, C., Straehle, C., McFadden, E., Dolci, M. S., and Gelber, R. D. 2005. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. *N. Engl. J. Med. 353*: 1659–1672.

Romond, E. H., Perez, E. A., Bryant, J., Suman, V. J., Geyer, C. E. Jr., Davidson, N. E., Tan-Chiu, E., Martino, S., Paik, S., Kaufman, P. A., Swain, S. V., Pisansky, T. M., Fehrenbacher, L., Kutteh, L. A., Vogel, V. G., Visscher, D. W., Yothers, G., Jenkins, R. B., Brown, A. M., Dakhil, S. R., Mamounas, E. P., Lingle, W. L., Klein, P. M., Ingle, J. N., and Wolmark, N. 2005. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. *N. Engl. J. Med. 353*: 1673–1684.

Slamon, D. J., Godolphin, W., Jones, L. A., Holt, H. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A. 1989. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science 244*: 707–712.

Tanner, M., Gancberg, D., Di Leo, A., Larsimont, D., Rouas, G., Piccart, M. J., Isola, J. 2000. Chromogenic in situ hybridization: a practical alternative for fluorescence in situ hybridization to detect HER-2/neu oncogene amplification in archival breast cancer samples. *Am. J. Pathol. 157*: 1467–1472.

Tanner, M., Isola, J., Wiklund, T., Erikstein, B., Kellokumpu-Lehtinen, P., Malmstrom, P., Wilking, N., Nilsson, J., and Bergh, J. 2006. Topoisomerase IIalpha gene amplification predicts favorable treatment response to tailored and dose-escalated anthracycline-based adjuvant chemotherapy in HER-2/neu-amplified breast cancer: Scandinavian Breast Group Trial 9401. *J. Clin. Oncol. 24*: 2428–2436.

Thor, A. D., Liu, S., Edgerton, S., Moore, D., Kasowitz, K. M., Benz, C. C., Stern, D. F., and DiGiovanna, M. P. 2000. Activation (tyrosine phosphorylation) of ErbB-2 (HER-2/neu): a study of incidence and correlation with outcome in breast cancer. *J. Clin. Oncol. 18*: 3230–3239.

Tse, C., Brault, D., Gligorov, J., Antoine, M., Neumann, R., Lotz, J. P., and Capeau, J. 2005. Evaluation of the quantitative analytical methods real-time PCR for HER-2 gene quantification and ELISA of serum HER-2 protein and comparison with fluorescence in situ hybridization and immunohistochemistry for determining HER-2 status in breast cancer patients. *Clin. Chem. 51*: 1093–1101.

Tubbs, R. R., Pettay, J. D., Roche, P. C., Stoler, M., Jenkins, R. B., and Grogan, T. M. 2001. Discrepancies in clinical laboratory testing of eligibility for trastuzumab therapy: apparent immunohistochemical false-positives do not get the message. *J. Clin. Oncol. 19*: 2714–2721.

Tubbs, R. R., Pettay, R. J., Swain, E., Roche, P. C., Powell, W., Hicks, D. G., and Grogan, T. 2006. Automation of manual components and image quantification of direct dual label fluorescence in situ hybridization (FISH) for HER2 gene amplification: a feasibility study. *Appl. Immunohistochem. Mol. Morphol. 14*: 436–440.

van 't Veer, L. J., Dai, H., van de Vijver, M. J., He, Y. D., Hart, A. A., Mao, M., Peterse, H. L., van der Kooy, K., Marton, M. J., Witteveen, A. T., Schreiber, G. J., Kerkhoven, R. M., Roberts, C., Linsley, P. S., Bernards, R., and Friend, S. H. 2002. Gene expression profiling predicts clinical outcome of breast cancer. *Nature 415*: 530–536.

Vinatzer, U., Dampier, B., Streubel, B., Pacher, M., Seewald, M. J., Stratowa, C., Kaserer, K., and Schreiber, M. 2005. Expression of HER2 and the coamplified genes GRB7 and MLN64 in human breast cancer: quantitative real-time reverse transcription-PCR as a diagnostic alternative to immunohistochemistry and fluorescence in situ hybridization. *Clin. Cancer Res. 11*: 8348–8357.

Wolff, A. C., Hammond, M. E., Schwartz, J. N., Hagerty, K. L., Allred, D. C., Cote, R. J., Dowsett, M., Fitzgibbons, P. L. Hanna, W. M., Langer, A., McShane, L. M., Paik, S., Pegram, M. D., Perez, E. A., Press, M. F., Rhodes, A., Sturgeon, C., Taube, S. E., Tubbs, R., Vance, G. H., van de Vijver, M., Wheeler, T. M., and Hayes, D. F. 2007. American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer. *J. Clin. Oncol. 25*: 118–145.

Yaziji, H., Goldstein, L. C., Barry, T. S., Werling, R., Hwang, H., Ellis, G. K., Gralow, J. R., Livingston, R. B., and Gown, A. M. 2004. HER-2 testing in breast cancer using parallel tissue-based methods. *JAMA 291*: 1972–1977.

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Multifocal or Multicentric Breast Cancer: Understanding Its Impact on Management and Treatment Outcomes

Julia L. Oh

DEFINITIONS OF MULTIFOCALITY AND MULTICENTRICITY

Multifocality and multicentricity (MFMC) are frequently used descriptors to assess the extent of disease in patients presenting breast cancer. The presence of two or more foci of cancer within the *same* breast quadrant is defined as multifocal, while the presence of two or more foci of cancer in *different* quadrants of the same breast is defined as multicentric. The nomenclature is widely used to describe multiple tumors diagnosed clinically on physical examination, on breast imaging studies including mammogram, ultrasound, and magnetic resonance imaging (MRI) or on pathologic analysis.

Holland *et al*. (1985) described multicentric breast cancers as typically exhibiting cancer foci > 5 cm apart and frequently associated with extensive intraductal carcinoma (EIC). Because *in situ* carcinoma is a precursor of invasive carcinoma, the presence of *in situ* disease in multiple separate cancer foci has led investigators to view such foci as polyclonal tumors versus those of monoclonal origin (Noguchi *et al*., 1994; Teixeira *et al*., 1997). However, unless clonal analysis is performed on each cancer foci, whether the tumors are of polyclonal or monoclonal origin, the disease cannot be assessed by spatial presentation alone.

IMPACT ON SURGICAL RESECTION

With the increasing use of whole breast ultrasound and MRI, clinical MFMC breast cancer is being diagnosed more commonly. Wilkinson *et al*. (2005) reported a 15% rate of MFMC breast cancer detection with mammography compared to 34% with the use of adjuvant bilateral breast ultrasound. In addition, Sardanelli *et al*. (2004) demonstrated superior sensitivity of breast MRI to detect MFMC breast cancer foci in women with particularly dense breasts when compared to mammography.

The diagnosis of clinical multifocal or multicentric breast cancer has an immense impact on the extent of surgical resection planned because the surgeon's goal is to remove all foci of cancer. In general, the surgical approach is used when two or more foci of cancer arise from the same quadrant of the breast (multifocal), and can be resected with a single incision; these patients are deemed suitable candidates for breast conservative surgery. However, in patients with *multicentric* disease with two or more foci of cancer located in different quadrants of the breast, a mastectomy remains the standard of care to ensure all cancer foci are removed. The highest standard of care should be taken to obtain negative surgical margins to optimize local control.

If the patient is treated with neoadjuvant chemotherapy, the same guidelines apply for surgical excision. One additional procedure necessary in patients receiving neoadjuvant chemotherapy is that each foci of cancer is marked under ultrasound guidance with a radiopaque marker to mark the tumor bed before any chemotherapy is initiated. This ensures that the surgeon will be able to identify and remove the entire tumor bed at the time of lumpectomy even if the patient achieves a clinical complete response to the chemotherapy.

IMPACT ON ADJUVANT RADIATION THERAPY

The impact of clinical MFMC breast cancer on adjuvant radiation therapy treatment decisions is more controversial. If the patient undergoes breast conservative surgery, postoperative adjuvant radiation to the intact breast is clearly indicated. However, when the clinical presentation precludes conservative surgery and a mastectomy is performed, postmastectomy radiation therapy (PMRT) is recommended only when the risk of locoregional recurrence is ~ 15% or greater. It is controversial whether patients with MFMC breast cancer are at greater risk of locoregional recurrence after a mastectomy, and therefore warrant postmastectomy radiation therapy.

The debate stems from an observed increased rate of axillary nodal positivity associated with MFMC disease. Andea *et al*. (2002) analyzed the relationship between aggregate tumor size and lymph node status in MFMC breast cancer and reported an increased odds ratio of 2.8 for positive axillary nodes in patients with multifocal lesions versus unifocal lesions ($p < 0.0001$). Coombs and Boyages (2005) analyzed 848 patients with breast cancer treated with breast conservative therapy and reported rates of axillary node involvement of 52.1% among the 49 women with multifocal breast cancer versus 37.5% in patients with unifocal tumors ($p = 0.007$). However, the multifocal group in this study had a statistically significant younger mean age of patients than the unifocal group that may have contributed to the increased incidence of positive axillary nodes.

Current staging rules guided by The American Joint Committee of Cancer (AJCC) 6th edition (2001) state that each patient should be staged by the diameter of the largest tumor focus and number of involved lymph nodes. This method of estimating survival has been criticized as inadequate as it fails to account for the aggregate tumor burden. These studies illustrate the ongoing belief that patients with MFMC breast tumors do

worse than their unicentric cohorts due to their observed increased rate of axillary nodal involvement; however, it should be noted that neither of these studies included patients treated with neoadjuvant chemotherapy or reported outcome data after appropriate treatment.

A recent report by Litton *et al*. (2007) analyzed 300 women 35 years old or younger treated from 1990 to 2002 and compared relapse-free survival (RFS) and overall survival (OS) between patients with pathologically diagnosed multifocal (N = 58) versus unicentric breast cancer. None of these patients received neoadjuvant chemotherapy. Seventy-six percent of patients received adjuvant systemic chemotherapy or endocrine therapy, of which 50% were anthracycline-based regimens. At a median follow-up of 43.9 months, the 5-year RFS was 57.1% versus 44.4% ($p = 0.36$) and OS was 67.3% versus 69.7% ($p = 0.70$) for multifocal versus unicentric tumors, respectively. This study illustrates that for pathologically diagnosed multifocal breast cancers treated with adjuvant chemotherapy, the RFS and OS are equivalent to those seen with unicentric tumors. Therefore, adjuvant radiation therapy beyond the standard recommendations is not indicated based on multifocality found at the time of surgery.

NEOADJUVANT CHEMOTHERAPY AND LOCOREGIONAL THERAPY

In patients who receive chemotherapy in the neoadjuvant setting, whether initial clinical MFMC diagnosed on breast mammogram and/or whole breast ultrasound, the independent risk factor for recurrence was questioned. The hypothesis was generated from reports that noted an increased incidence of axillary nodal involvement in patients with pathologically diagnosed multifocal or multicentric tumors. Did MFMC breast cancers have an increased risk of locoregional failure? If so, additional adjuvant locoregional treatments would be justified. To address this concern, Oh *et al*. (2006) compared the outcome between 97 patients with clinical MFMC breast cancer with 609 patients with unicentric cancer treated with doxorubicin-based neoadjuvant chemotherapy, followed by stage-appropriate local therapy. The patient groups were balanced with regard to patient age, nuclear grade, tumor stage, nodal stage, overall stage, and estrogen receptor status. At a mean follow-up of 66 months, the 5-year rate of locoregional failure was 7% and 10% in patients with MFMC and unicentric disease, respectively ($p = 0.78$). Five-year disease-free survival was 86% for patients with MFMC disease versus 78% for patients with unicentric disease ($p = 0.16$). Patients with MFMC disease demonstrated an 86% 5-year overall survival compared to 83% in the patients with unicentric disease, again demonstrating equally favorable outcome.

The report, therefore, refutes the notion of clinical MFMC breast cancer as an independent high-risk feature for locoregional recurrence in patients treated with doxorubicin-based neoadjuvant chemotherapy, followed by locoregional therapy. An important caveat to note is that all of the patients treated with neoadjuvant chemotherapy and breast conservative therapy had clinical *multifocal* disease only rather than multicentric disease. In contrast, all patients with *multicentric*

disease underwent a mastectomy after neoadjuvant chemotherapy regardless of response to chemotherapy. Separate analyses divided by locoregional therapy (breast conservative therapy, mastectomy alone, mastectomy plus postmastectomy radiation therapy) after neoadjuvant chemotherapy did not demonstrate any difference in locoregional control based on MFMC or unicentric disease; therefore, clinical MFMC is not a high-risk feature predictive of locoregional recurrence.

CONCLUSIONS ON MANAGEMENT

In summary, multifocality and multicentricity are widely accepted descriptors that describe numerous foci of cancer within the same breast located in the same or separate quadrants. Multiple foci of cancer can be noted on clinical exam, imaging with mammogram, ultrasound or MRI or on pathologic examination. Although there are data to support that pathologic MFMC breast cancers have a higher incidence of pathologically positive axillary nodes, it is controversial whether this has any clinical impact. Data on clinical outcome have failed to demonstrate inferior relapse-free survival in patients with MFMC versus unicentric tumors.

After treatment with neoadjuvant chemotherapy followed by locoregional therapy, patients with clinical MFMC disease should be expected to have equivalent locoregional control, disease-free survival and overall survival to their unicentric cohorts. Therefore, the data do not support altering the AJCC staging guidelines and we continue to stage MFMC breast tumors by the diameter of the largest single foci of cancer and extent of axillary nodal involvement. Standard guidelines for postmastectomy radiation therapy should apply to patients with clinical MFMC disease as the use of postmastectomy radiation therapy in otherwise early stage breast cancer patients will risk overtreating low-risk patients with subsequent detrimental impact on the timing of breast reconstruction. For patients with multifocal disease, who undergo breast conservative surgery, postoperative radiation therapy to the intact breast is warranted. There are to date no data on breast conservation therapy for clinically multicentric tumors.

REFERENCES

Andea, A.A., Wallis, T., Newman, L.A., Bouwman, D., Dey, J., and Visscher, D.W. 2002. Pathologic analysis of tumor size and lymph node status in multifocal/multicentric breast carcinoma. *Cancer 94*: 1383–1390.

Coombs, N.J., and Boyages, J. 2005. Multifocal and multicentric breast cancer: does each focus matter? *J. Clin. Oncol. 23*: 7497–7502.

Greene, F.L., Page, D.P., Fleming, I.D., Fritz, A.G., Balch, C.M., Haller, D.G., and Morrow, M. 2001. *American Joint Commission on Cancer Staging Handbook* (6th ed.): 256–281.

Holland, R., Veiling, S.H., Mrauunac, M., and Hendriks, J.H. 1985. Histologic multifocality of Tis, T_{1-2} breast carcinomas. Implications for clinical trials of breast-conserving surgery. *Cancer 56*: 979–990.

Litton, J.K., Eralp, Y., Gonzalez-Angulo, A.M., Broglio, K., Uyei, A., Hortobagyi, G.N., and Arun, B. 2007. Multifocal breast cancer in women < / = 35 years old. *Cancer 110*: 1445–1450.

Noguchi, S., Aihara, T., Koyama, H., Motomura, K., Inaji, H., and Imaoka S. 1994. Discrimination between multicentric and multifocal carcinomas of the breast through clonal analysis. *Cancer 74*: 872–877.

Oh, J.L., Dryden, M.J., Woodward, W.A., Yu, T.K., Tereffe, W., Strom, E.A., Perkins, G.H., Middleton, L., Hunt, K.K., Giordano, S.H., Oswald, M.J., Domain, D., and Buchholz, T.A.

2006. Locoregional control of clinically diagnosed multifocal or multicentric breast cancer after neoadjuvant chemotherapy and locoregional therapy. *J. Clin. Oncol. 24*: 4971–4975.

Sardanelli, F., Giuseppetti, G.M., Panizza, P., Bazzocchi, M., Fausto, A., Simonetti, G., Lattanzio, V., and Del Maschio, A. 2004. Sensitivity of MRI versus mammography for detecting foci of multifocal, multicentric breast cancer in fatty and dense breasts using the whole-breast pathologic examination as a gold standard. *AJR Am. J. Roentgenol. 183*: 1149–1157.

Teixeira, M.R., Pandis, N., Bardi, G., Andersen, J.A., Bohler, P.J., Qvist, H., and Heim, S. 1997. Discrimination between multicentric and multifocal breast carcinoma by cytogenetic investigation of macroscopically distinct ipsilateral lesions. *Genes Chromos. Cancer 18*: 170–174.

Wilkinson, L.S., Given-Wilson, R., Hall, T., Potts, H., Sharma, A.K., and Smith, E. 2005. Increasing the diagnosis of multifocal primary breast cancer by the use of bilateral whole-breast ultrasound. *Clin. Radiol. 60*: 573–578.

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Are Breast Cancer Survivors at Risk for Developing Other Cancers?

Jaime S. Raymond and Carol J. Rowland Hogue

SUMMARY

Second primary cancers occur in ~ 12% of women with an initial breast cancer diagnosis. This study assessed predictors of the risk of developing a second primary cancer after breast cancer. The analysis included 335,191 females, registered in the National Cancer Institute's Surveillance Epidemiology and End Results (SEER) database, who had been diagnosed with breast cancer. Observed numbers of subsequent cancers in the SEER database with a first breast cancer diagnosed from 1973 to 2000 were compared with the expected numbers based on age-adjusted incidence rates to calculate standardized incidence ratios. Kaplan-Meier curves were conducted to determine the median time until the second primary cancer diagnosis.

Average number of years until diagnosis varied by site and by age as well as median years until second cancer diagnosis. Most cancer risks decreased with age, but there was an increase in aging-related cancers such as lung cancer. The median years of follow-up were well beyond the 5-year mark. Breast cancer survivors should be advised of their increased risk for developing certain cancers in their lifetime.

INTRODUCTION

Approximately, 600,000 women in the United States are diagnosed with cancer in a given year, and 31% of them will be diagnosed with breast cancer. Breast cancer is the leading cancer for women, and deaths associated with breast cancer are second behind lung cancer, and account for ~ 15% of all cancer deaths (American Cancer Society, 2004). Survival rates (5, 10, and 20-year) continue to increase and many women will survive their breast cancer diagnosis and treatment (American Cancer Society, 2004; Rosen *et al.*, 1989; Jones and Raghavan, 1993).

Second primary tumors may develop in women previously diagnosed with breast cancer. Yet little is known regarding the risk of multiple primary tumors among breast cancer survivors. The purpose of this study was to examine whether women diagnosed

with breast cancer have an increased risk of developing a subsequent cancer compared with women of equal age without an initial breast cancer diagnosis. The current study reports the most common second primary tumors following breast cancer, the time to diagnosis for women with a second tumor and how overall survival is affected for these women.

MATERIALS AND METHODS

Population

The study population was derived from the SEER Cancer Registry database, which encompasses ~ 11% of the national cancer burden (Fritz and Ries, 1998; SEER*Stat software, 2004). All patients in this study were women in the SEER Cancer Registry diagnosed with breast cancer between January 1, 1973 and December 31, 2000. There were 335,191 women diagnosed with breast cancer (International Classification of Diseases for Oncology 2 [ICD-O-2] code C50) (Percy *et al*., 1990). Women were considered if the breast cancer was categorized as in situ or malignant and if the morphology was either ductal, lobular, or medullary neoplasm (ICD-O-2 codes 8500–8540). A total of 2,330 women developed a second cancer, but the information was never completely entered into the SEER database. These women were used in the survival analysis, because survival status information was provided, but these 2,330 women were excluded from the incidence ratio analyses. Another 3,177 women had missing follow-up time. These women were excluded from the survival analysis, but if they developed a second cancer simultaneously, they were then included in the incidence analyses. A total of 332,014 women were used in the survival analysis and were followed up until they were diagnosed with their second tumor, December 2000, or death before December 2000, whichever came first.

Definition of Second Primary Tumors

Multiple primary tumors are difficult to distinguish from metastatic tumors. The current study uses the SEER definition to define multiple primary tumors. The SEER definition takes into account, when determining multiple primary tumors, the site of the tumor, behavior of the second tumor, histology, date of diagnosis, and the laterality if it is a paired organ (Fritz and Ries, 1998). Using this definition, metastatic and recurrent tumors are excluded from multiple primary tumor counts. There are exceptions and factors such as histology, laterality, and the date of diagnosis that can alter the decision of determining multiple primary tumors with paired organs. The SEER definition of multiple primary tumors for the breast include the following criteria:

- A single tumor with one histologic type is regarded as a single primary tumor; a single tumor disposed with multiple histologic types is regarded as a single tumor.
- A new tumor that contains the same histology as a previous cancer and is diagnosed within 2 months of the previous cancer is regarded as a single primary unless determined to be a recurrent or metastatic cancer.
- Simultaneous multiple tumors with the same histologic type within the breast are regarded as a single tumor, and even

when different tumors have different behavior codes.
- Simultaneous bilateral involvement of the breasts with only one histology is regarded as a single primary tumor.
- Multiple tumors with the same histologic type appearing in the breast and in a different site are regarded as multiple primary tumors unless stated as metastatic tumors.
- Multiple tumors of different histologic types within the breast are counted as multiple primary tumors regardless of time.
- Multiple tumors of different histologic types appearing in the breast and in a different site are multiple primary tumors regardless of time (Fritz and Ries, 1998; Howe *et al*., 2003).

Analysis of Risk of Second Primary Tumors

The primary variable considered as the exposure in the first part of the analysis was the type of cancer the women developed after breast cancer. These women were then stratified into 10-year age groups for analysis. Age was determined by the age of each woman at the date of diagnosis for breast cancer. Data were analyzed using STATA software (STATA Corporation, 2002). Risks are considered significant when corresponding 95% confidence interval did not include the null value. The risk was calculated for 14 second primary tumors in addition to an overall risk for second primary tumors in 10-year age periods. These were selected because there were at least 100 cases to analyze or showed to be statistically significant.

To control for potential confounding of the differences in age distributions, age-adjusted incidence rates were used for comparison. Age/period-specific cancer incidence rates from the SEER database were applied to the cohort, to calculate the number of subsequent tumors that would be expected for each site. This number was compared with the observed number to obtain a standardized incidence ratio (SIR) estimate and 95% confidence intervals (CI) were calculated assuming a Poisson distribution. Those sites that were statistically significant or showed an association with breast cancer were listed.

The cohort includes women diagnosed at earlier ages who survive cancer to the next 10-year age group. For example, women diagnosed prior to age 30 are included in older age groups if they did not have a second primary tumor diagnosis prior to age 30. Subsequent data accumulate cohorts similarly.

Analysis of Time to Diagnosis for Women with Second Primary Tumor

For the women who were diagnosed with a second primary tumor, univariate analysis was performed using Kaplan Meier curves, to estimate the time function diagnosis. Curves were calculated for all sites combined and for specific sites with at least 100 cases, and women were divided into decades of age at breast cancer diagnosis, 20–29, 30–39, 40–49, 50–59, 60–69, and 70 and older. Several exposure variables were explored in univariate analyses. Variables of interest were grouped and coded accordingly. These include multiple status, grade, AJCC stage or summary staging, and race. All variables were significant in the univariate analysis and included in the multivariate analysis. Multivariate analysis was determined between groups assessed

by goodness of fit (GOF) methods (Kaplan and Meier, 1958; Lee, 1980; Snedecor and Cochran, 1980).

RESULTS

Of those diagnosed with breast cancer during 1973–2000, 40,068 women developed one additional cancer after breast cancer, 3,796 women developed two cancers, 351 women developed three cancers, 31 women developed four cancers, and three women developed five cancers. Of these women, 0.76% were diagnosed by age 29, 6.79% between 30 and 39, 19.72% between 40 and 49, 21.19% between 50 and 59, 22.34% between 60 and 69, and 29.20% at or after age 70. Second cancers were subsequently reported for 11.7% of the patients who were diagnosed with breast cancer before age 50, and 17% of patients diagnosed with breast cancer at or after age 50. Among women with a second tumor diagnosed, the mean time until the second cancer occurred was 6.2 years: 8 years for patients less than 50 years old and 5.7 years for women at or greater than age 50. Among women with a single breast cancer diagnosis, the average time from diagnosis of the breast cancer until death was 6.71 years for women less than 50 years old, and 4.5 years for women at or greater than age 50. 60.28% of the women were still alive at the end of follow-up.

The results of the standardized incidence ratio analysis are found in Table 41.1. For women diagnosed with breast cancer by age 29, the overall second primary tumor risk is large (SIR = 17.3, 95% CI (13.7, 21.6)), as is the risk of developing a second breast cancer (SIR = 478.5) or stomach cancer (SIR = 145.4). As age at primary diagnosis increases, the overall SIR's decrease until the eldest age group. Cancer sites with the highest ratios for women diagnosed with breast cancer before age 50 include bone, breast, leukemia, lung, and ovary. However, having a breast cancer diagnosis was protective against cancer of the cervix for these age groups. For women who did not develop breast cancer until at least 50 years old, the overall SIR for developing a second cancer by age 59 was 2.7, which is much lower than risks among some of the earlier age groups. Again, the overall ratios decrease with age. Cancer sites with the highest SIR's were breast, colon/rectum, corpus uteri, lung, ovary, skin, and thyroid. Among older women, an initial breast cancer diagnosis was not protective against cervical cancer. Overall, both excess risk and protective effects decreased with age for most of the 14 cancers examined. For example, the SIR for a second breast cancer was greatest for women 20–29 years of age (478.53) and decreased dramatically and consistently after age 30 (Table 41.1).

Table 41.2 presents the data comparing the SIR's of a second breast cancer diagnosis with a second non-breast cancer diagnosis within 10-year time intervals. For women diagnosed with breast cancer by age 29, their risk of developing a second primary tumor other than breast within 10 years of their initial diagnosis is large (SIR = 8.9, 95% CI (8.27, 9.60)), but their risk for developing a second breast cancer, within the same 10 years, is five and a half times greater (SIR = 48.4, 95% CI (46.5, 50.3)). But after 20 years, these same women actually showed a protective effect against both cancer groups, breast and other, compared to the general population (SIR = 0.2, 95% CI (0.2, 0.3),

TABLE 41.1. Standardized incidence ratios for a second primary cancer diagnosis by decade age at risk.

	20–29		30–39[a]		40–49		50–59		60–69		70+		50–59[b]		60–69[b]		70+[b]			Percent
Age/cancer	N	**SIR**	N	**SIR**	N	**SIR**	N	**SIR**	N	**SIR**	N	**SIR**	N	**SIR**	N	**SIR**	N	**SIR**	Total	%
All sites	78	**17.3**	1,037	**5.9**	4,547	**2.37**	2,968	**1.64**					4,720	**2.67**	10,393	**1.74**	20,506	**1.33**	44,249	
Breast	65	**478.5**	857	**21.8**	3,463	**4.14**	1,507	**2.19**	387	**1.45**			3,118	**4.63**	5,268	**2.83**	8,262	**2.11**	22,927	51.81
Other cancers	13	**4.4**	180	**1.3**			1,461	**1.3**	526	**0.9**	71	**0.8**	1,602	**1.3**	5,125	**1.3**	12,244	**1.1**	21,322	48.19
Bone	1	**20.1**	3	**8.2**			7	**4.39**							11	**2.17**			22	0.05
Brain	1	**9.3**																	1	0.00
Cervix uteri			39	**0.5**	140	**0.55**	57	**0.68**	8	**0.35**							160	**0.72**	404	0.91
Colon/ rectum									82	**0.77**	11	**0.54**	214	**1.42**	869	**1.18**	3,145	**1.07**	4,321	9.77
Connective tissue			6	**5.9**											49	**2.4**	105	**1.79**	160	0.36
Corpus uteri							189	**1.17**	49	**0.67**			207	**1.31**	679	**1.34**	1,273	**1.61**	2,397	5.42
Leukemia	3	**27.4**			69	**2.2**	72	**1.81**					64	**1.64**	259	**1.45**			467	1.06
Lung			17	**6.7**	127	**1.31**	228	**1.24**					303	**1.69**	978	**1.25**	1,854	**1.12**	3,507	7.93
Ovary	2	**16**	27	**5**	153	**1.9**	141	**1.77**					147	**1.89**	338	**1.47**	531	**1.11**	1,339	3.03
Pancreas			3	**6.8**									112	**4.25**			488	**0.88**	603	1.36
Skin			15				104	**1.63**	32	**1.48**			107	**1.71**	264	**1.76**	559	**1.58**	1,049	2.37
Stomach	1	**145.4**	1												11	**0.14**			13	0.03
Thyroid			17	**1.9**			46	**1.64**					61	**2.23**	77	**1.53**	113	**1.38**	314	0.71

All numbers represents an elevated risk that is statistically significant by 95% Confidence Intervals

[a]Women diagnosed prior to age 30 are included if they did not have a primary tumor diagnosis prior to age 30. Subsequent tables accumulate cohorts similarly

[b]The first diagnosis of breast cancer occurred at or after age 50.

TABLE 41.2. Standardized incidence ratios categorized by either a second breast cancer diagnosis or a second primary cancer other than breast cancer, by time intervals after first diagnosis of breast cancer.

	Less than 10 years after initial breast cancer				10–19 years after initial breast cancer				20–29 years after initial breast cancer			
	Second primary - Breast cancer		Second primary - Non-breast cancer		Second primary - Breast cancer		Second primary - Non-breast cancer		Second primary - Breast cancer		Second primary - Non-breast cancer	
Age	SIR	95% CI	SIR	95% CI	SIR	95% CI	SIR	95% CI	SIR	95% CI	SIR	95% CI
20–29	48.4	(46.5, 50.3)	8.90	(8.3, 9.6)	11.5	(10.6, 12.4)	1.46	(1.3, 1.2)	0.2	(0.2, 0.3)	0.5	(0.4, 0.6)
30–39	44.1	(42.3, 45.9)	2.79	(2.6, 3.0)	2.1	(1.9, 2.3)	1.35	(1.2, 1.5)	0.3	(0.2, 0.3)	0.3	(0.3, 0.4)
40–49	12.1	(11.6, 12.6)	4.08	(3.8, 4.3)	1.3	(1.2, 1.4)	0.88	(0.8, 1.0)	0.3	(0.2, 0.3)	0.0	(0.0, 0.05)
50–59	10.9	(10.6, 11.3)	4.75	(4.6, 5.0)	1.5	(1.4, 1.6)	0.99	(0.9, 1.1)	0.1	(0.1, 0.2)	0.2	(0.2, 0.2)
60–69	6.4	(6.2, 6.6)	2.99	(2.9, 3.1)	0.4	(0.3, 0.4)	0.50	(0.5, 0.5)	0.1	(0.1, 0.1)	0.1	(0.1, 0.2)
70+	1.8	(1.8, 1.9)	1.60	(1.5, 1.7)	0.2	(0.2, 0.2)	0.30	(0.3, 0.3)	0.1	(0.1, 0.1)	0.1	(0.1, 0.1)

SIR = 0.5, 95% CI (0.4, 0.6)). For women diagnosed with breast cancer at or after age 70, their risk for developing either a second breast cancer or a second primary cancer, other than breast within 10 years, is virtually the same (SIR = 1.8, 95% CI (1.8, 1.9) and SIR = 1.6, 95% CI (1.5, 1.7)). In general, most women had a more elevated risk of developing a second breast cancer, than a second primary cancer other than breast, but both risks were greater for these women than the general population. The risk reduced with time and age for all groups.

Table 41.3 summarizes data from Kaplan Meier Curves for time until second primary cancer diagnosis, stratified on age at diagnosis of the breast cancer and limited to the 12.3% of women who were diagnosed with a second primary tumor. The median years of follow-up until the second cancer is diagnosed ranged from 2.3 years for women over 70 developing cervix uteri to 18.08 for women 30–39 developing a second breast cancer. The difference in follow-up time for the age groups can be attributed to (1) a longer at-risk period for women < 50 years for developing the second cancer, and (2) the increased risk of developing some cancers at an advanced age.

Figure 41.1 illustrates the median ages at the time of the second cancer diagnosis for each of the 14 cancers. Cervix uteri cancer has the youngest median age with 59 years, while colon/rectum cancer has the oldest median age with 75 years.

Survival analysis was then constructed stratifying on the women who only developed breast cancer and those women who were diagnosed with breast cancer and a second primary cancer. Survival curves were adjusted for race and grade. In the 20–29 year old category, grade was categorized as an ordinal variable. Figure 41.2 illustrates survival analysis for women 20–29 years old diagnosed with breast

TABLE 41.3. Point estimates for overall time until second primary cancer diagnosis, stratified on age, and limited to the 12.3% of women who were diagnosed with a second primary tumor.

Age at diagnosis	75%	50%	25%
20–29	10.5	5.5	1.9
30–39	16.9	11.1	4.2
40–49	9.9	4.3	0.9
50–59	10.5	4.8	1.1
60–69	8.8	4.4	1.2
70+	6.0	2.7	0.4

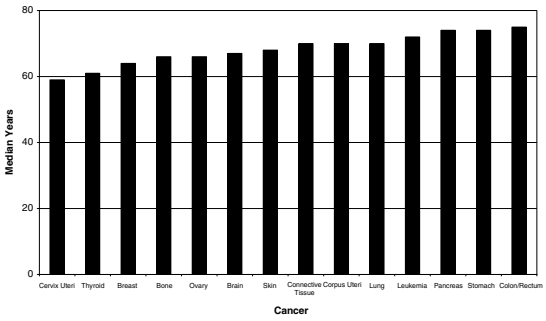

FIGURE 41.1. Median age at cancer diagnosis for second primary tumors

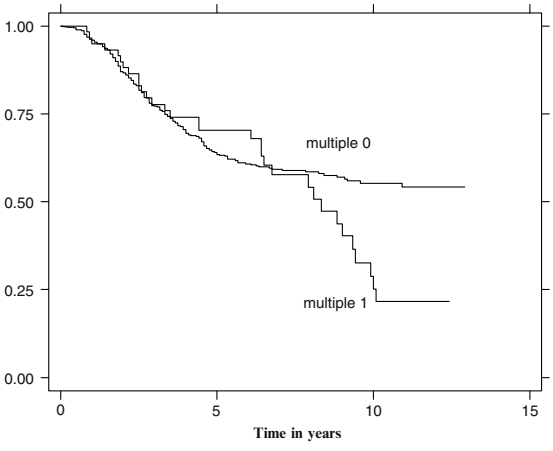

FIGURE 41.2. Survival analysis for women 20–29 years old diagnosed with breast cancer in or after 1988, stratified on multiple tumor status, controlling for race and grade

cancer in or after 1988, stratified on multiple tumor status, controlling for race (P = 0.621) and grade (P = 0.511). The figure shows women with multiple primary cancers had poorer survival than women with only a breast cancer diagnosis over time. Women in this young age group who had metastatic breast cancer have a 15-year survival probability of only 23.23%.

In general, overall survival was poorer for women with multiple primary tumors compared with women with no second tumors. In addition to second tumors, grade of the breast cancer, race, and extent of disease played a role in determining the survival for each age group. A woman with a higher-grade breast tumor followed by a second tumor had much poorer survival than a woman with a low-grade breast tumor followed by a second tumor. This is also true for extent of disease. Time also acted as a contributing factor to poorer survival.

DISCUSSION

Changes in both the breast cancer incidence and the mortality rates have been documented in the past 25 years (Young *et al.*, 2001). Evidence shows a fundamental increase in incidence, which is partly attributed to the improved screening and diagnostic techniques as well as to the changes in reproductive patterns, i.e., women waiting until later to have children. Mortality rates had been relatively constant from 1973–1990. Since 1990, mortality rates have decreased by 2.3% overall. This decrease was more pronounced in women diagnosed with breast cancer before age 50 than with women age 50 or older and have been attributed to the improvement of the various cancer treatments (Ries *et al.*, 2003). Recent data have shown an overall survival rate for women diagnosed with breast cancer at least 5 years prior to be as high as 87% but drops to 77% for the 10-year mark and to 52% by the 20-year mark (Ries *et al.*, 2003). Given the increase in incidence and survival, it is important to assess health risks for breast cancer survivors.

Using a standard method, population-based data were collected from high-quality registries that covered ~ 11% of the United States population. The study provided a unique opportunity to study a large number of women, over 335,000, across several years (1973–2000). This is the largest and most comprehensive study to date of cancer among breast cancer survivors. An international study that examined the risk of a second primary tumor following an initial breast cancer diagnosis, only assessed risk for two age groups: < 50 and ≥ 50 years old. The study also used the International Association for Research on Cancer definition of a second primary cancer, which is not the definition widely used in the United States (Chu *et al.*, 1999). Another study examined women developing sarcoma after breast cancer, using the Surveillance, Epidemiology, and End Results (SEER) program data from 1973–1997 (Yap *et al.*, 2002). The major drawback in this study was that it included only patients with invasive breast cancer.

This study found that 12.3% of breast cancer survivors were diagnosed with second primary malignant tumors among breast cancer survivors. Excess risks for 13 specific cancers were found, with the highest excess risks for breast. Among women with a second primary tumor, the time to second diagnosis varied by type

of tumor and age at diagnosis of the first tumor. The results in this study are comparable to other findings regarding second primary breast cancer (Berstein *et al*., 2003). There were a number of cancers that increased with age, mostly because almost all cancer risks increase with age, such as lung cancer.

These results may help to inform patients and clinicians regarding expected quality of life for breast cancer survivors (Cimprich *et al*., 2002). A greater understanding of the patient's prognosis would assist both the patients and the clinician in treatment options. For example, if a woman understands her risks for developing a second primary cancer, she may choose to alter her treatment to be more aggressive in hopes of minimizing her risk. She may also need to be counseled regarding preventable risk factors, such as weight gain, that have been associated premature mortality for women with a second breast cancer in other studies (Bernstein *et al*., 2002).

In this study, breast cancer survivors had the greatest risk for developing breast, bone, colon/rectal, connective tissue (sarcoma), leukemia, lung, ovarian, and thyroid cancer. There are several possible explanations for these correlations, including genetic mutations. Four possible genetic mutations have been identified which can aid in predicting which woman with breast cancer will develop multiple primary tumors. All four of these genetic mutations include breast cancer and other cancers. BRCA1 and BRCA2 are considered to be the breast cancer genes, but BRCA1 is also associated with ovarian cancer (Szabo and King, 1997). BRCA2 is associated with multiple primary tumors including breast, colorectal, ovarian, and other cancers. Ovarian cancer, such as breast cancer, is an estrogen-driven cancer. Thus estrogen is the most obvious link between the two cancers. However, the risk of ovarian cancer is also increased with the presence of the BRCA1 gene mutation (Szabo and King, 1997). Both BRCA1 and BRCA2 may bestow a heightened sensitivity to carcinogenic effects of radiation (Turner *et al*., 1999; Ford *et al*., 1994). Recently, studies have shown a possible association between lung cancer and BRCA1 and BRCA2 mutation (Bergfeldt *et al*., 2000).

Multiple primary cancers, including acute adult leukemia, have been associated with a family history of breast cancer (Rauscher *et al*., 2002). P53, known as Li-Fraumeni Syndrome, is associated with the development of cancer at a young age and is associated with breast cancer, sarcoma, brain cancer, and leukemia (Lee, 1980). Women with P-53 mutation have a lifetime probability of almost 100% for developing at least one of these cancers. Lastly the genetic mutation PTEN or Cowden Disease imports risk for both breast and thyroid cancer.

Breast cancer survivors appeared to have protection from developing cervical cancer. Low socioeconomic status is a risk factor for cervical cancer while high socioeconomic status is a risk factor for breast cancer. However, the causal relationship underlying these associations is not well understood. Breast cancer was associated with increased risk of connective tissue cancer for several of the age groups. Two possible explanations are: (1) many women with breast cancer undergo radiation, which increases a woman's risk for connective tissue cancer in the surrounding areas, and (2) some genetic mutations,

including Li-Fraumeni Syndrome are linked with both breast cancer and connective tissue cancer (Rubino *et al*., 2003; Malkin *et al*., 1990).

Radiation may also increase the risk of lung cancer (Roychoudhuri *et al*., 2004; Matesich and Shapiro, 2003). During the 1950s, most of the upper body was exposed to radiation as the treatment for breast cancer. This exposure included the thyroid, lungs, breasts, and chest wall. This excess risk should therefore decrease as the cohort exposed to high doses of radiation doses ages. Radiation or chemotherapy as a treatment option for breast cancer may be a risk factor for developing leukemia as a second primary cancer.

Among women who developed a second tumor, the time to cancer varied by age. Risk for cancer generally increases with age. This is most evident for the "older age" cancers such as colorectal, in which median years to second diagnosis decreased from 18 for the 20–29 year group to less than four for the 70+ year group. Other age-dependent cancers were ovarian (median years ranging from 11 to > 4) and pancreas (median years ranging from 15 to ~ 4). In the past, clinicians used as a general rule that if a woman is cancer free for 5 years after the initial diagnosis, then she is cured of cancer. This analysis shows that a sizeable proportion of women who developed a second tumor were cancer-free 5 years after their initial diagnosis. With the exception of the oldest group, the median time to diagnosis exceeded 5 years for the women who had a second tumor. For women who were diagnosed with breast cancer between the ages of 30 and 39, the median number of years until they were diagnosed with any second tumor was 11.42 years. This long lapse for the 30–39 year old age group is primarily due to the fact that the median number of years until a second breast cancer diagnosis was 18.08 years. It appears that the women in this age group are treated very aggressively and tend to have mastectomies performed on them. The only second cancer where the median number of years was < 5 was cancer of the cervix uteri, but this estimate is probably unrealistic because of the small number of women who developed cancer of the cervix uteri after breast cancer. On the other hand, connective tissue cancer had a median number of years until diagnosis of over 10 years, except for the 70+ age group.

This study has several limitations. While population-based cancer registries provide the opportunity to study these rare events, the registries lack some important variables such as smoking status, specific doses of treatment, and genetic make-up. Also, quality of data used for classifying multiple primary tumors is not under the control of the registries and may vary across site and over time. Even studies that used data sets known to be accurate found errors that affected the interpretation (Bergfeldt *et al*., 2000). Finally, missing and incomplete data may introduce measurement error. In this study, 5.7% of women known to have been diagnosed with a second tumor were excluded from the incidence risk analysis because of unknown diagnosis. If the distribution of the tumor sites in these women is substantially different from the distribution of known tumor sites, results for some of the more rare tumors may be biased. However, the number of unknown tumors is unlikely to have affected the risks from the more frequent tumors. Whatever bias, if present, is likely to be small, as there are striking similarities in results

from published studies of selected second tumors (Yap *et al*., 2002).

In conclusion, it is clear that some women who survive breast cancer are at increased risk of additional cancers. Excess risks attenuate with increasing age at first diagnosis and with increasing years since first diagnosis. Excess risk also varies by cancer type, consistent with existing causal hypotheses regarding genetics mutations and risk of aggressive radiation therapy. More research should focus on isolating the factors that may predispose a breast cancer survivor to the development of subsequent cancers.

REFERENCES

American Cancer Society 2004. Available at http://www.cancer.org/ Accessed January 9, 2004.

Bergfeldt, K., Silversward, C., Einhorn, S., and Hall, P. 2000. Overestimated risk of second primary malignancies in ovarian cancer patients. *Eur. J. Cancer 36*: 100–105.

Bernstein, J.L., Lapinski, R., Lynch, C., Holford, T., and Thompson, W.D. 2002. Factors influencing mortality among young women with second primary breast carcinoma. *Cancer 95*(10): 2051–2058.

Berstein, J.L., Lapinski, R.H., Takore, S.S., Doucette, J.T., and Thompson, W.D. 2003. The descriptive epidemiology of second primary breast cancer. *Epidemiology 14*(5): 552–558.

Chu, K.U., Turner, R.R., Hansen, N.M., Brennan, M.B., Bilchik, A., and Giuliano, A.E. 1999. Do all patients with sentinel node metastasis from breast carcinoma need complete axillary node dissection? *Ann. Surg. 229*(4): 536–541.

Cimprich, B., Ronis, D.L., and Martinez-Ramos, G. 2002. Age at diagnosis and quality of life in breast cancer survivors. *Cancer Pract. 10*(2): 85–93.

Ford, D., Easton, D.F., Bishop, D.T., Narod, S.A., and Goldgar, D.E. 1994. Risks of cancer in BRCA1 mutation carriers. *Lancet 343*: 692–695.

Fritz, A., and Ries, L. (Eds). 1998. *SEER Program Code Manual, 3rd edition*. Bethesda, MD: National Cancer Institute.

Howe, H.L., Weinstein, R., Hotes, J., Kohler, B., Roffers, S.D., and Goodman M.T. 2003. Multiple Primary Cancers of the Ovary in the United States, 1992–1997. *Supplement to Cancer*.

Jones, V.E., and Raghavan, D. 1993. Quantum leaps in treatment of high-risk breast cancer? Prove it! *Eur. J. Cancer 10*: 1488–1493.

Kaplan, E.L., and Meier, P. 1958. Non-parametric estimation from incomplete observations. *J. Am. Stat. Assoc. 53*: 457–481.

Lee, E.T. 1980. Statistical methods for survival analysis. Belmont, CA: Lifetime Learning Publications.

Malkin, D., Li, F.P., Strong, L.C., Fraumeni, J.F. Jr., Nelson, C.E., Kim, D.H., Kassel, J., Gryka, M.A., Bischoff, F.Z., and Tainsky, M.A. 1990. Germ line p53 mutations in a familial syndrome of breast cancer sarcomas, and other neoplasms. *Science 250*: 1233–1238.

Matesich S.M., and Shapiro C.L. 2003. Second cancers after breast cancer treatment. *Semin. Oncol. 30*(6): 740–748.

Percy, C., Van Holten, V., and Muir, C. (Eds). 1990. *International Classification of Disease for Oncology, 2nd edition* 1990 Geneva: World Health Organization.

Rauscher, G.H., Sandler, D.P., Poole, C., Pankow, J., Mitchell, B., Bloomfield, C.D., and Olshan, A.F. 2002. Family history of cancer and incidence of acute leukemia in adults. *Am. J. Epidemiol. 156*(6): 517–526.

Ries, L.A.B., Eisner, M.P., Kosary, C.L., Hankey, B.F., Miller, B.A., and Clegg, L. 2003. *SEER Cancer Statistics Review, 1975–2000*. Bethesda, MD: National Cancer Institute.

Rosen, P.R., Goshen, S., Saigo, P.E., Kinne, D.W., and Hellman, S. 1989. A long-term follow-up study of survival in stage I (T1N0M0) breast carcinoma. *J. Clin. Oncol. 7*: 355–366.

Roychoudhuri, R., Evans, H., Robinson, D., and Moller H. 2004. Radiation-induced malignancies following radiotherapy for breast cancer. *Br. J. Cancer 91*(5): 868–872.

Rubino, C., de Vathaire, F., Shamsaldin, A., Labbe, M., and Le, M.G. 2003. Radiation dose, chemotherapy, hormonal treatment and risk of second cancer after breast cancer treatment. *Br. J. Cancer 89*(5): 840–846.

Snedecor, G.W., and Cochran, W.G. 1980. *Statistical Methods, 7th edition*. Ames, IA: The Iowa State University Press.

Surveillance Research Program, National Cancer Institute SEER*Stat software 2004. (http://www.seer.cancer.gov/seerstat) version 5.0.

STATA Corporation 2002. STATA Statistics/Data Analysis, Version 7.0..

Szabo, C.I., and King, M.C. 1997. Population genetics of BRCA1 and BRCA2. *Am. J. Hum. Genet. 60*: 1013–1020.

Turner, B.C., Harrold, E., Matloff, E., Smith, T., Gumbs, A.A., Beinfield, M., Ward, B., Skolnick, M., Glazer, P.M., Thomas, A., and Haffy, B.G. 1999. BRCA1/BRCA2 germline mutations in locally recurrent breast cancer patients after lumpectomy and radiation therapy: Implications for breast-conserving management in patients with BRCA1/BRCA2 mutations. *J. Clin. Oncol. 17*: 3017–3024.

Yap, J., Chuba, P.J., Thomas, R., Aref, A., Lucas, D., Severson, R.K., and Hamre, M. 2002. Sarcoma as a second malignancy after treatment for breast cancer. *Int. J. Rad. Oncol. Biol. Physics 52*(5): 1231–1237.

Young, J.L. Jr., Roffers, S.D., Ries, L.A.G., Fritz, A.G., and Hurlbut, A.A. (Eds). 2001. *SEER Summary Staging Manual – 2000: Codes and Coding Instructions*. Bethesda, MD: National Cancer Institute, NIH Pub. No. 01–4969.

42
Distant Metastasis in Elderly Patients with Breast Cancer: Prognosis with Nodal Status

Anees B. Chagpar

INTRODUCTION

As our population ages, the number of elderly individuals diagnosed with breast cancer will continue to increase. Currently, the median age at diagnosis for breast cancer patients is 61 years (American Cancer Society, 2006), and women aged 75–79 years have the highest incidence rate of this disease. Despite the considerable number of elderly breast cancer patients being diagnosed today, these patients have traditionally been excluded from clinical trials (Goodwin *et al*., 1988; Trimble *et al*., 1994), and therefore controversy remains regarding what constitutes optimal therapy for this population. Certainly, the elderly form a distinct subgroup of patients, in whom tumor biology, comorbidities, and competing risks must be considered in weighing treatment decisions. However, with advances in medical management, our population continues to enjoy ever-increasing longevity, and therefore many elderly patients with breast cancer may still be expected to have a reasonable quantity and quality of life. Therefore, it behooves clinicians to consider a variety of factors influencing survival and progression to distant metastasis, and manage patients accordingly.

While it is well-known that lymph node status is one of the strongest predictors of prognosis, the need for lymph node evaluation in elderly patients has been a source of significant debate. Older literature points to the concept that axillary node dissection may lead to increased lymphedema in this population, and that adjuvant treatment may not change regardless of lymph node status. With the advent of sentinel node biopsy, a minimally invasive technique to accurately stage the axilla, the concept of lymph node evaluation in the elderly population with breast cancer is being revisited.

BREAST CANCER IN THE ELDERLY

The "elderly", variably defined in the literature as patients older than 65, 70 or 80

years of age, is becoming an increasing demographic population of breast cancer patients. Women aged 75–79 years have the highest incidence rate of breast cancer (496.6 cases per 100,000), and while the incidence rate of breast cancer has plateaued for all other age categories, the incidence rate of this disease continues to climb in those over the age of 50 (American Cancer Society, 2006). Despite being a significant subpopulation of breast cancer patients, the elderly remain understudied. They are less likely to be included in clinical trials, and it is difficult to extrapolate findings in younger patients to the elderly who have unique issues (both in terms of breast cancer tumor biology and other comorbidities and competing risks) which need to be considered in treatment planning.

Tumor Biology

It has been argued that elderly patients with breast cancer may have a biologically different disease than their younger counterparts (Djordjevic *et al*., 2004). A number of studies have found that older patients tend to have larger tumors at the time of diagnosis. For example, Djordjvic *et al*. (2004) found that 75% of patients younger than 65 years of age had tumors < 2 cm, whereas only 62% of patients 65 years of age and older had tumors that were < 2 cm. Similarly, Davis *et al*. (1985) found that 59% of patients 80 years of age or older presented with Stage II–IV breast cancer. Some authors, however, have found that Stage I lesions are equally common across age groups (Mueller *et al*., 1978), while others have found that elderly patients tend to have more localized tumors than younger patients (Herbsman *et al*., 1981). In a study by Hunt *et al*. (1980), the majority of elderly patients had larger local tumors, but did not have regional or distant metastasis, leading to the speculation that a "nonmetastasizing variant" of breast cancer may be more common in the elderly population.

In addition, older patients tend to have estrogen receptor positive (ER+) tumors, and may therefore be adequately treated with hormonal therapy. Djordjevic *et al*. (2004) found that 68% of older patients had ER+ disease, whereas only 28% of younger patients were ER+. Given that the majority of older patients have less aggressive, ER+ breast cancers that can be adequately treated with hormonal therapy, some authors have argued that lymph node evaluation in these patients is unnecessary as it will not change management. Therefore, consistently across studies, a significant minority of elderly patients have not been adequately staged as no form of lymph node evaluation has been performed.

Patient Comorbidities

While patient comorbidities are often cited as a factor influencing the decision not to pursue lymph node evaluation, some have found that less optimal surgery did not always correlate with the degree of comorbid conditions (Law *et al*., 1996; Greenfield *et al*., 1987). Some studies have demonstrated that morbidity of surgical interventions in this population is related primarily to the wound (Hunt *et al*., 1980; Djordjevic *et al*., 2004), and, therefore, the addition of sentinel node biopsy would be anticipated to add little to the morbidity of the surgical extirpation of the tumor. Other studies have found that morbidity

and mortality of breast cancer surgery are comparable between patients 80 years of age or older and their younger counterparts (Davis *et al*., 1985; Swanson *et al*., 1991). While elderly patients often face competing risks, it has been shown that their disease-specific survival is not significantly different from younger patients (Herbsman *et al*., 1981; Djordjevic *et al*., 2004; Crowe, Jr. *et al*., 1994). Therefore, chronologic age alone should not be a deterrent to appropriate treatment of breast cancer patients.

DETERMINANTS OF DISTANT METASTATIC DISEASE

It is well-accepted that breast cancer survival is determined primarily by distant metastatic disease. As most elderly patients have ER+ disease, and are managed with adjuvant hormonal therapy, there is a need to identify the determinants of early distant metastatic disease, the presence of which may precipitate the use of more aggressive systemic chemotherapy in patients deemed fit enough to tolerate such a regimen.

In a study of 938 patients ≥ 65 years of age treated in a multicenter prospective randomized controlled trial of tamoxifen versus toremifene for adjuvant therapy of breast cancer, it was found that 17 patients (1.8%) progressed to distant metastatic disease at a median time from diagnosis of 21 months (range: 3–50) (Chagpar *et al*., 2006). Eight of these patients (47.1%) died of their disease, with a median time to death of 22 months after metastatic disease. Factors predicting the development of metastatic disease in this cohort on univariate analysis included tumor size, number of positive nodes, lymphovascular invasion, nuclear grade, and progesterone status. On multivariate analysis, the number of positive lymph nodes (as a continuous variable) was the only independent factor which remained predictive of distant metastatic disease ($p = 0.029$). Further analysis demonstrated that patients with 4 or more positive nodes were significantly more likely to develop early metastatic disease than those with 0–3 positive nodes (OR: 20.304, 95% CI: 2.777–148.456, $p = 0.003$).

Similarly, a study of 181 node-positive patients aged 65–84 years treated with tamoxifen vs. placebo found that the number of positive nodes significantly affected the proportion of patients who were disease free at 10 years (Cummings *et al*., 1993). For example, in the tamoxifen arm alone, 61.9% of patients with 1–3 positive nodes were disease free at 10 years, compared with only 17.6% of patients who had 4 or more positive nodes. These data are echoed by survival data which found, in the tamoxifen treated arm, that patients with 4 or more positive nodes were nearly half as likely to be alive for 10 years than those with 1–3 positive nodes (35.8% vs. 61.0%).

It is not surprising that the number of positive lymph nodes is a key determinant of survival. In 5 previous National Surgical Adjuvant Breast and Bowel Project studies, the rate of distant metastasis in patients with 4 or more positive lymph nodes was found to be nearly twice that of patients who had only 1–3 positive nodes (Taghian *et al*., 2004). These data argue that it would behoove clinicians to consider lymph node status in determining the prognosis of elderly patients with breast cancer.

Others, however, have argued that lymph node status does not affect disease free survival in patients over 70 years of age. In a study of 378 patients over the age of 70, the only factor affecting 3- and 5-year survival rates was age (Newlin *et al*., 2002). These data, however, did not relate specifically to disease-specific survival. Interestingly, however, among all the factors analyzed, nodal status was the most significant, with node-positive patients having a worse 5-year survival than their node-negative counterparts (69% vs. 77%, $p = 0.0673$). One could speculate that if the study had increased power, and perhaps if the authors had analyzed the number of positive nodes rather than simply nodal status, a statistically significant association may have been found.

LYMPH NODE EVALUATION IN THE ELDERLY

It has been well-established that axillary node dissection alone does not improve survival (Fisher *et al*., 2002; Veronesi *et al*., 2002). However, it is also well-accepted that lymph node status is a significant factor affecting prognosis. Therefore, for the majority of breast cancer patients, lymph node evaluation is considered a standard part of breast cancer surgical management.

In the elderly population, lymph node evaluation is commonly omitted. In a study breast caner patients 70 years of age or older treated at a community hospital, it was found that complete pathologic staging was performed in only 64% of patients (Litvak and Arora, 2006). Similarly, in a study of 198 patients with breast cancer 80 years of age or older, histologic examination of the lymph nodes was not performed in 82 patients (41%) (Davis *et al*., 1985). In a study of 39 Japanese patients over 80 years of age, 6 (15.3%) did not have axillary staging (Morishita *et al*., 1997), and in a similar study of 325 Polish patients over 70 years of age, 50 (15.4%) did not have axillary staging (Nagadowska and Kulakowski, 1991).

Increasing age is associated with decreased odds of having lymph node evaluation independent of health status, patient preferences, clinical factors, and provider variables (Edge *et al*., 2002). Furthermore, increased age is associated with decreased adherence to breast cancer treatment guidelines independent of comorbidity score, clinical stage, and tumor characteristics (Giordano *et al*., 2005).

While a number of authors have found that lymph node evaluation significantly influences subsequent treatment decisions in the elderly population (e.g., McMahon *et al*., 2005), others point out that lymph node staging is not without morbidity. General anaesthesia is frequently required, and there is a risk of lymphedema, which may be significant for the elderly population. One study found that older patients had an increased risk of lymphedema after axillary surgery than younger patients (16% vs. 13%, $p = 0.02$) (Djordjevic *et al*., 2004). In addition, one study demonstrated that patients 67 years or older who underwent lymph node evaluation had three times the rate of arm complications 2 years post-treatment than those who did not have any axillary surgery (Mandelblatt *et al*., 2002). Furthermore, these arm sequelae resulted in lower physical and mental functioning.

With the advent of sentinel node biopsy, however, the risk of lymphedema and

other complications are significantly reduced while still preserving the ability to accurately stage the axilla (Veronesi *et al*., 2003). In addition, some authors have suggested that this technique can also be performed under local anaesthesia (van Berlo *et al*., 2003).

Sentinel Node Biopsy Technique

Sentinel node biopsy has become widely accepted as a minimally invasive method to stage the axilla in patients with breast cancer. This technique, which uses the injection of a radioactive colloid and/or a blue dye into the breast, allows for the tracking of the tracer to the first, or "sentinel", draining lymph nodes in the axilla (Figure 42.1). A number of studies have validated this technique, demonstrating that it is a safe, reliable and accurate means of staging the axilla (Veronesi *et al*., 2003).

Several authors have specifically evaluated the technique of sentinel node biopsy in the elderly. In the Mayo Clinic experience, for example, the overall sentinel node identification rate was not significantly different between patients < 70 years of age and those who were ≥ 70 (98.8% vs. 97.1%, $p = 0.11$) (McMahon *et al*., 2005). Furthermore, in their experience, knowledge of nodal status significantly impacted adjuvant therapy decisions. For example, patients ≥ 70 years of age were more likely to receive systemic cytotoxic chemotherapy if they were sentinel node-positive versus those who were sentinel node-negative (24.0% vs. 2.8%, $p < 0.01$). In addition, sentinel node status significantly correlated with the finding of systemic recurrence, with 8.2% of sentinel node positive patients progressing to distant metastatic disease, versus none of the sentinel node negative patients relapsing ($p < 0.01$).

The Milan group similarly found that sentinel node biopsy could be performed in patients ≥ 70 years of age with a 100% sentinel node identification rate (Gennari *et al*., 2004). Of the 241 patients in their study, 90 (37.3%) were found to have sentinel node metastasis, each of these patients had an axillary node dissection in the same surgical setting. No major surgical complications were noted in their cohort. Therefore, it appears that sentinel node biopsy is safe and reliable in the elderly population.

With the minimal morbidity of sentinel node biopsy, it would follow that most elderly patients may have lymph node evaluation in this manner. However, for those with exorbitant comorbidities, there may be a subpopulation in whom lymph node evaluation may not be warranted. Several small non-randomized studies found low rates of locoregional failure and no adverse effect on survival in elderly patients with small hormone-responsive

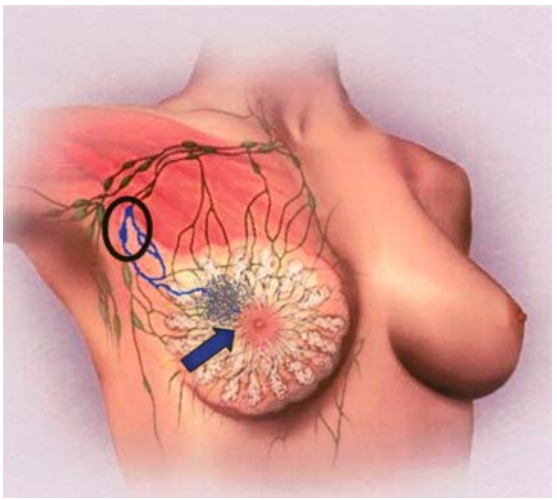

FIGURE 42.1. Arrow indicates injection site. SLNs (shown in blue) are circled. Non-SLNs are shown in green. Grey cloud indicates tumor.

tumors and clinically negative axillae in whom lymph node evaluation was omitted (Feigelson *et al*., 1996; Martelli *et al*., 2003). A randomized controlled trial comparing axillary dissection to no axillary dissection in patients 65–80 years of age with clinical T1N0 breast cancer found that 2/110 (1.8%) patients who did not undergo axillary dissection went on to develop clinically apparent axillary disease (Martelli *et al*., 2005). With a median follow-up of 60 months, the two groups were similar in terms of overall mortality, breast cancer mortality, and breast cancer events (including ipsilateral breast cancer recurrence, contralateral breast cancer and distant metastasis). While these results suggest that some elderly patients with low risk cancers may be spared lymph node evaluation, doing so could result in suboptimal local control and staging for some patients who may have benefited from axillary node dissection and systemic therapy.

In order to address the issue of predicting which elderly patients will have lymph node metastasis, a clinical prediction rule was recently presented (Chagpar *et al*., *American Society of Breast Surgeons*, 2007). This statistical model, based on a multivariate analysis, predicts the likelihood of lymph node metastasis in patients ≥ 70 years of age with hormonally sensitive tumors. The model, based on patient age, tumor size, and lymphovascular invasion, was found to predict a group of patients with a 5% likelihood of having lymph node metastasis. While this model was validated in an independent test set, and may be useful in predicting the likelihood of lymph node metastasis in elderly patients with significant comorbidities, it should not be used in lieu of sentinel node biopsy in patients at good operative risk.

In conclusion, the elderly represent a distinct subpopulation of breast cancer patients, who may have a unique tumor biology, and certainly present with a multitude of comorbidities which must be taken into account when planning management of their malignancy. However, while this population may have its own particular challenges, it has been well-established that the elderly are frequently undertreated, and this may have implications in terms of their overall survival and progression to distant metastatic disease.

One of the most significant predictors of early systemic metastasis in elderly patients is the number of positive lymph nodes. However, lymph node evaluation is frequently omitted in these patients. With the advent of sentinel node biopsy, the axilla may be accurately staged using a minimally invasive procedure with minimal if any morbidity. Therefore, the elderly should not be denied lymph node evaluation on the basis of chronological age alone. For those with significant comorbidities in whom sentinel node biopsy is deemed inappropriate, newer clinical prediction rules may provide some insight into the probability of lymph node metastasis in these elderly patients.

REFERENCES

American Cancer Society. 2006. Breast Cancer Facts & Figures 2005-2006. American Cancer Society, Atlanta.

Chagpar, A.B., McMasters, K.M., Martin, R.C., Thoene, C., Nurko, J.Y., and Edwards, M.J. 2006. Determinants of early distant metastatic disease in elderly patients with breast cancer. *Am. J. Surg. 192*: 317–321.

Crowe, J.P., Jr., Gordon, N.H., Shenk, R.R., Zollinger, R.M., Jr., Brumberg, D.J., and Shuck, J.M. 1994. Age does not predict breast cancer outcome. *Arch. Surg. 129*: 483–487.

Cummings, F.J., Gray, R., Tormey, D.C., Davis, T.E., Volk, H., Harris, J., Falkson, G., and Bennett, J.M. 1993. Adjuvant tamoxifen versus placebo in elderly women with node-positive breast cancer: long-term follow-up and causes of death. *J. Clin. Oncol. 11*: 29–35.

Davis, S.J., Karrer, F.W., Moor, B.J., Rose, S.G., and Eakins, G. 1985. Characteristics of breast cancer in women over 80 years of age. *Am. J. Surg. 150*: 655–658.

Djordjevic, N., Karanikolic, A., and Pesic, M. 2004. Breast cancer in elderly women. *Arch. Gerontol. Geriatr. 39*: 291–299.

Edge, S.B., Gold, K., Berg, C.D., Meropol, N.J., Tsangaris, T.N., Gray, L., Petersen, B.M., Jr., Hwang, Y.T., and Mandelblatt, J.S. 2002. Patient and provider characteristics that affect the use of axillary dissection in older women with stage I-II breast carcinoma. *Cancer 94*: 2534–2541.

Feigelson, B.J., Acosta, J.A., Feigelson, H.S., Findley, A., and Saunders, E.L. 1996. T1 breast carcinoma in women 70 years of age and older may not require axillary lymph node dissection. *Am. J. Surg. 172*: 487–489.

Fisher, B., Jeong, J.H., Anderson, S., Bryant, J., Fisher, E.R., and Wolmark, N. 2002. Twenty-five-year follow-up of a randomized trial comparing radical mastectomy, total mastectomy, and total mastectomy followed by irradiation. *N. Engl. J. Med. 347*: 567–575.

Gennari, R., Curigliano, G., Rotmensz, N., Robertson, C., Colleoni, M., Zurrida, S., Nole, F., de Braud, F., Orlando, L., Leonardi, M.C., Galimberti, V., Intra, M., Veronesi, P., Renne, G., Cinieri, S., Audisio, R.A., Luini, A., Orecchia, R., Viale, G., and Goldhirsch, A. 2004. Breast carcinoma in elderly women: features of disease presentation, choice of local and systemic treatments compared with younger postmenopasual patients. *Cancer 101*: 1302–1310.

Giordano, S.H., Hortobagyi, G.N., Kau, S.W., Theriault, R.L., and Bondy, M.L. 2005. Breast cancer treatment guidelines in older women. *J. Clin. Oncol. 23*: 783–791.

Goodwin, J.S., Hunt, W.C., Humble, C.G., Key, C.R., and Samet, J.M. 1988. Cancer treatment protocols. Who gets chosen? *Arch. Intern. Med. 148*: 2258–2260.

Greenfield, S., Blanco, D.M., Elashoff, R.M., and Ganz, P.A. 1987. Patterns of care related to age of breast cancer patients. *J. Am. Med. Assoc. 257*: 2766–2770.

Herbsman, H., Feldman, J., Seldera, J., Gardner, B., and Alfonso, A.E. 1981. Survival following breast cancer surgery in the elderly. *Cancer 47*: 2358–2363.

Hunt, K.E., Fry, D.E., and Bland, K.I. 1980. Breast carcinoma in the elderly patient: an assessment of operative risk, morbidity and mortality. *Am. J. Surg. 140*: 339–342.

Law, T.M., Hesketh, P.J., Porter, K.A., Lawn-Tsao, L., McAnaw, R., and Lopez, M.J. 1996. Breast cancer in elderly women: presentation, survival, and treatment options. *Surg. Clin. North Am. 76*: 289–308.

Litvak, D.A., and Arora, R. 2006. Treatment of elderly breast cancer patients in a community hospital setting. *Arch. Surg. 141*: 985–990.

Mandelblatt, J.S., Edge, S.B., Meropol, N.J., Senie, R., Tsangaris, T., Grey, L., Peterson, B., Hwang, Y.T., and Weeks, J.C. 2002. Sequelae of axillary lymph node dissection in older women with stage 1 and 2 breast carcinoma. *Cancer 95*: 2445–2454.

Martelli, G., Miceli, R., De Palo, G., Coradini, D., Salvadori, B., Zucali, R., Galante, E., and Marubini, E. 2003. Is axillary lymph node dissection necessary in elderly patients with breast carcinoma who have a clinically uninvolved axilla? *Cancer 97*: 1156–1163.

Martelli, G., Boracchi, P., De Palo, M., Pilotti, S., Oriana, S., Zucali, R., Daidone, M.G., and De Palo, G. 2005. A randomized trial comparing axillary dissection to no axillary dissection in older patients with T1N0 breast cancer: results after 5 years of follow-up. *Ann. Surg. 242*: 1–6.

McMahon, L.E., Gray, R.J., and Pockaj, B.A. 2005. Is breast cancer sentinel lymph node mapping valuable for patients in their seventies and beyond? *Am. J. Surg. 190*: 366–370.

Morishita, Y., Tsuda, H., Fukutomi, T., Mukai, K., Shimosato, Y., and Hirohashi, S. 1997. Clinicopathological characteristics of primary breast cancer in older geriatric women: a study of 39 Japanese patients over 80 years old. *Jpn. J. Cancer Res. 88*: 693–699.

Mueller, C.B., Ames, F., and Anderson, G.D. 1978. Breast cancer in 3,558 women: age as a significant determinant in the rate of dying and causes of death. *Surgery 83*: 123–132.

Nagadowska, M., and Kulakowski, A. 1991. Breast cancer in elderly women: characteristics of the disease. *Eur. J. Surg. Oncol. 17*: 609–614.

Newlin, M.E., Reiling, R., and Nichols, K. 2002. Necessity of axillary dissection in elderly women with early breast cancer. *World J. Surg. 26*: 1239–1242.

Swanson, R.S., Sawicka, J., and Wood, W.C. 1991. Treatment of carcinoma of the breast in the older geriatric patient. *Surg. Gynecol. Obstet. 173*: 465–469.

Taghian, A., Jeong, J.H., Mamounas, E., Anderson, S., Bryant, J., Deutsch, M., and Wolmark, N. 2004. Patterns of locoregional failure in patients with operable breast cancer treated by mastectomy and adjuvant chemotherapy with or without tamoxifen and without radiotherapy: results from five National Surgical Adjuvant Breast and Bowel Project randomized clinical trials. *J. Clin. Oncol. 22*: 4247–4254.

Trimble, E.L., Carter, C.L., Cain, D., Freidlin, B., Ungerleider, R.S., and Friedman, M.A. 1994. Representation of older patients in cancer treatment trials. *Cancer 74*: 2208–2214.

van Berlo, C.L., Hess, D.A., Nijhuis, P.A., Leys, E., Gerritsen, H.A., and Schapers, R.F. 2003. Ambulatory sentinel node biopsy under local anaesthesia for patients with early breast cancer. *Eur. J. Surg. Oncol. 29*: 383–385.

Veronesi, U., Cascinelli, N., Mariani, L., Greco, M., Saccozzi, R., Luini, A., Aguilar, M., and Marubini, E. 2002. Twenty-year follow-up of a randomized study comparing breast-conserving surgery with radical mastectomy for early breast cancer. *N. Engl. J. Med. 347*: 1227–1232.

Veronesi, U., Paganelli, G., Viale, G., Luini, A., Zurrida, S., Galimberti, V., Intra, M., Veronesi, P., Robertson, C., Maisonneuve, P., Renne, G., De Cicco, C., De Lucia, F., and Gennari, R. 2003. A randomized comparison of sentinel-node biopsy with routine axillary dissection in breast cancer. *N. Engl. J. Med. 349*: 546–553.

43
Concomitant Use of Tamoxifen with Radiotherapy Enhances Subcutaneous Breast Fibrosis in Hypersensitive Patients

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BACKGROUND

The efficacy of radiotherapy (RT) in the treatment of malignant disease is limited by the need to avoid severe and nonreversible late damage to normal tissues. Nevertheless, the positive impact of RT in several tumors such as breast cancer makes its use inevitable. Indeed, postoperative RT decreases the risk of locoregional recurrence and is associated with improved survival in high-risk premenopausal and postmenopausal breast cancer patients given adjuvant chemotherapy or tamoxifen (TAM), respectively (Nielsen *et al*., 2006).

The use of adjuvant TAM in postmenopausal breast carcinoma patients as an adjunct to primary surgery is well established. The benefits from this treatment have been shown in lymph node-negative as well as lymph node-positive patients, both in terms of a prolonged recurrence-free survival and an increase in overall survival, especially in women presenting with estrogen receptor-positive tumors (EBCTC Group, 2005).

The interaction of TAM and RT remains poorly defined. TAM appears to exert its cytostatic activity at least partly through competitive inhibition at the estrogen receptor, resulting in segregation of cells into G_0/G_1 phase of the cell cycle (Osborne *et al*., 1983). Because relatively less radiosensitivity has been observed in early G_1, a hypothetical concern is raised with TAM whether its combination with radiotherapy results in the radioprotection of tumor clonogens of hormonally responsive and unresponsive breast carcinoma cells at dose levels typical of those used clinically (Wazer *et al*., 1989). However, in this study, the cell cultures were grown in a medium containing phenol red and fetal bovine serum, two sources of exogenous estrogenic compounds. This fact complicates the interpretation of the resultant radiation survival curves. In contrast to these reports, no significant differences were observed in terms of radiosensitivity for estradiol-stimulated or 4-hydroxytamoxifen-inhibited cultures plated under growth-stimulating conditions immediately after irradiation

or following an additional 24 h under estrogen-free conditions (Sarkaria *et al*., 1994). Clearly, under defined hormonal conditions, no protective effect of the active TAM metabolite, 4-hydroxytamoxifen, was observed. In addition, irradiation and TAM may modify the estrogen and progesterone receptor content in the cytosol in breast cancer cells and that could explain their change in radiation sensitivity (Paulsen *et al*., 1996). Results from a study in tumor-bearing rats (Kantorowitz *et al*., 1993) receiving fractionated RT, TAM, or both showed that, in contrast to *in vitro* results, the combination treatment reduced the probability of subsequent tumor development.

In so far as the preclinical data with breast cancer cells can be extrapolated to the clinical situation, no alteration in responsiveness would be expected following TAM exposure. Although no randomized trials have been designed to address specifically the effect of concurrent TAM on the response to conventional RT, results from clinical trials, which included treatment arms with and without TAM, suggest that no deleterious consequences accompanied TAM treatment (Whelan and Levine, 2005). However, TAM has been reported to lead to worse cosmesis in women who underwent conservative surgery, RT, and had received TAM (Wazer *et al*., 1992) but not in others (Fowble *et al*., 1996; Taylor *et al*., 1995).

Our goal in this study was to evaluate the relationship between the concomitant administration of TAM and adjuvant RT and the risk of developing subcutaneous fibrosis after conservative or radical surgery in breast cancer patients. The analysis was based on patients participating in a large prospective study of 399 patients where we evaluated the correlation between the level of radiation-induced apoptosis of CD4 and CD8 lymphocytes and late side effects (Azria *et al*., 2004a; Ozsahin *et al*., 2005).

CONCURRENT TAMOXIFEN AND RADIOTHERAPY

Tamoxifen is a competitive anti-estrogen for mammary estrogen cell receptors, which prevents the stimulating mitogenic effect of circulating estrogens. It is now no longer controversial that TAM decreases local recurrence and increases global survival rates (EBCTC Group, 2005). Combined treatment of TAM and RT as an adjuvant treatment has been debated for many years but little data have been published.

Preclinical Data

Few data have been published concerning interaction between TAM and RT (Azria *et al*., 2004b). Part of the cytostatic activity of TAM seems to be the competitive inhibition of estrogen receptors with accumulation of the treated cells in the G0/G1 phase (Osborne *et al*., 1983). The early G1-phase of the cell cycle is recognized as an intermediate radiosensitive phase. Therefore, Wazer *et al*. (1989) suggested the potential radioprotector role of TAM. Nevertheless, the presence of fetal calf serum and phenol red in the culture medium made the analyses difficult, because these agents act as exogenous estrogen inductors and may distort the biological effects of TAM. Conversely, Sarkaria *et al*. (1994) did not observe any significant difference in terms of cell survival between RT alone and combined treatment with the active metabolite of TAM: 4-hydroxytamoxifen.

Furthermore, under optimal conditions for cell culture, there were no observations made of any protective effects of 4-hydroxytamoxifen. In addition, Paulsen *et al*. (1996) reported that RT and TAM may modify the estrogen and progesterone receptors content in cytosol in breast cancer cells. Hormonotherapy may alter the radiation sensitivity, even in estrogen receptor-negative cells, suggesting that hormonal agents may act both via receptor and nonreceptor binding mechanisms. Furthermore, Schmidt-Ullrich *et al*. (1994) demonstrated that after fractioned RT (2–50 Gy), the level of mRNA of estrogen receptors was diminished by two-thirds in the (MCF-7) breast cancer cell line. This 'mechanism' would be the reason behind the limited inhibiting effect of TAM on cell proliferation. These results are, however, too few in the literature and would warrant further studies to be validated. Kantorowitz *et al*. (1993) have demonstrated *in vivo* the ability of combined RT (45 Gy in 25 fractions) + TAM (500 mg/kg by s.c., injections the days of irradiation) to slow 1-methyl-1-nitrosourea- induced mammary tumor progression in rats. Furthermore, repopulation of (MCF-7) estrogen-dependant mammary cancer cells is considerably diminished between two fractions of RT under conditions of estrogen restriction. This strengthens the idea that anti-estrogens, which inhibit tumor repopulation between RT fractions, could induce a relative radiosensitivity.

Clinical Results

Efficacy of Combined TAM-RT Treatment

Several trials have reported the advantage in terms of efficacy of the association of RT with TAM in adjuvant hormonal receptors positive (HR+) breast cancers. In the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14 trial 2,644 patients who suffered from node negative HR+ nonmetastatic breast cancer were randomized between TAM and placebo during a 5-year period. Among them, 1,062 patients received a conservative surgery followed by locoregional RT. All patients started TAM before receiving RT and were thus treated with concurrent TAM and RT. After a 10-year follow up, 10.3% of local recurrences were observed in the placebo arm vs 3.4% in the TAM arm (Fisher *et al*., 1996).

In the NSABP (B-21) trial 1,000 patients with early breast cancer < 1 cm were randomized after conservative surgery between three arms: TAM alone (arm A) vs RT + placebo (arm B) vs RT + TAM (arm C). For majority of patients, RT and TAM were administered concurrently. After 8 years of follow up, local recurrence rates were 16.5%, 9.3%, and 2.8% in the A, B, and C arm, respectively (Fisher *et al*., 2002).

These two randomized studies demonstrated the superiority of combined RT and TAM on local control over RT alone as adjuvant treatment for localized breast cancer after conservative surgery, and do not support the potential radioprotective role of TAM suspected in the *in vitro* findings.

Recently, in view of the continued uncertainty of the optimal scheduling of TAM and RT in women with early breast cancer, the results of three studies were published (Ahn *et al*., 2005; Harris *et al*., 2005; Pierce *et al*., 2005). All three studies used a retrospective cohort design to compare the effects of concurrent TAM and RT with the effects of RT followed sequentially by TAM. Nevertheless, several comments are needed concerning this unresolved question (Azria *et al*.,

2005b; Whelan and Levine, 2005) as the results of a well-conducted randomized trial are not available. None of these studies showed any significant difference in the risk of ipsilateral breast tumor recurrence, disease-free survival, or overall survival in patients receiving concomitant versus sequential TAM and RT. Briefly, Ahn *et al*. (2005) identified 495 patients treated with concurrent TAM and RT (n = 254) or RT followed sequentially by TAM (n = 241). This study observed no significant difference in any analyzed endpoints but showed imbalances in the length of follow-up and in the treatment groups for important prognostic factors such as age and number of patients who received chemotherapy. Although this was the largest of the three studies, the sample size was not sufficient to draw definitive conclusions. Harris *et al*. (2005) compared 174 patients treated with concurrent TAM and RT, with 104 patients treated with RT followed sequentially by TAM. No significant differences in all analyzed endpoints were observed. However, important differences between the treatment groups were noted particularly the age of patients and the number of those who received chemotherapy. Pierce *et al*. (2005) identified two cohorts of patients from a randomized trial of different adjuvant systemic therapy regimens. Patients were randomly assigned to cyclophosphamide, doxorubicin, and fluorouracil (CAF), CAF followed by TAM; cyclophosphamide, methotrexate, and fluorouracil (CMF); or CMF followed by TAM. Patients treated with breast-conserving surgery were treated with RT either before adjuvant chemotherapy or after chemotherapy was completed. Of the 1,345 patients randomly assigned to a chemotherapy regimen followed by TAM, only 23% were treated with breast-conserving therapy or RT. Of these, 202 patients received concurrent TAM and RT and 107 received RT followed sequentially by TAM. Again, no differences were noted in all analyzed endpoints between RT followed sequentially by TAM and concurrent TAM and RT. However, important imbalances were observed between the groups which render difficult definitive conclusions (Pierce *et al*., 2005). It is thus reassuring that TAM given concomitantly with RT does not exert a radioprotective effect in a clinical setting. Therefore, preclinical data obtained with breast cancer cells have to be extrapolated cautiously to the clinical situation.

Toxicities of Combined TAM-RT Treatment

The most reported toxicities after concurrent TAM/RT treatment are subcutaneous and lung fibroses. However, data remains very contradictory. Subcutaneous fibrosis after concurrent TAM/RT treatment following surgery is the most described in the literature. Three studies (Fowble *et al*., 1996; Taylor *et al*., 1995; Wazer *et al*., 1992) have not detected any significant increase in subcutaneous fibrosis following concurrent TAM/RT treatment. Criteria for evaluating fibrosis and cosmetic results for the breast are complex and not well standardized. Among the three trials, only Wazer *et al*. (1992) demonstrated poorer breast cosmetic results, but the difference was not statistically significant (p = 0.062), probably due to the low number of included patients (53). Fowble *et al*. (1996) did not report esthetic alteration in patients treated with concurrent TAM/RT after conservative surgery. However, among the 154 patients only 23

received concurrent TAM/RT treatment (28). Finally, Taylor *et al.* (1995) did not find any alteration in cosmetic outcome of the operated breast in patients receiving TAM/RT treatment. However, in this study, the description of the treatment sequence was ambiguous (52).

In our study (Azria *et al.*, 2004a), 90 out of 147 patients treated with adjuvant RT after breast-conserving surgery, received a daily dose of TAM (20 mg) before starting RT. Fibrosis-free survival was 51% in the RT/TAM group vs 80% ($p = 0.029$) in the RT alone group. Furthermore, this difference was more pronounced for those at risk of developing late side-effects. More details are described in the next part of this review.

Few papers reported an increased risk of lung fibrosis with concurrent TAM/RT treatment. Bentzen *et al.* (1996) showed an increased in-field lung fibrosis after total mastectomy. However, the analysis only concerned 38 patients evaluated by conventional radiography. More recently, Koc *et al.* (2002) confirmed a higher risk of lung fibrosis after concurrent TAM/RT treatment in 74 patients treated with $Cobalt^{60}$ RT after mastectomy. In a biological point of view, TAM was reported to stimulate *in vitro* the secretion of transforming growth factor β (TGF-β) by the fibroblasts. There are also arguments in favor of the participation of TGF-β in the postradiation changes of healthy tissue (Canney and Dean, 1990). However, because fibrosis in the breast take years to stabilize, populations at high risk of late postradiation lesions will see their lesions worsen even if TAM is prescribed after RT. The secretion of TGF-β induced by TAM and RT seems to have an additive effect (Martin *et al.*, 1993).

TAMOXIFEN AND RADIOTHERAPY IN HYPERSENSITIVE PATIENTS

Our goal in this study was to evaluate the relationship between the concomitant administration of TAM and adjuvant RT and the risk of developing subcutaneous fibrosis after conservative or radical surgery in breast cancer patients. The analysis was based on patients participating in a large prospective study of 399 patients in which we evaluated the correlation between the level of radiation-induced apoptosis of CD4 and CD8 lymphocytes and late side effects (Azria *et al.*, 2004a; Ozsahin *et al.*, 2005).

Methodology

All patients included in the KFS 00539-9-1997/SKL 00778-2-1999 prospective study (Ozsahin *et al.*, 2005) were used in evaluating the predictive value of CD8 T-lymphocyte apoptosis on the development of radiation-induced late side effects, notably fibrosis. Among the 399 patients, 147 women presented with breast cancer. Our objective was to assess, in this population, whether the risk of developing subcutaneous fibrosis after conservative or radical surgery and adjuvant RT was increased by the concomitant administration of TAM.

Radiation-induced Apoptosis

Heparinized whole blood (7 ml) was obtained from consenting patients participating in the study, diluted 1:10 in RPMI 1640 medium (Life Technologies, Basel, Switzerland) containing 20% fetal bovine serum (Readysysteme, Zürich, Switzerland), and was divided into two 3.5-ml aliquots and placed in 25-cm^2 (60 ml)

flasks. These aliquots were irradiated at room temperature with 0- and 8-Gy using an Oris IBL 137 cesium source (CIS-Bio International, Gif-sur-Yvette, France) at a dose rate of 2.67 cGy s^{-1}. Following irradiation, the preparations were incubated at 37°C in 5% CO_2. After 48 h, the contents of each flask were distributed into four 5-ml test tubes and then centrifuged at 1,300 rpm for 5 min at room temperature. Most of the supernatant was aspirated and the pellet resuspended in ≈200 µl of the remaining solution. Ten microliters of FITC-conjugated anti-CD8 monoclonal antibody were added (Becton-Dickinson, Basel, Switzerland). Following incubation for 20 min at room temperature, 4 ml of 1:10 diluted lysis solution (Becton Dickinson, Basel, Switzerland) was added to the suspension, and the specimens were left for 10 min at room temperature in the dark to promote lysis of erythrocytes. The cells were then centrifuged at 1,450 rpm for 5 min, the supernatant was aspirated, and the cells were washed once with 4 ml phosphate-buffered saline (PBS; Becton Dickinson, Basel, Switzerland). After another round of centrifugation (1,450 rpm for 5 min), the supernatant was aspirated. The cells were resuspended in 200 µl of FACSFlow (Becton Dickinson, Basel, Switzerland) phosphate buffer to which 5 µl of propidium iodide (PI) stock (1 mg/ml in PBS) was added to stain the DNA. Then, 50 µl of RNase stock solution (1 mg/ml) was added, and the samples were incubated at room temperature for 5 min before flow cytometric measurement. Samples were measured using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA) with a 488 nm, 15 mW argon-ion laser (Coherent, Santa Clara, CA). Data analysis was performed via a two-step procedure using the CellQuest software (Becton-Dickinson, Basel, Switzerland) on a Macintosh computer. Data from each lymphocyte sample were acquired immediately after the staining procedure. Four-parameter acquisition permitted discrimination of the different subpopulations of lymphocytes. Forward and side light scattering and stain-induced fluorescence at two different wavelengths (530 nm green, and 640 nm red) were simultaneously measured from each cell. Using forward scatter (FSC) vs. side scatter (SSC) dot plots, three subpopulations of leukocytes (mono-, granulo-, and lymphocytes) as well as the cell debris could be distinguished, and the lymphocytes were selected. After staining the cells with FITC-conjugated antibodies (green fluorescence) to identify lymphocyte cell-type and PI (red fluorescence) to quantify cellular DNA content, the CD8-positive cells were identified by simultaneous measurement of the two laser-induced fluorescent signals. Apoptotic lymphocytes were defined as those cells staining positively for their cell-type-specific antibodies, and displaying reduced DNA content and cell size. These lymphocytes were previously examined for apoptotic cells by the TUNEL assay. Data for at least 10,000 cells/sample were acquired.

Treatment Modalities

All patients had staging investigations including chest X-ray, bone scan, and liver enzymes to rule out metastatic disease at diagnosis. Initial values of age, TNM 2002 staging according to the American Joint Committee on Cancer staging system for breast cancer, histopathology, type of surgery, margins, and menopausal status were noted. Surgical treatment consisted of breast-conserving surgery (any type) or

mastectomy and axillary dissection in 118 (80.3%) and 29 patients, respectively.

In those patients having breast-conserving surgery, irradiation was delivered to the breast and, when indicated, to the regional lymphatics. Treatment portals consisted of opposing tangential fields using cobalt or 6–18 MV photons. Both tangential fields were treated daily. A physical lead block or asymmetrical collimation was used for half-beam blocking. The breast dose, routinely prescribed to the midline, was 50 Gy at 2 Gy fractions, with a varying percentage of compensating filters and/or bolus. All patients received a 16-Gy boost (20 Gy in the case of suspicious surgical margins) to the primary tumor bed using 6–15 MeV electrons.

The 29 mastectomy patients received chest-wall irradiation using opposing tangential fields with cobalt or 6–18 MV photons using half-beam blocking. Both tangential fields were treated daily. The chest wall dose was 50 Gy at 2 Gy fractions using compensating filters and/or bolus (one third to half of the treatment). A 4–6 MeV electron boost of 10–16 Gy was given to the surgical scar in high-risk patients. Nodal irradiation, when indicated (mostly supraclavicular and internal mammary nodes), was given at a total dose of 50 Gy in 25 fractions. Supraclavicular lymph nodes were treated with a split anterior oblique (10–15°) beam at a dose of 50 Gy calculated at the depth of 3 cm. Internal mammary nodal irradiation in some patients was administered using a separate anterior field, namely, 25 Gy at a 4-cm depth with 6 MV photons, followed by 25 Gy at the 100% isodose line using tailored electrons (mostly 12 MeV) according to the position of the internal mammary lymph nodes as assessed on the CT-scan. When indicated, axillary lymph nodes were included in the anterior supraclavicular field, and the missing axillary midline dose was completed using a posterior axillary field.

Post- or perimenopausal women who were shown to have estrogen receptor and/or progesterone receptor-positive tumors (ER and/or PgR = 10 fmol/mg cytosol protein; or = 10% of the tumor cells positive by an immunocytochemical assay) were prescribed 20 mg of tamoxifen (TAM) daily. In all cases, TAM therapy was initiated the month before or the day of the start of RT. Premenopausal women (n = 11) with receptor positive tumors were given 20 mg of tamoxifen daily for 5 years and an LHRH analog monthly for at least 2 years. None of the patients who received TAM received adjuvant chemotherapy.

Women who were shown to have estrogen receptor- and progesterone receptor- negative tumors with axillary lymph node metastasis received six cycles of chemotherapy with cyclophosphamide, fluorouracil, and methotrexate, or with cyclophosphamide and adriamycin. No treatment with concomitant chemotherapy and irradiation was given. Typically, the patients began RT 3 weeks after the completion of chemotherapy.

Radiation-induced Assessment of Side Effects

During treatment, acute toxicity was evaluated according to WHO and CTC-NCI v2.0 criteria. All patients were visited every 6 months for 2 years. During the follow-up visits, late side effects were graded according to the RTOG/EORTC scale. The time at which the maximal grade of late side effects was observed, i.e., before 2 years had elapsed, was retained for analysis (RTOG-1). Patients were revaluated for

late side effects at 2 years (RTOG-2) by a second physician (D.A.). The assesment of toxicity was blinded to treatment.

Statistical Analysis

Data were summarized by frequencies and percentages for categorical variables and by means, standard deviations, median, and range for continuous variables. Three categories of absolute change in the percent CD8 cells in apoptosis before and after exposure to 8 Gy of irradiation were constructed around the median value. The Kruskal-Wallis test was used to compare the continuous variables, and the chi-square test was used to compare the categorical variables between the two groups of patients with or without TAM.

All survival estimations were computed from the date of start of radiotherapy. Overall survival, relapse-free survival (RFS), complication-free survival (CFS), and complication-relapse-free survival (CRFS) curves were estimated by the Kaplan-Meier method using the following first event definitions, death for overall survival, local or distant recurrence or death for RFS, grade 2 or 3 fibrosis for CFS, and any event for CRFS. The median follow-up was also estimated by the Kaplan-Meier method.

For overall survival, patients alive at the last follow-up visit were censored. For RFS, patients alive and relapse-free were censored at the last follow-up visit. For CFS, patients alive who never experienced a grade 2 or more fibrosis were censored at the last follow-up visit. Patients who relapsed before a grade 2 or greater fibrosis were censored at the time of relapse. For CRFS, patients alive and relapse-free who never experienced a grade 2 or greater fibrosis were censored at the last follow-up visit. The log-rank test was used to identify significant categorical variables for each of the survival curves. A step-wise Cox proportional hazards regression model was used for multivariate analysis. A *P*-value less than 0.05 was considered statistically significant. The data were expressed as means ±95% confidence intervals. All statistical tests were two-sided.

Competing risk methodology was used to estimate the cumulative incidence of each first failure type, grade 2 or greater fibrosis, and relapse. These estimates may be different from those obtained from the inverse Kaplan-Meier survival function estimates since the event-time distributions of each failure type was taken into account rather than censored independently of the other event.

RESULTS

Patient Characteristics

Patient characteristics of the 147 patients are presented in Table 43.1. There were significantly more patients 60 years or older in the group that received TAM than in the group that did not receive TAM (49% vs. 30%). Patients who received TAM were significantly more likely to have had pathologically positive axillary lymph nodes (47.1% vs. 28.6%), larger tumor size (41% vs. 32% ≥ T2), and more invasive lobular carcinoma histopathologic subtypes (20% vs. 12%). No difference was identified regarding margin measurements. Most of the patients were post-menopausal (87%) with no difference between the two groups. The extent of surgery applied to both groups was similar with 80% of patients having had breast-conserving surgery.

TABLE 43.1. Patient characteristics.

	No tamoxifen N = 57 (%)	Tamoxifen N = 90 (%)	All patients N = 147 (%)	*P* value
Age, year				
Mean (SD)	55.5 (1.61)	58.7 (1.21)	57.4 (11.78)	
Median (range)	55.0 (26–80)	59.0 (35–82)	57.0 (26–82)	
< 60	40 (70.2)	46 (51.1)	86 (58.5)	
≥ 60	17 (29.8)	44 (48.9)	61 (41.5)	0.022
Histopathology				
IDC	36 (63.2)	67 (74.5)	103 (70.1)	
ILC	7 (12.3)	18 (20.0)	25 (17.0)	
DCIS	10 (17.5)	3 (3.3)	13 (8.8)	
Others	4 (7.0)	2 (2.2)	6 (4.1)	0.008
TNM[c]				
T in situ	10 (17.9)	2 (2.3)	12 (8.4)	
T1	28 (50.0)	49 (56.3)	77 (53.8)	
T2	10 (17.9)	27 (31.0)	37 (25.9)	
T3	4 (7.1)	4 (4.6)	8 (5.6)	
T4	4 (7.1)	5 (5.8)	9 (6.3)	0.005[a]
N N0	40 (71.4)	46 (52.9)	86 (60.1)	
N1	12 (21.4)	39 (44.8)	51 (35.7)	
N2	2 (3.6)	–	2 (1.4)	
N3	2 (3.6)	2 (2.3)	4 (2.8)	0.027[b]
M M0	56 (100.0)	87 (100.0)	143 (100.0)	
M1	–	–	–	–
Type of surgery				
Mastectomy	12 (21.1)	17 (18.9)	29 (19.7)	
Conservative	45 (78.9)	73 (81.1)	118 (80.3)	0.748
Tumorectomy	31 (54.4)	49 (54.4)	80 (54.4)	
Quadrantectomy	14 (24.5)	24 (26.7)	38 (25.9)	0.931
Margins				
Clear	52 (91.2)	86 (95.6)	138 (93.9)	
Positive or close	5 (8.8)	4 (4.4)	9 (6.1)	0.286
Menopausal status				
Pre	81 (4.0)	11 (12.2)	19 (12.9)	
Peri or post	49 (86.0)	79 (87.8)	128 (87.1)	0.750

IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; DCIS, ductal carcinoma in situ

[a]Test on Tis/T1/T2/T3+T4.

[b]Test on N0 vs N1+N2+N3.

[c]Initial TNM was not available for four patients but these patients completed their treatments and continued to be visited at each medical evaluation. None of them were M1 during the follow-up visits.

Treatment Delivery

The radiation therapy characteristics are presented in Table 43.2. All but two patients (99%) received a dose rate of 2 Gy per fraction. The intensity of RT administered was similar for the two groups with no significant difference in the total dose of radiation, type of energy (cobalt or X-rays) delivered, volume of the irradiated breast, or the calculated dose at the surface of the breast. Median treatment duration was 47 days (range 17–70 days).

All patients receiving TAM were hormone receptor positive, and none received adjuvant chemotherapy. Chemotherapy was administered for hormone receptor-negative patients with positive axillary nodes and who were younger than 65 years old (12 patients, 21%). The CD8-radiation-induced apoptosis characteristics are

TABLE 43.2. Treatment delivery and characteristics of CD8 radiation-induced apoptosis.

	No tamoxifen N = 57	Tamoxifen N = 90	All patients N = 147	*P* value
Duration, days				
Mean (SD)	45.4 (0.99)	46.9 (0.70)	46.3 (7.02)	
Median (range)	46.0 (17–57)	48.0 (23–70)	47.0 (17–70)	0.187
Dose (Gy)				
< 35	1 (1.7%)	1 (1.1%)	2 (1.4%)	
50	8 (14.0%)	4 (4.4%)	12 (8.2%)	
60	1 (1.7%)	1 (1.1%)	2 (1.4%)	
66	42 (73.7%)	80 (88.9%)	122 (82.9%)	
≥ 68	5 (8.8%)	4 (4.4%)	9 (6.1%)	
Dose/fraction (Gy)				
1.8				
2	56 (98.2%)	1 (1.1%)	1 (0.7%)	
3	1 (1.8%)	89 (98.9%)	145 (98.6%)	
	–	– 1 (0.7%)		
Energy				
Breast-conserving surgery				
$Cobalt^{60}$	33 (73.3%)	47 (64.4%)	80 (67.8%)	
X6 (MV)	12 (26.7%)	26 (35.6%)	38 (32.2%)	0.312
Mastectomy				
$Cobalt^{60}$	6 (50.0%)	10 (58.8%)	16 (55.2%)	
X6 (MV)	6 (50.0%)	7 (41.2%)	13 (44.8%)	0.638
Volume of the irradiated breast (ml)				
Breast-conserving surgery	N = 45	N = 73	N = 118	
Mean (SD)	1134.4 (323.84)	1323.7 (613.56)	1251.5 (528.81)	
Median (range)	1071.0 (602.3–2018.3)	1224 (480–4032)	1127.0 (480–4032)	0.059
Mastectomy	N = 12	N = 17	N = 29	
Mean (SD)	911.2 (394.52)	1075.9 (455.6)	1007.8 (431.9)	
Median (range)	831.4 (514.3–1930.5)	966.9 (675–2268)	900.0 (514.3–2268)	0.320
Dose of the surface of the breast (Gy)				
Breast-conserving surgery	N = 45	N = 73	N = 118	
Mean (SD)	92.3 (10)	95.1 (6.6)	94.1 (8.1)	
Median (range)	94.1 (50.6–104.7)	95.4 (68.6–105.8)	95.3 (50.6–105.8)	0.067
Mastectomy	N = 12	N = 17	N = 29	
Mean (SD)	95.4 (7.76)	94.6 (7.4)	94.9 (7.44)	
Median (range)	95.1 (83.2–107.2)	95.1 (79.1–107.2)	95.1 (79.1–107.2)	0.762
CD8 (before RT*, %)				
Mean (SD)	9.1 (1)	7.9 (0.73)	8.4 (7.18)	0.134
Median (range)	6.5 (1.3–38.2)	5.6 (0.8–35.3)	6.3 (0.8–38.2)	
CD8 (after 8 Gy, %)				
Mean (SD)	31.9 (1.74)	26.6 (1.1)	28.7 (11.8)	0.024
Median (range)	29.7 (10.2–69.8)	26.2 (5.8–59.6)	28.2 (5.8–69.8)	
CD8 Difference, %				
Mean (SD)	22.9 (1.31)	18.7 (1.03)	20.3 (9.96)	
Median (range)	21.8 (6.2–51.9)	17.6 (3.4–55.7)	20.0 (3.4–55.7)	0.008
CD8 (%) ≤ 16	13 (22.8%)	37 (41.1%)	50 (34%)	
16–24	21 (36.8%)	31 (34.4%)	52 (35.4%)	
> 24	23 (40.4%)	22 (24.4%)	45 (30.6%)	0.041

* Radiotherapy

presented in Table 43.2. The overall mean difference (± standard deviation) before and after radiotherapy was 20.3 (± 9.96) with a statistically significant difference observed between the two groups, 18.7 and 22.9 in the TAM and no TAM groups, respectively. Significantly more patients included in the TAM group had CD8

radio-induced apoptosis ≤ 16% (41%) than patients not receiving TAM (23%).

Acute Toxicity

All patients experienced at least a grade 1 acute WHO side effect with 23.1% grade 3 of breast skin toxicity. No difference between the TAM+ and TAM- groups was observed. According to the CTC-NCI v2.0 classification, only five patients (3.4%) experienced grade 3 radiation dermatitis with no statistical difference between the two groups. No grade 4 toxicity was observed. Finally, neither WHO nor NCI-CTC v2.0 acute toxicities were correlated with CD8 radiation-induced apoptosis.

Relapse-free and Overall Survival

The median follow-up was 29 months (range: 23–79). Ten patients relapsed (6.8%), five of whom died (3.4%). The 3-year survival rate and the relapse-free survival rates were 97% (95% CI 88–99%) and 91% (95% CI 81–95%), respectively.

Late Side Effects

One patient was not evaluated for late side effects before 2 years because of early relapse. Four patients were followed up for less than 2 years and were not clinically examined for late side effects. A total of 135 patients (92.5%) had at least a grade 1 RTOG side effect before the first 2 years of follow-up. Four patients treated with TAM experienced early grade 3 toxicities: three subcutaneous fibrosis and one telangectasia. Among these four patients, all remained grade 3 at 2 years, and none relapsed. Thirty-six patients had grade 2 subcutaneous toxicity before 2 years. Among these patients and at 2 years, 29 remained grade 2, two decreased to grade 1, five increased to grade 3. At 2 years, 129 patients (90.2%) had at least grade 1 toxicity. Fourteen patients experienced 16 grade 3 skin and/or subcutaneous side effects within 2 years, 11 subcutaneous fibrosis and five skin side effects. Among them, two patients had both grade 3 skin and subcutaneous side effects.

Complication-Relapse-free Survival (CRFS)

Complication-Relapse-free Survival rates were similar for all patient characteristics except for treatment with TAM and CD8 radiation-induced apoptosis. There was a statistically significant difference at 3 years in terms of CRFS rates: 48% (95% CI 37.2–57.6%) vs. 66% (95% CI 49.9–78.6%) in the TAM and no-TAM groups, respectively. In each of these groups, CRFS rates were significantly lower for patients with low levels of CD8 radiation-induced apoptosis, 20% (95% CI 10–31.9%), 66% (95% CI 51.1–77.6%), and 79% (95% CI 55–90.9%) for CD8 ≤ 16%, 16–24%, and > 24%, respectively.

Complication-free Survival

Complication-free Survival rates were similar for all patient characteristics except for treatment with TAM and CD8 radiation-induced apoptosis. There was a statistically significant difference in CFS rates at 2 years, 51% (95% CI 40–61%) vs 80% (95% CI 67–89%) in the TAM and no-TAM groups, respectively. In each of these groups, the CFS rates were significantly lower for patients with low levels of CD8 radiation-induced apoptosis: 23% (95% CI 12–36%), 76% (95% CI 61–85%), and 91% (95% CI 78–97%) for CD8 ≤ 16%, 16–24%, and > 24%, respectively (Figure 43.1). A multivariate analysis using the Cox proportional hazards regression model showed a significant increase in the risk of grade 2 or greater

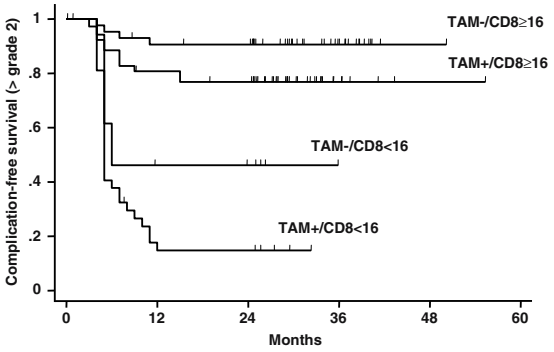

FIGURE 43.1. Complication-free > grade 2 curves according to CD8 radiation-induced apoptosis and concomitant tamoxifen with radiation therapy

fibrosis in the group of patients treated with TAM, with a hazard ratio of 2.1 (95% CI 1.08–4.12, $P = 0.029$), as well as in the group of patients considered as potentially more radiosensitive (CD8 apoptosis ≤ 16). The incidence of grade 2 or greater fibrosis was higher and at the limit of statistical significance in the group of patients with CD8 apoptosis ≤ 16 treated with TAM, 31 out of 37 (84%) compared to no-TAM, 7 out of 13 (54%). No grade 3 side effects were observed for patients with CD8 > 24%.

The 2-year complication-relapse-free survival rate was 54%, indicating that 46% of patients experienced either a grade 2 or 3 RTOG fibrosis, or relapse as a first event before 2 years. For example, the 2-year cumulative incidence rates in the group of patients with CD8 T-lymphocyte apoptosis ≤ 16 and treated with TAM was 83% for grade 2 or more fibrosis and 3% for relapse, which adds up to an overall incidence of 86%, the complement of the CRFS rate. In all patients, the relapse components were similarly distributed between the three categories of CD8, with an estimated cumulative incidence of 4%, 7.7%, and 2.2%, respectively.

DISCUSSION AND PERSPECTIVES

The concept that the inherent radiosensitivity of both normal cells and tumor cells varies from one individual to another is well established. This is clinically relevant because large patient-to-patient variation in radiation morbidity has been documented, even after RT with a fixed dose-fractionation schedule (Bentzen, 2006). The data published so far on the cellular and molecular factors underlying acute or late tissue reactions appeared to be contradictory and suggest that there is no clear-cut relationship between cellular radiosensitivity and the risk of acute or late reactions; consequently, no test has been recommended up to now for predicting the risk or the severity of late reactions in breast cancer (Ho *et al.*, 2006). To confirm our first preclinical and retrospective studies on the correlation of radiation-induced CD4 and CD8 T-lymphocyte apoptosis (RTLA) and late side effects after RT, we assessed prospectively RTLA by the prediction of individual intrinsic radiosensitivity of 399 consenting patients treated with curative RT for miscellaneous cancers (Ozsahin *et al.*, 2005). RTLA significantly predicted grades 2 and 3 late effects ($P < 0.0001$). Considering grade 3 late toxicity, patients with late effects ($n = 25$) showed CD4 or CD8 radiation-induced apoptosis below the median ($P < 0.0001$). The area under the curve of the receiver-operator characteristic curves of CD4 and CD8 apoptosis considered separately or CD4 and CD8 analyzed together were 0.84, 0.89, and 0.92, respectively. To our knowledge, this is the first rapid predictive test based on lymphocyte apoptosis confirmed prospectively in a large number of patients.

We considered CD8 more sensitive and more specific than CD4 T-lymphocyte apoptosis. We therefore analyzed in the present study, the subgroup of 147 women who were treated for breast cancer by RT with (concomitant) or without TAM, and stratified by CD8 radiation-induced apoptosis. Our finding of the influence of TAM on subcutaneous fibrosis, particularly in radiosensitive patients, is not supported by the results of any other published studies and reinforces the idea of the impact of a biological assay in any future clinical trial in radiotherapy.

It is not clear from our study whether the predominant effect of TAM is on the induction of RT injury or whether it is through a post-RT modification of the processing of RT injury in the tissue. We recommend delaying the start of TAM after completion of RT without reduced efficacy for the patients (Delozier *et al*., 2000), but interactions between both treatments may occur, even if they are separated in time.

Among the interesting questions arising from this study are whether subcutaneous fibrosis might be prevented, or at least reduced. First, our predictive radiation-induced lymphocyte apoptosis assay seems to be highly specific and sensitive to discriminate subgroups of patients as a function of their intrinsic radiosensitivity. Further prospective studies are still necessary before using this test in routine daily practice. Second, preliminary results have shown that TGF-β antagonists may inhibit or reduce the action of this growth factor (Delanian *et al*., 2003). The significant reduction of chronic RT damage obtained with the pentoxifylline and alpha-tocopherol combination does not support the concept that established RT sequelae such as radiation-induced subcutaneous fibrosis are irreversible. Third, evidence from the updated analyses of the ATAC and BIG trials support the use of aromatase inhibitors such as anastrozole or letrozole for the adjuvant treatment of early breast cancer in postmenopausal women (Coates *et al*., 2007; Howell *et al*., 2005). Our data show that in radiosensitive patients, TAM should be delayed after completion of RT. Another approach could be to replace TAM by an aromatase inhibitor. This type of molecule has yet to be tested concomitantly with RT in a clinical setting. Recently, we demonstrated the radiosensitization of breast cancer cells transfected with the aromatase gene by the non-steroidal aromatase inhibitor letrozole (Azria *et al*., 2005a). We have just finished the inclusions in a phase II randomized evaluating toxicities of concurrent or sequential letrozole and RT in adjuvant early-breast cancer treatment. We conclude that the concomitant use of TAM with RT is significantly associated with the incidence of subcutaneous fibrosis but not telangiectasia. In patients receiving adjuvant hormonal treatment, TAM and RT should only be administered concomitantly with caution to radiosensitive patients.

REFERENCES

Ahn, P.H., Vu, H.T., Lannin, D., Obedian, E., DiGiovanna, M.P., Burtness, B., and Haffty, B.G. 2005. Sequence of radiotherapy with tamoxifen in conservatively managed breast cancer does not affect local relapse rates. *J. Clin. Oncol. 23*: 17–23.

Azria, D., Gourgou, S., Sozzi, W.J., Zouhair, A., Mirimanoff, R.O., Kramar, A., Lemanski, C., Dubois, J.B., Romieu, G., Pelegrin, A., and Ozsahin M. 2004a. Concomitant use of tamoxifen with radiotherapy enhances subcutaneous breast fibrosis in hypersensitive patients. *Br. J. Cancer 91*: 1251–1260.

Azria, D., Lemanski, C., Zouhair, A., Gutowski, M., Belkacemi, Y., Dubois, J.B., Romieu, G., and Ozsahin, M. 2004b. Adjuvant treatment of breast cancer by concomitant hormonotherapy and radiotherapy: state of the art. *Cancer Radiother. 8*: 188–196.

Azria, D., Larbouret, C., Cunat, S., Ozsahin, M., Gourgou, S., Martineau, P., Evans, D.B., Romieu, G., Pujol, P., and Pelegrin, A. 2005a. Letrozole sensitizes breast cancer cells to ionizing radiation. *Breast Cancer Res. 7*: R156–R163.

Azria, D., Pelegrin, A., Dubois, J.B., Mirimanoff, R.O., and Ozsahin, M. 2005b. Radiation therapy and tamoxifen: concurrent or sequential? It's no longer the question! *J. Clin. Oncol. 23*: 4239–4241.

Bentzen, S.M. 2006. Preventing or reducing late side effects of radiation therapy: radiobiology meets molecular pathology. *Nat. Rev. Cancer 6*: 702–713.

Bentzen, S.M., Skoczylas, J.Z., Overgaard, M., and Overgaard, J. 1996. Radiotherapy-related lung fibrosis enhanced by tamoxifen. *J. Natl. Cancer. Inst. 88*: 918–922.

Canney, P.A., and Dean, S. 1990. Transforming growth factor beta: a promotor of late connective tissue injury following radiotherapy? *Br. J. Radiol. 63*: 620–623.

Coates, A.S., Keshaviah, A., Thurlimann, B., Mouridsen, H., Mauriac, L., Forbes, J.F., Paridaens, R., Castiglione-Gertsch, M., Gelber, R.D., Colleoni, M., Lang, I., Del Mastro, L., Smith, I., Chirgwin, J., Nogaret, J.M., Pienkowski, T., Wardley, A., Jakobsen, E.H., Price, K.N., and Goldhirsch, A. 2007. Five years of letrozole compared with tamoxifen as initial adjuvant therapy for postmenopausal women with endocrine-responsive early breast cancer: update of study BIG 1–98. *J. Clin. Oncol. 25*: 486–492.

Delanian, S., Porcher, R., Balla-Mekias, S., and Lefaix, J.L. 2003. Randomized, placebo-controlled trial of combined pentoxifylline and tocopherol for regression of superficial radiation-induced fibrosis. *J. Clin. Oncol. 21*: 2545–2550.

Delozier, T., Switsers, O., Genot, J.Y., Ollivier, J.M., Hery, M., Namer, M., Fresney, M., Kerbrat, P., Veyret, C., de Lafontan, B., Janvier, M., and Mace-Lesech, J. 2000. Delayed adjuvant tamoxifen: ten-year results of a collaborative randomized controlled trial in early breast cancer (TAM-02 trial). *Ann. Oncol. 11*: 515–519.

Early Breast Cancer Trialists' Collaborative Group 2005. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet 365*: 1687–1717.

Fisher, B., Dignam, J., Bryant, J., DeCillis, A., Wickerham, D.L., Wolmark, N., Costantino, J., Redmond, C., Fisher, E.R., Bowman, D.M., Deschenes, L., Dimitrov, N.V., Margolese, R.G., Robidoux, A., Shibata, H., Terz, J., Paterson, A.H., Feldman, M.I., Farrar, W., Evans, J., and Lickley, H.L. 1996. Five versus more than five years of tamoxifen therapy for breast cancer patients with negative lymph nodes and estrogen receptor-positive tumors. *J. Natl. Cancer Inst. 88*: 1529–1542.

Fisher, B., Anderson, S., Bryant, J., Margolese, R.G., Deutsch, M., Fisher, E.R., Jeong, J.H., and Wolmark, N. 2002. Twenty-year follow-up of a randomized trial comparing total mastectomy, lumpectomy, and lumpectomy plus irradiation for the treatment of invasive breast cancer. *N. Engl. J. Med. 347*: 1233–1241.

Fowble, B., Fein, D.A., Hanlon, A.L., Eisenberg, B.L., Hoffman, J.P., Sigurdson, E.R., Daly, M.B., and Goldstein, L.J. 1996. The impact of tamoxifen on breast recurrence, cosmesis, complications, and survival in estrogen receptor-positive early-stage breast cancer. *Int. J. Radiat. Oncol. Biol. Phys. 35*: 669–677.

Harris, E.E., Christensen, V.J., Hwang, W.T., Fox, K., and Solin, L.J. 2005. Impact of concurrent versus sequential tamoxifen with radiation therapy in early-stage breast cancer patients undergoing breast conservation treatment. *J. Clin. Oncol. 23*: 11–16.

Ho, A.Y., Atencio, D.P., Peters, S., Stock, R.G., Formenti, S.C., Cesaretti, J.A., Green, S., Haffty, B., Drumea, K., Leitzin, L., Kuten, A., Azria, D., Ozsahin, M., Overgaard, J., Andreassen, C.N., Trop, C.S., Park, J., and Rosenstein, B.S. 2006. Genetic predictors of adverse radiotherapy effects: the Gene-PARE project. *Int. J. Radiat. Oncol. Biol. Phys. 65*: 646–655.

Howell, A., Cuzick, J., Baum, M., Buzdar, A., Dowsett, M., Forbes, J.F., Hoctin-Boes, G., Houghton, J., Locker, G.Y., and Tobias, J.S. 2005. Results of the ATAC (Arimidex, Tamoxifen,

Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. *Lancet 365*: 60–62.

Kantorowitz, D.A., Thompson, H.J., and Furmanski, P. 1993. Effect of conjoint administration of tamoxifen and high-dose radiation on the development of mammary carcinoma. *Int. J. Radiat. Oncol. Biol. Phys. 26*: 89–94.

Koc, M., Polat, P., and Suma, S. 2002. Effects of tamoxifen on pulmonary fibrosis after cobalt-60 radiotherapy in breast cancer patients. *Radiother. Oncol. 64*: 171–175.

Martin, M., Lefaix, J.L., Pinton, P., Crechet, F., and Daburon, F. 1993. Temporal modulation of TGF-beta 1 and beta-actin gene expression in pig skin and muscular fibrosis after ionizing radiation. *Radiat. Res. 134*: 63–70.

Nielsen, H.M., Overgaard, M., Grau, C., Jensen, A.R., and Overgaard, J. 2006. Study of failure pattern among high-risk breast cancer patients with or without postmastectomy radiotherapy in addition to adjuvant systemic therapy: long-term results from the Danish Breast Cancer Cooperative Group DBCG 82 b and c randomized studies. *J. Clin. Oncol. 24*: 2268–2275.

Osborne, C.K., Boldt, D.H., Clark, G.M., and Trent, J.M. 1983. Effects of tamoxifen on human breast cancer cell cycle kinetics: accumulation of cells in early G1 phase. *Cancer Res. 43*: 3583–3585.

Ozsahin, M., Crompton, N.E., Gourgou, S., Kramar, A., Li, L., Shi, Y., Sozzi, W.J., Zouhair, A., Mirimanoff, R-O., and Azria, D. 2005. CD4 and CD8 T-Lymphocyte Apoptosis Can Predict Radiation-Induced Late Toxicity: a prospective study in 399 patients. *Clin. Cancer Res. 11*: 7426–7433.

Paulsen, G.H., Strickert, T., Marthinsen, A.B., and Lundgren, S. 1996. Changes in radiation sensitivity and steroid receptor content induced by hormonal agents and ionizing radiation in breast cancer cells in vitro. *Acta Oncol. 35*: 1011–1019.

Pierce, L.J., Hutchins, L.F., Green, S.R., Lew, D.L., Gralow, J.R., Livingston, R.B., Osborne, C.K., and Albain, K.S. 2005. Sequencing of tamoxifen and radiotherapy after breast-conserving surgery in early-stage breast cancer. *J. Clin. Oncol. 23*: 24–29.

Sarkaria, J.N., Miller, E.M., Parker, C.J., Jordan, V.C., and Mulcahy, R.T. 1994. 4-Hydroxytamoxifen, an active metabolite of tamoxifen, does not alter the radiation sensitivity of MCF-7 breast carcinoma cells irradiated in vitro. *Breast Cancer Res. Treat. 30*: 159–165.

Schmidt-Ullrich, R.K., Valerie, K.C., Chan, W., and McWilliams, D. 1994. Altered expression of epidermal growth factor receptor and estrogen receptor in MCF-7 cells after single and repeated radiation exposures. *Int. J. Radiat. Oncol. Biol. Phys. 29*: 813–819.

Taylor, M.E., Perez, C.A., Halverson, K.J., Kuske, R.R., Philpott, G.W., Garcia, D.M., Mortimer, J.E., Myerson, R.J., Radford, D., and Rush, C. 1995. Factors influencing cosmetic results after conservation therapy for breast cancer. *Int. J. Radiat. Oncol. Biol. Phys. 31*: 753–764.

Wazer, D.E., Tercilla, O.F., Lin, P.S., and Schmidt-Ullrich, R. 1989. Modulation in the radiosensitivity of MCF-7 human breast carcinoma cells by 17B-estradiol and tamoxifen. *Br. J. Radiol. 62*: 1079–1083.

Wazer, D.E., DiPetrillo, T., Schmidt-Ullrich, R., Weld, L., Smith, T.J., Marchant, D.J., and Robert, N.J. 1992. Factors influencing cosmetic outcome and complication risk after conservative surgery and radiotherapy for early-stage breast carcinoma. *J. Clin. Oncol. 10*: 356–363.

Whelan, T., and Levine, M. 2005. Radiation therapy and tamoxifen: concurrent or sequential? That is the question. *J. Clin. Oncol. 23*: 1–4.

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Malignant Phyllodes Tumor of the Breast: Is Adjuvant Radiotherapy Necessary?

Aaron P. Brown, David K. Gaffney, and O. Kenneth Macdonald

INTRODUCTION

Phyllodes tumor of the breast is a rare fibroepithelial tumor that accounts for < 1% of all primary breast neoplasms (Macdonald *et al*., 2006; Parker and Harries, 2001). Chelius described this tumor in 1827 and in 1838 Johannes Müller first coined the term *cystosarcoma phyllodes*. Müller (1838) described the tumor as a fleshy, grayish-white lesion with leaf-like (*phyllos* in Greek) papillary projections of connective tissue. The term *cystosarcoma phyllodes*, however, may be misleading because the lesion typically follows a more benign course than most other soft tissue sarcomas.

Phyllodes tumors actually represent a broad spectrum of fibroepithelial breast diseases ranging from the more common benign lesions (estimated 35–64% of phyllodes tumors) to the rare malignant neoplasms capable of rapid growth and metastasis (Reinfuss *et al*., 1996; Salvadori *et al*., 1989). The first malignant phyllodes tumor was noted in 1931 by Lee and Pack who published a series of 111 patients, one of which had metastatic cystosarcoma phyllodes to the lung (Lee and Pack, 1931). Since Müller's description, > 60 synonyms have been reported. In 1982 the World Health Organization (WHO) designated *phyllodes tumor* as the appropriate nomenclature, and adopted the recommendation of Azzopardi *et al*. (1979) to sub-classify phyllodes tumors histologically as benign, borderline, and malignant (WHO, 1982). An extensive description of the subtleties related to histology is beyond the scope of this chapter but the interested reader would do well to read the summary penned by Parker and Harries (2001) and chapters by Tan *et al*., and Esposito and Dabbs in this volume.

CLINICAL PRESENTATION AND DIAGNOSIS

Patient Characteristics

Phyllodes tumor accounts for < 1% of all primary breast neoplasms, and the incidence of malignant phyllodes tumor has been estimated to be ~ 2.1–3.1 cases per million women (Bernstein *et al*.,

1993). The majority of phyllodes tumors arise in women of age 35–55 years, 20 years later on average than most fibroadenomas (Chaney *et al*., 2000; Macdonald *et al*., 2006). However, phyllodes tumor has been diagnosed over a broad range of ages, occurring in the elderly and in young women. The youngest patient on record to have malignant phyllodes tumor was diagnosed at age 6 (Sasa *et al*., 1995).

There are notable differences in presentation among ethnic groups. According to one population-based study in the United States, incidence is the highest and most rapidly increasing for Latina immigrants (Bernstein *et al*., 1993). Also, a higher incidence has been reported in Asian populations with a younger age at diagnosis (25–30 years) (Bernstein *et al*., 1993). Although extremely rare, phyllodes tumors have been reported in men, and the term phyllodes tumor is also used to describe epithelial-stromal tumors of the prostate (Chen *et al*., 2005a; Konstantakos and Graham, 2003).

Triple Assessment

Triple assessment through clinical examination, radiological imaging and histological analysis with fine needle aspiration cytology (FNAC) and/or core biopsy is the fundamental basis for nonoperative evaluation of breast masses. Preoperative diagnosis of phyllodes tumor is challenging because of poor diagnostic accuracy in all three methods of triple assessment, whether individually or in combination (Jacklin *et al*., 2006).

Clinical Examination

Clinically, phyllodes tumor usually presents as a painless breast mass that is smooth, rounded, and multinodular. Masses can grow as large as 45 cm, but on average masses are 4–6 cm in diameter (Fajdic *et al*., 2007; Parker and Harries, 2001). Ulceration can occur. Many grow rapidly, which does not necessarily indicate malignancy. They are found more commonly in the upper outer quadrant with equal propensity to occur in either breast (Macdonald *et al*., 2006). Clinicians should entertain the diagnosis of phyllodes tumor with any rapidly growing, but clinically benign breast lump in a woman over 35 years of age.

Radiological Imaging

Mammography and ultrasonography are the mainstays of breast mass imaging. Mammography commonly shows a large, well-circumscribed oval or lobulated mass with rounded borders that may show a lucent halo or microcalcifications. Ultrasonography findings include an inhomogeneous, solid mass with low-level internal echoes, smooth walls and good through transmission (Jacklin *et al*., 2006). A cyst within a solid lesion on ultrasound is highly suggestive of phyllodes tumor (Jorge Blanco *et al*., 1999). Other imaging techniques being investigated include MRI, proton magnetic resonance spectroscopy, and scintimammography (Jacklin *et al*., 2006). At present, phyllodes tumors cannot be reliably distinguished from other breast lesions through radiological studies, nor can malignant and benign lesions be differentiated.

Histological Analysis

Methods of obtaining cellular material for analysis of breast masses include (FNAC) and core biopsy. Cytological features of phyllodes tumor have been well described, but FNAC still maintains a low overall accuracy of ~ 63% (Jacklin *et al*., 2006).

Although not as well studied, core biopsy may have a higher accuracy for diagnosis of phyllodes tumor (Jacklin *et al*., 2006) reviewed the literature on diagnosis of phyllodes tumor and they suggest that core biopsy is the most appropriate study for preoperative diagnosis of phyllodes tumor, being both cost-effective and minimally invasive when compared with excisional biopsy. They propose an algorithm using clinical findings, imaging, and FNAC as criteria to perform preoperative core biopsy.

Markers of cell proliferation have recently been investigated in phyllodes tumors to enhance diagnosis and estimation of prognosis. The most studied methods include flow cytometric determination of the S-Phase Fraction (SPF)/DNA ploidy, p53 expression, and MIB-1 index (Jacklin *et al*., 2006). Results are conflicting, but examples of possible clinical utility using these findings include an association of p53 expression and malignancy, and the inverse association of the MIB-1 index and overall survival (Dacic *et al*., 2002; Niezabitowski *et al*., 2001). The role of these findings is not well-defined and investigation in these areas is ongoing.

PROGNOSIS

Local recurrence is common in phyllodes tumors, occurring in ~ 15% of patients, with lower rates (6–10%) seen in patients with benign histology and higher rates in malignant phyllodes tumor (20–65%) (Asoglu *et al*., 2004; Parker and Harries, 2001; Reinfuss *et al*., 1996). There are various contradicting opinions regarding what factors predispose to local recurrence, but two features that appear to be strongly associated with local recurrence are stromal overgrowth and positive or encroaching (< 1 cm) margins after excision (Asoglu *et al*., 2004; Mangi *et al*., 1999; Pandey *et al*., 2001; Reinfuss *et al*., 1996; Salvadori *et al*., 1989). Phyllodes tumors spread hematogenously. Lymph node enlargement occurs in ~ 20% of patients with malignant phyllodes tumor, but this is likely due to necrotic tumor or other factors because the incidence of lymph node metastasis is extremely low, usually < 5% (Asoglu *et al*., 2004; Macdonald *et al*., 2006; Mangi *et al*., 1999; Reinfuss *et al*., 1996; Salvadori *et al*., 1989). Distant metastasis in malignant phyllodes tumor occurs in ~ 25–40% of patients (Asoglu *et al*., 2004; Parker and Harries, 2001; Reinfuss *et al*., 1996). The most common sites of metastasis are lungs/pleura, bone, and abdominal viscera. Metastatic lesions are aggressive and deadly. Chen *et al*. (2005b) found that factors associated with a high risk for metastasis includes an infiltrating tumor margin, severe stromal overgrowth, atypia and cellularity. Stromal overgrowth is a particularly poor prognostic finding (Asoglu *et al*., 2004; Chaney *et al*., 2000; Chen *et al*., 2005b; Reinfuss *et al*., 1996).

Survival estimates following diagnosis of phyllodes tumor vary widely within the literature due to the challenges of studying a rare tumor. Reported overall 5 year survival for malignant phyllodes tumor ranges from 54–88%, and overall 10 year survival from 23–77% (Asoglu *et al*., 2004; Chaney *et al*., 2000; Macdonald *et al*., 2006; Mangi *et al*., 1999; Pandey *et al*., 2001). The largest and most comprehensive analysis of survival was done using the surveillance, epidemiology, and end results (SEER) database from the National Cancer Institute (Macdonald *et al*., 2006).

Overall survival for 821 patients, 5, 10, and 15 years after diagnosis of malignant phyllodes tumor was 84%, 77%, and 73%, respectively. Moreover, cause-specific survival for 5, 10, and 15 years after diagnosis was 91%, 89%, and 89% respectively. Thus, women after diagnosis of malignant phyllodes tumor can enjoy a relatively good prognosis with proper treatment.

TREATMENT

Surgery

Malignant phyllodes tumor is often classified as a primary breast sarcoma, due to similarities in presentation, tumor size, and prognosis (Confavreux *et al*., 2006; Zelek *et al*., 2003). For this reason there are many similarities in treatment principles. As in breast sarcoma, the cornerstone of therapy for phyllodes tumor is surgery. As previously mentioned, phyllodes tumors have the tendency to recur, especially in excisions with positive or close margins. Studies vary widely in their opinions of the best surgical strategy. It is widely accepted that enucleation is inadequate, even for benign lesions (Reinfuss *et al*., 1996; Salvadori *et al*., 1989). For this reason, wide local excision (WLE) with margins of viable breast tissue greater than 1 cm is recommended for benign phyllodes tumors (Chen *et al*., 2005b; Reinfuss *et al*., 1996). For unexpected benign lesions where a limited resection has already been performed, some advocate a "wait and see" policy, without immediate revision, with other authors advocating simple mastectomy for benign lesions > 5 cm in diameter (Salvadori *et al*., 1989).

For malignant phyllodes tumor, the accepted practice in the past was mastectomy which is still supported by some authors (Mangi *et al*., 1999; Norris and Taylor, 1967; Salvadori *et al*., 1989). However, as early as 1975, surgeons began to apply a more conservative approach, finding no difference in local recurrence or mortality between WLE and mastectomy. The conservative approach recommended includes WLE, with simple mastectomy only in lesions too large to achieve a margin > 1 cm (Asoglu *et al*., 2004; Chen *et al*., 2005b; Reinfuss *et al*., 1996; Salvadori *et al*., 1989; Zelek *et al*., 2003). The aforementioned SEER study found that mastectomy was more commonly performed in prior decades, in older women and in women with larger or locally aggressive tumors (Macdonald *et al*., 2006). This study confirmed prior reports that found no significant difference in cause-specific survival among women who received mastectomy and those who received local excision. In summary, optimal surgical therapy is achieved by excising the tumor with an adequate margin of healthy breast tissue. This is done by wide local excision or mastectomy on a case-by-case basis, taking into consideration tumor size, location, histological findings, and the wishes of the patient.

Surgical Technique

The particular surgical approach that one elects to pursue is dependent on the ultimate aim regarding the preservation or *en bloc* removal of the intact breast. The absolute and relative contraindications that exist regarding the application of breast conserving strategies in invasive epithelial malignancies of the breast do not necessarily apply to malignant phyllodes tumor, insofar as many approach this tumor from a soft

tissue sarcoma perspective. Nevertheless, techniques for breast conserving wide local excision or lumpectomy in epithelial malignancies can be similarly applied in excising phyllodes tumors of the breast. Likewise, the techniques for mastectomy or the entire removal of the breast would not be expected to differ between the two histologies.

The goal of wide local excision or lumpectomy is to remove the malignancy in its entirety while maintaining an aesthetically acceptable breast. Cosmetically speaking, the surgical approach is best made with a curvilinear or transverse incision in the upper breast or curvilinear or radial incision in the lower breast following the contours of the breast directly over the mass. In order to maintain breast contours following excision, narrow skin flaps and removal of subcutaneous fat should be avoided, remembering that skin flaps should be raised only for the purpose of accessing the tumor. Exquisite hemostasis is essential as post-operative hematomas can be devastating to the central goal of conserving cosmetic appearance. The surgical approach should be carefully planned with the goal of removing adequate margins around the primary mass, as discussed previously. Furthermore, the specimen should be removed *in toto* and not be transected *in situ*. Suture can be used to orient the specimen with respect to its position in the breast and intra-operative gross or microscopic frozen sections can be evaluated for margin adequacy.

Multiple approaches to removing the entire breast are recognized and include modified radical mastectomy and total mastectomy. The modified radical mastectomy consists of the complete removal of all breast tissue, the underlying fascia of the pectoralis major muscle, and the axillary contents. This technique is accomplished by an ellipsoid transverse excision that extends from the medial chest, encompassing the nipple-areolar complex, to the latissimus dorsi muscle. The extent of breast tissue removal extends superiorly to the inferior boarder of the clavicle and inferiorly to the superior extent of the rectus sheath. With a skin-sparing technique, a similar amount of breast tissue and axillary contents can be removed but the skin is preserved by an incisional technique in which the underlying breast tissue is separated from the overlying subcutaneous tissues of the skin. The remaining skin can then be used in breast reconstruction. Total mastectomy consists of the entire removal of the breast tissue including the axillary tail of Spence while preserving the pectoral muscles and axillary contents. Like lumpectomy, the prevention of post-operative hematoma and subsequent sequelae is best accomplished primarily with exquisite hemostasis. Closed suction drains are often left temporarily in the post-operative period in the axilla and under the skin flap to assist in preventing large fluid collections.

Radiotherapy

Radiotherapy has been effective at decreasing recurrence of invasive and noninvasive breast carcinoma following breast conserving surgery, as well as low and high grade sarcomas (Fisher *et al*., 1989; Pisters *et al*., 1996; Veronesi *et al*., 1993). Yet, for many years, little had been published regarding the use of adjuvant radiotherapy in phyllodes tumor, probably because many believed that phyllodes tumor to be resistant to radiation therapy, despite some

early reports of success with radiotherapy (Hopkins *et al*., 1994). The study of radiotherapy for malignant phyllodes tumor of the breast is particularly challenging because of small numbers of patients. Nevertheless, some attempts have been made to define the role of adjuvant radiotherapy in phyllodes tumor.

Two studies have specifically examined radiotherapy in malignant phyllodes tumor (Pandey *et al*., 2001; Soumarova *et al*., 2004). Pandey *et al*. (2001) included 37 patients with malignant phyllodes tumor, 25 of which were irradiated. Patients who received adjuvant radiotherapy experienced a nonsignificant benefit in disease-free survival at 5 years (hazard ratio of 0.6). Soumarova *et al*. (2004) studied 25 patients with malignant phyllodes tumor, 17 of which were irradiated. They observed a decrease in local recurrence following adjuvant radiotherapy. Both authors recommend adjuvant radiotherapy following excision of any malignant phyllodes tumor. Other authors agree that adjuvant radiotherapy can be justified for any malignant phyllodes tumor, and may be useful in benign or borderline lesions that exhibit poor prognostic indicators (positive or encroaching margins, recurrent disease, large size, and tumors exhibiting severe stromal overgrowth) (Cohn-Cedermark *et al*., 1991; Mangi *et al*., 1999; Ray-Coquard *et al*., 2004; Tan *et al*., 2006; Zelek *et al*., 2003).

Two larger studies investigated adjuvant radiotherapy in primary breast sarcoma including multiple patients with malignant phyllodes tumor. Their results were conflicting. McGowan *et al*. (2000) found a trend towards a longer event-free rate in the patients who had received radiotherapy ($p = 0.06$). However, Confavreux *et al*. (2006) found that there was no significant difference in local recurrence or survival among irradiated and non-irradiated cohorts. They recommend following sarcoma clinical practice guidelines, emphasizing the importance of achieving a negative margin, while minimizing the usefulness of adjuvant radiotherapy. Cohn-Cedermark *et al*. (1991) found no significant increase in metastasis-free survival in 24 irradiated patients. Other authors conclude that adjuvant radiotherapy is unnecessary and provides no increase in overall survival (McGowan *et al*., 2000). The SEER study by Macdonald *et al*. (2006) reported adjuvant radiotherapy as an independent predictor of worse cause-specific survival, but few women in the series received adjuvant radiotherapy which dramatically underpowered the analysis. Additionally, the SEER database does not contain information regarding local recurrence, or adequacy and quality of radiation therapy.

Radiation Technique

The most common approach to radiating an intact breast following breast conservation surgery is with two tangential fields. These matched fields are targeted obliquely across the thorax of the affected breast and are created to include the entirety of the breast tissue on the ipsilateral side while minimizing exposure to the adjacent and underlying normal soft tissues (Figure 44.1). Women are generally treated in a supine position with the ipsilateral arm raised above the head with a pillow, custom fitted body mold, or commercially available "wing board" to support the raised arm. Prior to treatment, simulation occurs where the borders of the breast tissue are demarcated using radio-opaque

wires, and images of the breast in the treatment position are obtained, whether under fluoroscopy or with computed tomography (CT). Multiple techniques have been described that ensure a non-divergent deep beam through the soft tissues of the thorax. Optimally, 1–2 cm of lung parenchyma is included to ensure adequate coverage of the breast tissue that overlies the sloping thoracic contour. Custom cut blocks or field design using multi-leaf collimators can be employed to block critical normal structures, such as the heart, without compromising breast or tumor cavity coverage.

Partial breast irradiation (PBI) is currently being studied in a randomized controlled setting within the United States. PBI consists of treating the post-operative cavity, following breast conserving surgery, with an additional margin of breast tissue. The relative advantages and disadvantages of this technique is beyond the scope of this chapter; however, the radiotherapeutic approach to soft tissue sarcomas is reminiscent of the PBI technique. Adjuvant or neoadjuvant radiotherapy for soft tissue sarcomas incorporates treating the tumor or tumor disease site with an additional margin, dismissing the need to radiate an entire extremity or body region where the tumor may reside. Similarly, malignant phyllodes tumor could potentially be addressed, radiating the area of the surgical cavity or tumor without radiating the entire breast. Intensity modulated radiotherapy (IMRT) or 3-D conformal radiotherapy could be employed to optimize dose delivery to the target while minimizing dose to the adjacent, normal tissues at risk (Figure 44.2). Currently, there is no literature available addressing this technique for malignant phyllodes tumor.

There is minimal literature available regarding the appropriate radiation dose in the treatment of malignant phyllodes tumor. Reported doses of radiation in the available literature range from 36–70 Gy. One study reported a statistical benefit associated with the appropriate radiation dose. McGowan *et al*. (2000) reported that the median normalized radiation dose in

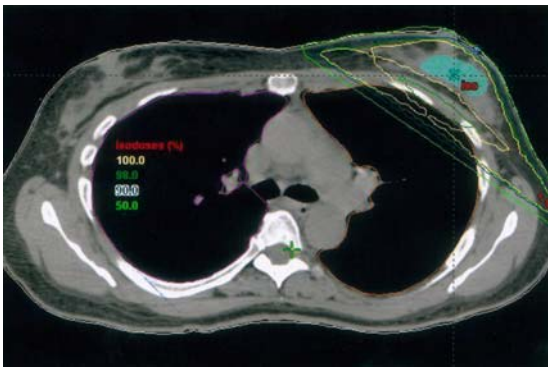

FIGURE 44.1. Treatment planning images at the same level of the breast following lumpectomy for whole breast radiotherapy. The lumpectomy cavity as contoured on computed tomography (CT) is in blue, and calculated isodose coverage is shown as tangential irradiation.

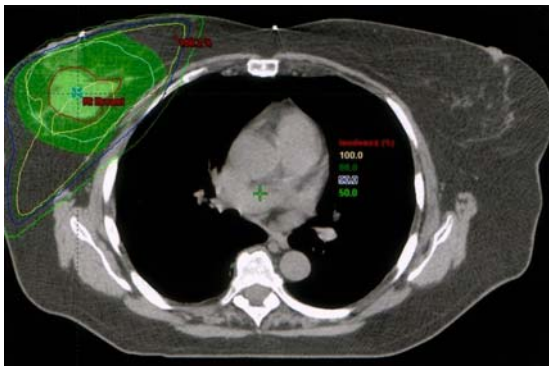

FIGURE 44.2. Treatment planning images at the same level of the breast following lumpectomy for partial breast radiotherapy. The lumpectomy cavity as contoured on computed tomography (CT) (a) and with calculated isodose coverage with multi-field, noncoplanar intensity modulated irradiation (b)

their 26 patients was 48 Gy. Patients receiving > 48 Gy had significantly higher cause-specific survival than those who received less than 48 Gy. However, those results are likely to be biased because there were a significantly greater number of low grade tumors in the high dose cohort compared to the low dose group. They conclude by recommending treatment of malignant phyllodes tumor with conservative surgery followed by post-operative radiation of at least 60 Gy to the tumor bed.

In conclusion, there is a paucity of relevant literature regarding the use of adjuvant radiotherapy in malignant phyllodes tumor. For patients who desire the most aggressive treatment to prevent local recurrence and prolong survival, post-operative adjuvant radiotherapy may be warranted. On a case-by-case basis, adjuvant radiotherapy may be particularly useful for patients with positive or encroaching margins, recurrent disease, and with evidence of severe stromal overgrowth. However, radiotherapy cannot be routinely recommended due to the lack of evidence that it prolongs survival or prevents local recurrence. More robust data is needed before firm recommendations can be made. One group is currently accruing patients for a prospective investigation of the effectiveness of adjuvant radiotherapy after resection of borderline and malignant phyllodes tumor (Tan *et al*., 2006).

Chemotherapy

In spite of the belief that distant metastases are resistant to chemotherapy, there have been reports of a survival benefit with radiochemotherapy in patients with distant metastasis (Burton *et al*., 1989; Hawkins *et al*., 1992; Mangi *et al*., 1999; Reinfuss *et al*., 1996). Most agree though that there is no established role for adjuvant chemotherapy in phyllodes tumor or primary breast sarcoma. In a review of 12 prospective studies involving chemotherapy for primary breast sarcoma, only 2 studies demonstrated a survival benefit (McGrath *et al*., 1995). The only role currently recommended for adjuvant chemotherapy in breast sarcoma is for high grade or large lesions.

Chemotherapy Technique

An extensive description of the methods of chemotherapy delivery is beyond the scope and intent of this chapter, particularly due to the myriad agents that could potentially be applied, and also owing to their relative inefficacy in malignant phyllodes tumors of the breast. Nevertheless, some generalities should be mentioned regarding the chemotherapeutic approach. Firstly, a basic understanding of the pharmacokinetics and pharmacodynamics of any particular therapeutic agent is essential to delivering it for optimal benefit. Second, principles of absorption, distribution, metabolism, and excretion that can be unique to each agent must be respected in planning methods of delivery, route, dose, and frequency of administration. Depending on the agent, central venous access, peripheral venous access, or intact swallowing function would be important in selecting the route and method of delivery. Baseline renal and hepatic function, with an understanding of an agent's metabolism and active, inactive and toxic metabolites, should be adequately evaluated prior to initiating a course of chemotherapy. Decisions regarding dose intensity are routinely developed with careful consideration of

an agent's metabolic properties and route of excretion, paying particular attention to a patient's baseline physiologic function. Third, a thorough evaluation of the patient, paying particular attention to those organs at greatest risk from a particular agent, precedes the delivery of chemotherapy, yet this will still not necessarily predict or preempt both acute and chronic toxicities. Last, dose modification and therapeutic adaptations should be applied appropriately in response to excess toxicity with the occasional need to supplement a patient's innate physiology with intravenous fluids, growth-factors, antibiotics, or other drugs as necessary.

In summary, malignant phyllodes tumor is an extremely rare breast disease, making it difficult to study and develop clear guidelines for diagnosis and treatment. It should be considered in the differential diagnosis of any rapidly growing breast mass in women ages 35–55. Evaluation consists of standard triple assessment by clinical examination, radiological imaging and histological analysis by core biopsy. Treatment of malignant phyllodes tumor is challenging due to elevated rates of local recurrence and distant metastasis. Optimal therapy requires excision of the tumor with an adequate margin of healthy breast tissue either by wide local excision or mastectomy. Surgical management should be determined on a case-by-case basis, taking into consideration tumor size, location, histological findings and the wishes of the patient. Adjuvant radiotherapy may be useful, particularly in patients with positive or encroaching margins, recurrent disease, and with evidence of severe stromal overgrowth. However, radiotherapy cannot be routinely recommended due to the lack of definitive evidence that it prolongs survival or prevents local recurrence. There is no established role for chemotherapy in the treatment of malignant phyllodes tumor. With proper treatment, women can enjoy an optimistic prognosis with 10-year overall survival of 77%, and 10-year cause-specific survival of 89%.

REFERENCES

Asoglu, O., Ugurlu, M.M., Blanchard, K., Grant, C.S., Reynolds, C., Cha, S.S., and Donohue, J.H. 2004. Risk factors for recurrence and death after primary surgical treatment of malignant phyllodes tumors. *Ann. Surg. Oncol. 11*: 1011–1017.

Azzopardi, J.G., Ahmed, A., and Millis, R.R. 1979. Problems in breast pathology. *Major Probl. Pathol. 11*: i–xvi, 1–466.

Bernstein, L., Deapen, D., and Ross, R.K. 1993. The descriptive epidemiology of malignant cystosarcoma phyllodes tumors of the breast. *Cancer 71*: 3020–3024.

Burton, G.V., Hart, L.L., Leight, G.S., Jr., Iglehart, J.D., McCarty, K.S., Jr., and Cox, E.B. 1989. Cystosarcoma phyllodes. Effective therapy with cisplatin and etoposide chemotherapy. *Cancer 63*: 2088–2092.

Chaney, A.W., Pollack, A., McNeese, M.D., Zagars, G.K., Pisters, P.W., Pollock, R.E., and Hunt, K.K. 2000. Primary treatment of cystosarcoma phyllodes of the breast. *Cancer 89*: 1502–1511.

Chen, T.A., Chou, J.M., Sun, G.H., and Lee, H.S. 2005a. Malignant phyllodes tumor of the prostate. *Int. J. Urol. 12*: 1007–1009.

Chen, W.H., Cheng, S.P., Tzen, C.Y., Yang, T.L., Jeng, K.S., Liu, C.L., and Liu, T.P. 2005b. Surgical treatment of phyllodes tumors of the breast: retrospective review of 172 cases. *J. Surg. Oncol. 91*: 185–194.

Cohn-Cedermark, G., Rutqvist, L.E., Rosendahl, I., and Silfversward, C. 1991. Prognostic factors in cystosarcoma phyllodes. A clinicopathologic study of 77 patients. *Cancer 68*: 2017–2022.

Confavreux, C., Lurkin, A., Mitton, N., Blondet, R., Saba, C., Ranchere, D., Sunyach, M.P., Thiesse, P., Biron, P., Blay, J.Y., and Ray-Coquard, I. 2006. Sarcomas and malignant phyllodes

tumours of the breast–a retrospective study. *Eur. J. Cancer 42*: 2715–2721.

Dacic, S., Kounelis, S., Kouri, E., and Jones, M.W. 2002. Immunohistochemical profile of cystosarcoma phyllodes of the breast: a study of 23 cases. *Breast J. 8*: 376–381.

Fajdic, J., Gotovac, N., Hrgovic, Z., Kristek, J., Horvat, V., and Kaufmann, M. 2007. Phyllodes tumors of the breast diagnostic and therapeutic dilemmas. *Onkologie 30*: 113–118.

Fisher, B., Redmond, C., Poisson, R., Margolese, R., Wolmark, N., Wickerham, L., Fisher, E., Deutsch, M., Caplan, R., and Pilch, Y. 1989. Eight-year results of a randomized clinical trial comparing total mastectomy and lumpectomy with or without irradiation in the treatment of breast cancer. *N. Engl. J. Med. 320*: 822–828.

Hawkins, R.E., Schofield, J.B., Wiltshaw, E., Fisher, C., and McKinna, J.A. 1992. Ifosfamide is an active drug for chemotherapy of metastatic cystosarcoma phyllodes. *Cancer 69*: 2271–2275.

Hopkins, M.L., McGowan, T.S., Rawlings, G., Liu, F.F., Fyles, A.W., Yeoh, J.L., Manchul, L., and Levin, W. 1994. Phylloides tumor of the breast: a report of 14 cases. *J. Surg. Oncol. 56*: 108–112.

Jacklin, R.K., Ridgway, P.F., Ziprin, P., Healy, V., Hadjiminas, D., and Darzi, A. 2006. Optimising preoperative diagnosis in phyllodes tumour of the breast. *J. Clin. Pathol. 59*: 454–459.

Jorge Blanco, A., Vargas Serrano, B., Rodriguez Romero, R., and Martinez Cendejas, E. 1999. Phyllodes tumors of the breast. *Eur. Radiol. 9*: 356–360.

Konstantakos, A.K. and Graham, D.J. 2003. Cystosarcoma phyllodes tumors in men. *Am. Surg. 69*: 808–811.

Lee, B.P. and Pack, G.T. 1931. Giant intracanalicular fibro-adenomyxoma of the breast: the so-called cystosarcoma phyllodes mammae of Johannes Muller. *Amer. J. Cancer 15*: 2583.

Macdonald, O.K., Lee, C.M., Tward, J.D., Chappel, C.D., and Gaffney, D.K. 2006. Malignant phyllodes tumor of the female breast: association of primary therapy with cause-specific survival from the surveillance, epidemiology, and end results (SEER) program. *Cancer 107*: 2127–2133.

Mangi, A.A., Smith, B.L., Gadd, M.A., Tanabe, K.K., Ott, M.J., and Souba, W.W. 1999. surgical management of phyllodes tumors. *Arch. Surg. 134*: 487–492; discussion 492–483.

McGowan, T.S., Cummings, B.J., O'Sullivan, B., Catton, C.N., Miller, N., and Panzarella, T. 2000. An analysis of 78 breast sarcoma patients without distant metastases at presentation. *Int. J. Radiat. Oncol. Biol. Phys. 46*: 383–390.

McGrath, P.C., Sloan, D.A., and Kenady, D.E. 1995. Adjuvant therapy of soft-tissue sarcomas. *Clin. Plast. Surg. 22*: 21–29.

Müller, J. 1838. Uber den feineran Bau and die Forman der krankhaften Geschwilste. *1*: 54–57.

Niezabitowski, A., Lackowska, B., Rys, J., Kruczak, A., Kowalska, T., Mitus, J., Reinfuss, M., and Markiewicz, D. 2001. Prognostic evaluation of proliferative activity and DNA content in the phyllodes tumor of the breast: immunohistochemical and flow cytometric study of 118 cases. *Breast Cancer Res. Treat. 65*: 77–85.

Norris, H.J. and Taylor, H.B. 1967. Relationship of histologic features to behavior of cystosarcoma phyllodes. Analysis of ninety-four cases. *Cancer 20*: 2090–2099.

Pandey, M., Mathew, A., Kattoor, J., Abraham, E.K., Mathew, B.S., Rajan, B., and Nair, K.M. 2001. Malignant phyllodes tumor. *Breast J. 7*: 411–416.

Parker, S.J. and Harries, S.A. 2001. Phyllodes tumours. *Postgrad. Med. J. 77*: 428–435.

Pisters, P.W., Harrison, L.B., Leung, D.H., Woodruff, J.M., Casper, E.S., and Brennan, M.F. 1996. Long-term results of a prospective randomized trial of adjuvant brachytherapy in soft tissue sarcoma. *J. Clin. Oncol. 14*: 859–868.

Ray-Coquard, I., Thiesse, P., Ranchere-Vince, D., Chauvin, F., Bobin, J.Y., Sunyach, M.P., Carret, J.P., Mongodin, B., Marec-Berard, P., Philip, T., and Blay, J.Y. 2004. Conformity to clinical practice guidelines, multidisciplinary management and outcome of treatment for soft tissue sarcomas. *Ann. Oncol. 15*: 307–315.

Reinfuss, M., Mitus, J., Duda, K., Stelmach, A., Rys, J., and Smolak, K. 1996. The treatment and prognosis of patients with phyllodes tumor of the breast: an analysis of 170 cases. *Cancer 77*: 910–916.

Salvadori, B., Cusumano, F., Del Bo, R., Delledonne, V., Grassi, M., Rovini, D., Saccozzi, R., Andreola, S., and Clemente, C. 1989. Surgical treatment of phyllodes tumors of the breast. *Cancer 63*: 2532–2536.

Sasa, M., Morimoto, T., Ii, K., Tsuzuki, H., Kamamura, Y., Komaki, K., Uyama, T., and

Monden, Y. 1995. A malignant phyllodes tumor of the breast in a 6-year old girl. *Breast Cancer 2*: 71–75.

Soumarova, R., Seneklova, Z., Horova, H., Vojkovska, H., Horova, I., Budikova, M., Ruzickova, J., and Jezkova, B. 2004. Retrospective analysis of 25 women with malignant cystosarcoma phyllodes–treatment results. *Arch. Gynecol. Obstet. 269*: 278–281. Epub 2004 Mar 2006.

Tan, E.Y., Tan, P.H., Yong, W.S., Wong, H.B., Ho, G.H., Yeo, A.W., and Wong, C.Y. 2006. Recurrent phyllodes tumours of the breast: pathological features and clinical implications. *ANZ. J. Surg. 76*: 476–480.

Veronesi, U., Luini, A., Del Vecchio, M., Greco, M., Galimberti, V., Merson, M., Rilke, F., Sacchini, V., Saccozzi, R., Savio, T., et al. 1993. Radiotherapy after breast-preserving surgery in women with localized cancer of the breast. *N. Engl. J. Med. 328*: 1587–1591.

WHO 1982. Histological typing of breast tumors. *Tumori 68*: 181–198.

Zelek, L., Llombart-Cussac, A., Terrier, P., Pivot, X., Guinebretiere, J.M., Le Pechoux, C., Tursz, T., Rochard, F., Spielmann, M., and Le Cesne, A. 2003. Prognostic factors in primary breast sarcomas: a series of patients with long-term follow-up. *J. Clin. Oncol. 21*: 2583–2588.

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Locally Advanced Breast Cancer: Multidrug Resistance

Can Atalay

INTRODUCTION

Breast cancer is the most common type of cancer encountered in women and the second leading cause of death after lung cancer. Thus, breast cancer is a serious health concern for all women. Fortunately, a high proportion of breast cancer patients are diagnosed at an early stage due to an increase in the screening programs and the awareness of the public worldwide. However, breast cancer is still diagnosed as a locally advanced disease in 10–15% of the patients. Although surgery is the mainstay of the treatment for breast cancer, chemotherapy is initially utilized in locally advanced cases. In this group of patients, resistance to chemotherapy is the main obstacle for the success of treatment. In order to avoid unnecessary side effects and to improve outcome of chemotherapy and to save time and money, new molecular markers for the prediction of response to chemotherapy are necessary. The subject of drugs used to treat breast cancer is discussed by McCubrey and colleagues in this volume.

LOCALLY ADVANCED BREAST CANCER

Locally advanced breast cancer (LABC) comprises a large spectrum of disease presentation in the clinic. Mainly, LABC includes bulky primary tumors and/or extensive lymphadenopathy. According to the latest edition of tumor, node, and metastases (TNM) classification, LABC includes patients with T3 (> 5 cm) or T4 tumors (chest wall fixation or skin edema or ulceration and/or satellitosis) and N2/N3 disease (matted axillary lymph nodes and/or internal mammary lymph node metastases or isolated supraclavicular lymph node metastases) (Sobin and Wittekind, 2002).

Patients with the features of LABC are usually not good candidates for surgery at the time of diagnosis because obtaining tumor free margins or dissecting macroscopic lymph nodes invading axillary vessels is not always possible without increased morbidity or even mortality. The early results of the treatment of LABC starting with surgery were dismal due to high local recurrence rates and short

survival. This group of patients were designated as inoperable cases depending on the results of early studies. For this reason, alternative approaches to surgery were sought to treat patients with LABC.

Systemic treatment methods, namely chemotherapy and hormonetherapy, were prefferred at the beginning of treatment over surgery due to their theoretical advantages. Upfront systemic therapy eliminates the possible micrometastatic disease and depicts the *in vivo* efficacy of the chemotherapy regimen utilized. The detection of clinical resistance to chemotherapy drugs during treatment gives the chance to change the drugs earlier or apply preoperative radiotherapy.

Although long-term survival could be achieved in patients with breast cancer by utilizing surgery, chemotherapy, radiotherapy and hormonetherapy in combination, there are clear differences in the survival rates of early and LABC patients. Five-year survival rates are 85–95% for stage I patients, whereas this decreases to 50% for stage III LABC patients. Anthracyclines increase tumor regression rate and patient survival when given in the neoadjuvant setting. Taxanes had additional beneficial effect on tumor response and patient survival. Clinical complete response rate increased from 40% to 65% whereas pathologic complete response rate increased from 13.7% to 25.6% with taxanes (Bear *et al*., 2003). These results also supported the extensive use of neoadjuvant chemotherapy for early stage breast cancer to increase the number of breast conserving surgery.

MULTIDRUG RESISTANCE

Multidrug resistance (MDR) is a significant challenge to overcome in the treatment of cancer. This type of resistance develops against various drugs with different structure and mechanism of action such as anthracyclines, taxanes, epipodophyllotoxins, and vinca alkaloids used in breast cancer treatment (Ambudkar *et al*., 2003). The members of ATP-binding cassette (ABC) transporter family play a pivotal role in MDR. ABC transporter family has 48 different members identified up to date in humans and these are divided into seven subfamilies, ABC-A to ABC-G based on the similarities of gene sequence. ABC proteins consist of four domains, two transmembrane domains (TMD) and two nucleotide-binding domains (NBD). If these four domains are components of a single polypeptide chain, it is called a full transporter, whereas if they are present within two seperate proteins they are called half transporters. Half transporters have to form homo- or hetero-dimers to be functionally active.

ABC transporters mainly function as efflux pumps located at the cell membranes as well as in the cytoplasm, and they have physiologic roles in secretion and excretion of various substances in the body and protection of vital organs. ABC transporters are highly expressed in the brush border of intestinal cells, the biliary canalicular membrane of hepatocytes, the luminal membrane in proximal tubules of the kidney, in the endothelial cells of the brain capillaries, and in the epithelial cells of the choroid plexus. Basically, these efflux pumps couple the energy derived from ATP hydrolysis to perform the transmembrane movement of their substrates actively against a concentration gradient. ATP hydrolysis takes place at NBD, while the substrate binds to TMDs. Although the precise mechanism of drug efflux is unknown,

there are at least three proposed models: the classical model, the hydrophobic vacuum cleaner model, and the flippase model (Atalay, 2007). In the classical model, two TMDs are organized to form a pore in the membrane and the drugs are expelled out of the cell through these pores. In the hydrophobic vacuum cleaner model, drugs are detected and expelled as they enter the cell membrane as if a hydrophobic vacuum cleaner operates within the membrane. According to the flippase model, the substrate binding site is accessible from the inner side of the cell membrane. The hydrophobic portion of the substrate faces the hydrophobic core of the membrane and the charged portion toward the polar cytosolic part of the membrane. Thus, the substrate diffuses through the membrane until it binds to a site on the transporter in the inner leaflet of the lipid bilayer. The transporter then flips the substrate into the outer leaflet of the membrane using ATP energy.

Although there are 48 members of the ABC transporter family, only a few of these proteins are involved in the anticancer drug resistance. There are certain criteria to demonstrate the involvement of an ABC transporter in resistance to anticancer drugs;

1. The expression of the transporter in tumor cells must be at the same level as shown *in vitro* to give resistance to the drugs in question.
2. The level of expression of the transporter should correlate with the degree of drug resistance.
3. The development of drug resistance should be parallel to the increased expression of the transporter during the treatment.
4. Known inhibitors of the transporters should reverse the drug resistance.
5. Use of these inhibitors with chemotherapy should result in survival advantage (Ambudkar *et al*., 2003).

Among the members of the ABC transporter family, ABCB1 (MDR1), ABCC1 (MRP1), and ABCG2 (BCRP) fulfill most of the above mentioned criteria and their role in drug resistance in cancer patients has been extensively studied.

ABCB1 (MDR1) Protein

There are 11 members of the B subfamily of ABC transporters. ABCB1 is the first discovered and the most extensively studied membrane transporter (Juliano and Ling, 1976). It is encoded by the multidrug resistance gene 1 (*mdr1*) which comprises of 28 exons ranging in size from 49 to 587 bp and is localized to chromosome 7q21. ABCB1 is a typical full transporter protein that is 190 kDa and composed of 1,280 amino acids, although both half and full transporters are present in the ABCB protein subfamily. It is also named as P-glycoprotein (Pgp) and consists of two homologous halves, each containing a TMD with six helices and a NBD which comprises of the highly conserved parts of the protein for ATP binding. It is located in the apical/luminal membrane in polarized cells. ABCB1 protein transports neutral and cationic hydrophobic molecules. It is expressed in certain tissues with barrier function such as liver, kidney, small and large intestine, and brain, whereas it is not present in breast tissue (Ambudkar *et al*., 2003). Besides functioning in barriers, it has a physiologic role in the protection of the cells and organs against toxic compounds and metabolites.

ABCC1 (MRP1) Protein

There are 13 members of the C subfamily of ABC transporters. ABCC1 is the first

discovered member of the multidrug resistance associated proteins (MRP) subfamily of ABC transporter family (Cole *et al*., 1992). There are two structurally different groups in ABCC subfamily. ABCC4 and ABCC5 are full transporters, whereas other ABCCs have a third TMD composed of five transmembrane helices, an extra intracellular loop, and an extracellular N-terminus distinguishing them from other proteins. ABCC1 is 170 kDa consisting of 1,531 amino acids. The amino acid sequence has 15% similarity to ABCB1 in humans. *ABCC1* gene is located on the chromosome 16. In normal tissues, MRPs are located in the cytoplasm functioning as carriers of various molecules to the organelles. In contrast, MRPs are known to be located both in the cytoplasm and on the cellular membrane in cancer cells. ABCC1 functions as a glutathione, glucuronide, and sulfate conjugate pump and confers resistance against drugs such as anthracyclines, epipodophyllotoxins, vinca alkaloids, camptothecins, and methotrexate, whereas, in contrast to ABCB1, taxane resistance has not been reported for ABCC1. Numerous reports have documented the expression of *ABCC1* in cancers such as leukemia, breast, and colorectal cancers that are treated with anthracyclines, camptothecins, and etoposide (Burger *et al*., 2003). For this reason, it is possible to infer that ABCC1 contributes to the inherent resistance of cancers where it is expressed. Unlike ABCB1 that transports hydrophobic drugs, ABCC1 protein can transport hydrophilic molecules, lipophilic organic anions, and neutral drugs conjugated with glutathione, glucuronide, or sulfate. ABCC1 protein functions as a basolateral transporter and moves compounds away from luminal surfaces into tissues below the basement membrane.

ABCG2 (BCRP) Protein

The G subfamily of ABC transporters comprises five half-transporters functioning as homo-dimers bridged by disulfide bonds. *ABCG2* was first cloned from the placenta and the drug resistant MCF-7 breast cancer cell lines not expressing either *ABCB1* or *ABCC1* and termed as breast cancer resistance protein (BCRP) (Doyle *et al*., 1998). Its gene is located on chromosome 4q22 and spans over 66 kb consisting of 16 exons ranging from 60 to 532 bp and 15 introns. The full-length cDNA has 2,418 nucleotides and codes for a protein consisting of 655 amino acids. The molecular mass of the ABCG2 protein is ~ 72 kDa. ABCG2 is normally localized in the placenta, bile canaliculi, colon, small bowel, and brain microvessel endothelium, and has a protective role in the organism similar to most of the ABC transporters. It functions as a high capacity drug transporter which is capable of transporting large, hydrophobic, both positively and negatively charged molecules, including cytotoxic compounds such as mitoxantrone, doxorubicin, daunorubicin, and topotecan.

MULTIDRUG RESISTANCE IN LOCALLY ADVANCED BREAST CANCER

In all types of cancers, drug resistance can either be intrinsic or acquired. In intrinsically resistant tumors, MDR related genes are constitutively expressed and these are the malignancies originating from tissues where these genes play a role in secretion

or protection. On the other hand, acquired resistance develops due to either the selection of drug resistant clones during chemotherapy or a real induction of MDR related genes in cancer cells. In cancer patients with induced type of resistance to chemotherapy, later in the course of the disease, the treatment of the recurrent or the metastatic disease effectively with chemotherapy can be difficult. Breast cancer is moderately responsive to chemotherapy and obtaining a sustained cure with the combined use of surgery, chemotherapy, radiotherapy, and hormonetherapy is not always possible because local or distant disease recurrences are observed many years after the treatment of the primary tumor when chemoresistance becomes a real challenge to overcome, and considering that the probability of drug resistance may help to select the appropriate drugs in advance. Administering chemotherapy regimens, excluding the drugs associated with MDR, such as anthracyclines, taxanes, or vinca alkaloids to the patients expressing MDR related genes might be a logical solution to overcome the drug resistance in breast cancer.

The relationship between MDR and clinical response to chemotherapy or patient survival has been previously studied in breast cancer with conflicting results. There are a few reasons for this discrepancy. Previous studies labeled the patients with recurrent or metastatic disease as clinically resistant, and their tissue samples were analyzed retrospectively for MDR related genes. This method selects the patients with intrinsic resistance with a higher probability of treatment failure. In addition, MDR related genes were not frequently studied in patients with LABC receiving neoadjuvant chemotherapy, and tissue samples were generally obtained prior to chemotherapy. This type of study design can only show the prevalence of MDR related genes in LABC pointing out again to the intrinsic properties of the tumor. Even if tumor samples were evaluated after neoadjuvant chemotherapy, the value of this research method would be less because prechemotherapy status of MDR genes remains unknown. As a result, detecting the patients with induced gene expression during chemotherapy is a better way to understand the role of MDR genes in breast cancer treatment. For this purpose, at least two tissue samples must be studied, one obtained before and the other after chemotherapy. In addition, the drugs known to be transported by MDR related pumps should be used for treatment. In this way, it will be possible to prove the exact role of MDR related genes in clinical resistance and patient survival in breast cancer.

In previous studies, various molecular methods such as Northern blotting, *in situ* hybridization, and RT-PCR were used for the detection of MDR related gene expressions and immunohistochemistry (IHC) was the prefferred method for protein analyses (Atalay *et al.*, 2006). Besides, the patients selected for the studies formed heterogenous groups including early, locally advanced stage, or metastatic breast cancer patients. In addition, the studies usually evaluated a single sample obtained from the patient either at the time of diagnosis or before initiating chemotherapy for metastatic disease. In this context, the reported values of *ABCB1* gene expression ranged between 15–100%. RT-PCR is the most sensitive method, and even a few cancer cells with *ABCB1* expression can be detected. *ABCB1* gene

expression was present in half of the untreated patients with primary breast cancer using Northern blotting technique, whereas the expression rate was > 60% with RT-PCR. These moderate to high rates of *ABCB1* gene expression are in accordance with the clinical observation of a moderate response to chemotherapy in breast cancer.

In addition to evaluating MDR genes, protein products of these genes in breast cancer tissues should be indicated in order to verify the expression of the genes involved in drug resistance. Pgp, protein product of *ABCB1* gene, was evaluated in the previous studies using IHC that was prefferred over molecular detection methods for its widespread availability, lower cost, ease of application, and possibility for retrospective analysis of paraffin embedded tissues, despite its lower sensitivity compared to RT-PCR. Various monoclonal antibodies were utilized to detect Pgp, and in a study comparing the accuracy of these antibodies, JSB1 was found to be superior to other antibodies (Linn *et al*., 1997). Pgp positivity was reported to change between 0–100%, and the variability in the patient groups and the monoclonal antibodies could be the reason for this wide range of results. In LABC patients, Pgp positivity was similar to primary breast cancer patients despite the studies reporting higher Pgp positivity in LABC and higher MRP1 positivity in primary breast cancer (Linn *et al*., 1997).

Studies of the *ABCC1* gene expression in breast cancer patients are fewer than those on *ABCB1* gene probably due to relatively recent discovery of *ABCC* gene subfamily. *ABCC1* expression in breast cancer patients was higher than *ABCB1* expression and ranged between 70–100% with RT-PCR. Similarly, various monoclonal antibodies such as MRPr1, MRPm6, QCRL-1, and QCRL-3 were utilized for the detection of MRP1, the protein product of *ABCC1* gene. The detection rate of MRP1 in breast cancer tissues varied between 18–100% depending on the antibody used and patient groups studied.

In recent years, ^{99m}Tc-MIBI scintimammography has evolved as a noninvasive imaging modality to determine the response to chemotherapy in breast cancer patients (Alonso *et al*., 2002; Sciuto *et al*., 2002). ^{99m}Tc-MIBI is widely used for myocardial perfusion imaging and is taken up by breast carcinomas due to increased blood flow, proliferative and mitochondrial activity in the tumors. ^{99m}Tc-MIBI is also a transport substrate for Pgp and this property enables non-invasive assessment of Pgp expression. ^{99m}Tc-MIBI scintimammography was found to be useful for the prediction of the response to chemotherapy comprising especially of anthracyclines and taxanes (Takamura *et al*., 2001). Thus, ^{99m}Tc-MIBI scintimammography may be suitable in the future for wide spread use due to its advantages over laborous methods such as IHC or RT-PCR.

EFFECTS OF MDR ON CLINICAL RESPONSE TO CHEMOTHERAPY

The effect of MDR related genes on clinical response to chemotherapy was evaluated in a number of previous studies. Presence of an overt association between MDR and clinical response to chemotherapy cannot be easily proven due to the problems mentioned in the previous section. LABC patients were included

in the majority of these studies as a part of the whole study population. However, there are also other studies evaluating only LABC patients (Arnal *et al*., 2000; Atalay *et al*., 2006). In addition, patients treated with neoadjuvant chemotherapy, either in early or locally advanced stage of breast cancer, were included together in a few studies which was helpful seeing the induction of MDR related genes during chemotherapy (Chevillard *et al*., 1997; Vargas-Roig *et al*., 1999).

ABCB1 and *ABCC1* genes and their protein products, Pgp and MRP1, were evaluated either seperately or in combination. Pgp positivity changed between 26–85% in chemotherapy naive LABC patients (Verrelle *et al*., 1991; Rudas *et al*., 2003), whereas *ABCB1* gene expression was present in 12–95% of the patients (Arnal *et al*., 2000; Atalay *et al*., 2006). On the other hand, MRP1 positivity was detected in 20–68% of LABC patients before chemotherapy, and *ABCC1* gene expression rate was similar (68%) (Rudas *et al*., 2003; Atalay *et al*., 2006). Although anthracyclines or taxanes are known to be involved in the induction of MDR and were the main components of chemotherapy protocols in most of the studies, they were utilized for the treatment of only two thirds of the patients in a few studies (Chevillard *et al*., 1997; Rudas *et al*., 2003).

Tumor samples from LABC patients were evaluated either before or after chemotherapy, or both before and after chemotherapy. A single tumor sample was obtained in most of the studies; however, 22.5–85% of LABC patients had their tumor samples studied both before and after chemotherapy (Ro *et al*., 1990; Chung *et al*., 1997). There are also other studies designed with the purpose of detecting the induction of MDR related genes during chemotherapy and samples were obtained sequentially before and after chemotherapy in all patients (Arnal *et al*., 2000; Atalay *et al*., 2006).

Verrelle *et al*. (1991) reported for the first time a direct relation between *ABCB1* overexpression and anthracycline resistance in untreated LABC patients. Similarly, Botti *et al*. (1993) studied LABC patients before chemotherapy and observed that all patients with recurrent disease had Pgp positivity, whereas 43% of the patients with no evidence of recurrence stained negative for Pgp. These studies supported a decreased clinical response in the presence of Pgp positivity. Ro *et al*. (1990) studied LABC patients treated with neoadjuvant chemotherapy by obtaining post-chemotherapy tissue samples, and clinical response was inversely correlated with Pgp positivity. Because the adjacent normal breast tissue also stained positive for Pgp, it is hard to differentiate whether the drug resistance is intrinsic or induced type. In contrast, Dixon *et al*. (1992) found all breast cancer tissue samples unstained for Pgp after chemotherapy which may be the result of not choosing anthracyclines or taxanes in the treatment.

When the previous studies evaluating Pgp positivity both before and after chemotherapy in LABC were examined in detail, both an increase and a decrease in the number of Pgp positive patients were encountered. This supports the idea that studying a single tissue sample will not be enough to determine the role of MDR genes in clinical drug resistance. Vargas-Roig *et al*. (1999) detected Pgp positivity in 59% (22/37) of the patients before chemotherapy, and 43% had persistent Pgp positivity

after chemotherapy, whereas 16% became Pgp negative. Interestingly, Pgp induction was not observed in any of the patients during chemotherapy and, in accordance with this finding, Pgp positivity was found to be unrelated to clinical response. Linn *et al*. (1997) included 40 LABC patients, and only 17 had both pre- and post-chemotherapy tissue samples. In this study, both Pgp and MRP1 were evaluated simultaneously. Pgp positivity was detected in 64% of patients before chemotherapy and decreased to 57% after chemotherapy in contrast to the expectation of Pgp induction; however, MRP1 positivity increased from 20% to 56% after chemotherapy. As a result, no correlation between both Pgp and MRP1 and clinical response to chemotherapy was observed. However, both of these studies included early stage breast cancer patients as well as LABC patients whose tumors may show a different biological behaviour than LABC patients. Pgp and MRP1 positivity were higher in LABC and early stage breast cancer, respectively, supporting this hypothesis.

On the other hand, Rudas *et al*. (2003) included 80 patients with LABC and 68 (85%) had samples taken both before and after chemotherapy. Anthracyclines and taxanes were administered to 66% of the patients and they reported a significant change in Pgp positivity during chemotherapy, which increased from 55% to 100%. Similarly, MRP1 positivity was 62% before chemotherapy, and it increased to 88% after chemotherapy resulting in a significant difference. Although a significant increase in both Pgp and MRP1 positivity was detected indicating *ABCB1* and *ABCC1* gene inductions during chemotherapy, no correlation with the clinical response was reported. The underlying reason for this result was that Pgp or MRP1 positivity either before or after chemotherapy was evaluated seperately in relation to clinical response to chemotherapy. The patient groups with or without gene induction during chemotherapy were not compared directly with each other.

While studies are planned with the intention to prove the induction of MDR related genes in tumor tissue, induction of these genes in normal breast tissue adjacent to tumor tissue is a possibility. Arnal *et al*. (2000) studied *ABCB1* gene expression both in tumor and normal breast tissues before and after chemotherapy. They detected the expression of *ABCB1* gene in 95% (38/40) of tumor tissues and 80% (32/40) of normal breast tissues before chemotherapy including LABC and inflammatory cancer. After administering a doxorubicin containing chemotherapy regimen, *ABCB1* gene expression significantly increased both in tumor and normal breast tissues. However, no significant difference in *ABCB1* gene expression was detected between clinically responsive and unresponsive patients which may be explained with the high expression rate of *ABCB1* gene before chemotherapy. Similarly, Schneider *et al*. (2000) studied 48 patients with LABC and found no correlation between *ABCB1* gene and clinical response.

In contrast to these studies, *ABCB1* gene played a key role in the development of clinical resistance to chemotherapy in other studies. Chevillard *et al*. (1997) sequentially evaluated 73 breast cancer patients treated with neoadjuvant chemotherapy obtaining tissue samples with fine needle aspiration once before chemotherapy and twice during chemotherapy (day 7 and 28). Although 13.7% (10/73) of the

patients had LABC, this study is valuable for depicting the role of *ABCB1* gene induction during chemotherapy. Patients with induced or increased *ABCB1* gene expression were unresponsive to chemotherapy. However, in the group of patients with *de novo ABCB1* expression which remained constant during chemotherapy, clinical response was affected only in patients treated with anthracyclines. Early initiation of *ABCB1* gene expression at the end of the first week of chemotherapy cannot be merely explained by selection of resistant cancer cells. In addition, this study has shown a concordance between RT-PCR and IHC in the majority of cases.

Chung *et al*. (1997) studied LABC patients including inflammatory breast cancer. Pgp positivity was evaluated in 40 patients and 23 of them had samples obtained before and after treatment. The effect of chemotherapy drugs in tumor tissues was graded from low to high. Slight microscopic changes such as swelling of cytoplasm with pyknosis of nuclei being low grade whereas massive coagulation necrosis with only a few or no cancer cells grouped in high grade. Microscopically evaluated drug effect was high in Pgp negative patients and all clinically complete responders were also in this group.

Atalay *et al*. (2006) included a homogenous group of patients with LABC (stage IIIB) in their study, taking tumor samples both before and after chemotherapy, and treated them utilizing anthracyclines which are known to induce expression of MDR genes. They reported a correlation between *ABCB1* gene induction during chemotherapy and clinical resistance. Patients had lower *ABCB1* gene expression (12%) before chemotherapy and the expression significantly increased (24%) after chemotherapy. In contrast to *ABCB1*, induction of *ABCC1* gene in the same group of patients did not have a significant influence on clinical resistance, most probably due to the higher expression rate before chemotherapy (68% pre- and 84% post-chemotherapy).

Recently, the role of single nucleotide polymorphism (SNP) in MDR related genes became the subject of research in this field. SNPs can impair the proper functioning of ABC transporter proteins leading to a change in response to chemotherapy. Normally T/T genotype is present at position 3435 in exon 26 of *ABCB1* gene and T base is replaced with C base resulting in SNP. Although this SNP did not cause any variability in amino acid sequence of Pgp, Kafka *et al*. (2003) reported a decrease in clinical response to anticancer drugs from 87% to 62% in LABC patients with C/T genotype. Further research is required to define the role of SNPs detected in MDR related genes in the development of drug resistance.

In the studies evaluating ^{99m}Tc-MIBI scintimammography, an inverse correlation between sestamibi uptake and *ABCB1* expression was reported previously, whereas a similar correlation for *ABCC1* is doubtful. In LABC, patients with higher uptake are more likely to respond to neoadjuvant chemotherapy (Alonso *et al*., 2002; Sciuto *et al*., 2002). When tumor to normal tissue ratio of MIBI uptake was evaluated, Takamura *et al*. (2001) found that Pgp positivity was significantly higher in patients with low tumor to normal tissue ratio of MIBI uptake. In contrast, no relation was detected between *ABCB1* gene expression and MIBI uptake in the same patient group.

Previous clinical studies have reported conflicting results regarding the role

of MDR related genes and proteins in drug resistance in breast cancer. Since a substantial heterogeneity was observed across individual studies, additional well-designed studies are needed to assess the importance of MDR related genes in breast cancer. However, Trock *et al*. (1997) stated in their meta-analysis that there is no evidence to support the assumption that *ABCB1* expression has no role in breast cancer and that in tumors where expression is detectable, this contributes to MDR phenotype. In contrast, chemoresistance is not detected in all of the patients expressing MDR related genes. There is evidence in the previous studies indicating that in some patients gene expression may decrease or even stop during chemotherapy (Linn *et al*., 1997; Vargas-Roig *et al*., 1999). If gene expression is evaluated only prior to or after chemotherapy, it is not possible to detect the exact changes occuring in gene expression during chemotherapy. The patients responding to chemotherapy with MDR related gene expression before chemotherapy may well have paused gene expression during chemotherapy which may be confusing in clinical practice.

EFFECTS OF MDR ON SURVIVAL

The impact of MDR related genes on patient survival in breast cancer was reported in a few studies. The results of these studies suffer from various shortcomings due to the differences in study populations (early stage vs locally advanced disease), the use of different types of chemotherapy regimens (CMF vs. CAF/CEF), and the detection methods used (RT-PCR vs. IHC). Supporting the role of *ABCB1* gene, previous studies treating LABC patients with anthracyclines reported a shorter survival for patients with *ABCB1* gene expression. In the study by Botti *et al*. (1993), all patients with recurrent disease were Pgp positive before chemotherapy. However, this observation reflected a poorer survival in intrinsically resistant patients because all samples belonged to prechemotherapy period.

In the study by Chung *et al*. (1997), as mentioned before, histopathologic drug effect was evaluated after chemotherapy and drug effect was more prominent in Pgp negative patients. Thus, Pgp negative patients presenting with a high drug effect had a lower disease recurrence rate and, in addition, a marginal survival benefit. On the other hand, Linn *et al*. (1997) studied both Pgp and MRP1 and reported a decrease in overall survival (OS) only if Pgp and p53 were expressed together. Rudas *et al*. (2003) found an inverse correlation between MRP1 positivity and progression-free survival due to an increase in the number of distant metastases in MRP1 positive patients. In contrast to studies reporting a survival disadvantage in patients with MDR related gene expression, no significant effect of *ABCB1* gene on survival was found in other studies. Although Arnal *et al*. (2000) evaluated LABC patients both before and after chemotherapy using RT-PCR, they did not look for *ABCB1* gene induction during chemotherapy. Their analyses sought for a correlation between survival and gene expression either before or after chemotherapy. Vargas-Roig *et al*. (1999) included early stage breast cancer patients in addition to LABC patients. These patients may behave clinically different than LABC patients, which might be the reason for not

finding a significant effect of MDR genes on survival.

When the studies showing no significant effect of MDR related genes on survival were examined, generally there was also no correlation between MDR related gene expression and clinical response to chemotherapy. Poor clinical response to neoadjuvant chemotherapy in LABC results in further progression of the tumor. Because local response to anticancer drugs in the breast reflects the systemic response, the probability of developing distant metastases increases in clinically unresponsive patients, and this leads to decreased disease-free survival (DFS) and OS in patients. On the other hand, multidrug resistant phenotype may be a predictor of aggressive behaviour in tumors resulting in shorter DFS and OS in patients in which case the effect of switching to a new chemotherapy protocol will be minimal. Although it is not definitely known which mechanism plays the principal role in poor survival, development of clinical resistance to drugs seems like a more plausible explanation.

MODULATION OF ABC TRANSPORTERS

Reversal of MDR in cancer cells has been a subject of many clinical and laboratory studies. Inhibitors or modulators of ABC transporters have been used to decrease drug extrusion from the cancer cells and to increase the therapeutic effect of anticancer drugs. Some of these modulators are, in fact, substrates of these transporters and they possibly interfere with drug binding to ABC transporters by competitive inhibition or by causing conformational changes in the transporter proteins. Because ABCB1 is the first discovered ABC transporter with an established role in drug resistance in cancer cells, most of the modulators were developed against this protein. However, in recent years, research is also continuing for modulators specific for ABCC1 and ABCG2 proteins.

There are three generations of ABCB1 (Pgp) modulators. The first generation of modulators was primarily developed to treat various other disease states. Calcium channel blocker, verapamil and immunosuppressive agent, cyclosporin A, were studied extensively. Because these drugs were not developed with the intent of reversing MDR, they are less potent, not selective, and have adverse side effects. The higher *in vivo* concentrations of the first generation of drugs required to overcome ABCB1 resistance increased their toxicity. However, cyclosporin A and verapamil were tested in clinical trials and the addition of verapamil to chemotherapy regimen increased survival in breast cancer patients (Belpomme *et al*., 2000).

The second generation of modulators had an increased potency for reversal of MDR with less adverse side effects. Valspodar (PSC833), a structural analogue of cyclosporin A, lacks the immunosuppressive effect and is ten fold more potent than cyclosporin A, whereas R-isomer of verapamil, dexverapamil, has less cardiac effects. On the other hand, second generation of modulators are not fully specific to only one ABC transporter. Valspodar was tested in a phase II clinical trial in patients with metastatic breast cancer, and the moderate treatment results did not improve the outcome compared to anticancer drugs alone (Carlson *et al*., 2006).

In order to increase the specificity and potency even further, the third generation

of modulators were developed. Zosuquidar (LY335979), tariquidar (XR9576), elacridar (GF120918), laniquidar (R101933), and OC144-093 are specific for Pgp inhibition and have no effect on MRP1. Some of these third generation modulators were evaluated in clinical trials. Tariquidar (XR9576) is an anthranilic acid derivative and showed only a limited activity in advanced breast cancer patients in a phase II trial (Pusztai *et al*., 2005). The efficacy of biricodar was also evaluated against breast cancer in a phase II study with a modest activity (Toppmeyer *et al*., 2002). In contrast, dofequidar was tested recently in a randomized controlled trial including 221 patients with advanced or recurrent breast cancer in combination with cyclophosphamide, doxorubicin, and 5-fluorouracil. It was found to be effective in premenapausal stage IV patients with no prior therapy (Saeki *et al*., 2007).

There are numerous ABCC1 modulators such as verapamil and quinoline derivatives, flavonoids, raloxifene, toremifene, and disulfiram. Flavonoids are naturally occurring polyphenols and have been explored as modulators of ABCB1 mediated MDR. However, synthetic analogue flavopiridol has been introduced as an ABCC1 modulator with a higher activity than flavonoids. Although flavonoids are safe and relatively nontoxic in the clinical setting, their interaction with many different enzymes might lead to adverse effects. Raloxifene is a nonsteroidal estrogen receptor mixed agonist/antagonist and a series of raloxifene analogues were discovered with both *in vitro* and *in vivo* activities modulating ABCC1-mediated MDR. Similar to raloxifene, toremifene, an anti-estrogenic compound with triphenylethylene structure, has also been shown to render the mutidrug resistant cancer cells sensitive to cytotoxic drugs. Besides, disulfiram, a drug used for the treatment of chronic alcoholism, has been reported to be effective on ABCB1 and ABCC1 mediated MDR, inhibiting the maturation of the transporter by decreasing the glycosylated form at the cell surface and the ATP hydrolysis (Sauna *et al*., 2004).

Although ABCG2 is the most recently discovered ABC transporter related to drug resistance, several molecules effective in modulating its function have been reported. Elacridar (GF120918), a third generation ABCB1 protein modulator, is also effective on ABCG2. Imatinib, a tyrosine kinase inhibitor originally used to treat gastrointestinal stromal tumors and leukemias, has been shown to inhibit ABCG2 mediated drug resistance. Flavonoids and their synthetic derivatives also increased the drug sensitivity of the tumor cells with ABCG2 mediated MDR. Curcumins purified from turmeric, a tropical plant, are reported recently to modulate the function of ABCB1, ABCC1, and ABCG2 (Chearwae *et al*., 2006). Fumitremorgin C (FTC), a mycotoxin produced by *Aspergillus fumigatus*, and its analogue Ko143 showed an inhibitory effect against ABCG2 in *in vitro* studies; however, its neurotoxicity prohibited its clinical use (Allen *et al*., 2002). Similar to FTC, tryprostatin A, a fungal secondary metabolite, has also been shown to overcome MDR in cancer cells without any cytotoxicity (Woehlecke *et al*., 2003).

FUTURE PERSPECTIVES

Development of drug resistance during chemotherapy is the leading cause of treatment failure and decreased survival in cancer patients. There is a tremendous

amount of work to be done to overcome the drug resistance, but a real success has not been achieved up to date due to the presence of many different mechanisms taking part in drug resistance. An ideal way to overcome MDR will be the development of anticancer agents that do not interact with any of the ABC transporters. NSC73306, a thiosemicarbazone derivative, was demonstrated to selectively kill the cancer cells with intrinsic or induced ABCB1 function indirectly overcoming drug resistance (Ludwig *et al*., 2006). However, the extremely wide recognition pattern of ABC transporters makes pharmacological modulation the first choice, but the effectivity observed in preclinical studies did not translate into a success in clinical applications. In addition, modulation or inhibition of ABC transporters with various agents has resulted in adverse effects due to toxicity to nontumoral tissues expressing physiologic levels of ABC transporters.

In the future, strategies based on cancer prevention, including the replacement of genes with SNPs by wild-type genes or decreasing the expression of these genes, should have a priority in research field, and these strategies will spare healthy individuals from the deleterious consequences of cancer. For this purpose, MDR related genes can be manipulated at transcriptional or translational level. Studies are being conducted to reveal the control mechanisms of MDR genes. Manipulating the overexpressed genes in cancer cells will allow the ABC transporters to continue their physiologic functions in normal tissues. Phospholipase C gamma and mitogen-activated protein kinase pathways seem appropriate for manipulation to inhibit the transcriptional activity of *ABCB1* gene (Yang *et al*., 2001). In addition, ABC transporter genes can be blocked at the transcriptional level through the use of RNA interference techniques. Introduction of small specific interfering RNA (siRNA) duplex in multidrug resistant cancer cells suppresses *ABCB1* and *ABCG2* genes, resulting in elevated levels of drug accumulation (Ee *et al*., 2004). In addition, specific antisense oligonucleotides whose base arrangement sequence is complementary to a fragment of ABC transporter gene are used for preventing the expression of these genes. Oligonucleotides targeted to *ABCB1* and *ABCC1* genes are capable of forming either a stable triplex with the gene or a stable duplex with a portion of mRNA (Kang *et al*., 2004). Using specifically designed ribozymes to cleave the mRNA of *ABCB1* and *ABCC1* genes was also effective to overcome MDR in *in vitro* studies (Nagata *et al*., 2002). However, reversal of MDR using siRNA, antisense oligonucleotides, or ribozymes is a cumbersome method because the majority of the tumor cells has to be transfected and the delivery of these small molecules to the tumor site cannot be easily accomplished. Functions of ABC transporter proteins can be decreased further by increasing the degradation of these proteins by ubiquitin-proteasome pathway. Because Pgp is a polyubiquitinated protein, polyubiquitination starts the cellular signal that results in the degradation of this protein (Burger and Seth, 2004).

Synthetic peptides resembling the transmembrane helices of the transporters are other tools used for the inhibition of ABC transporter proteins. These peptides decrease the transporter function presumably by disrupting its structure. They are reported to be effective on ABCB1, ABCC1, ABCC2, and ACBG2 proteins. Reversins are peptide derivatives consisting

of naturally occuring L-amino acids which can effectively inhibit the expulsion of cytotoxic drugs at low concentrations. They are capable of reversing MDR without causing serious side-effects in normal tissues and without irreversibly disrupting the physiological functions of ABCB1 transporter.

A general consensus stating that the treatment of cancer should be individualized in the forecoming years according to the biological characteristics of the tumor looks plausible. Developments in microarray technology enable us to plan the individualized treatments. Recently, overexpression of 16 different members of ABC transporter gene family was detected in breast cancer cell lines using microarray technology. Because targeted therapies resulting in individualized treatment methods are the subject of ongoing research, attacking MDR related proteins by means of specific molecules is logical. For this purpose, targeted vaccines were prepared from the peptides derived from the extracapsular loop of Pgp containing several epitopes conjugated to fatty acid molecules. These peptides induce anti-Pgp antibodies in the body when they are given to the patients. However, these peptides must be contained in liposomes to protect them from degradation in the body before reaching the targeted cancer tissues.

In conclusion, development of MDR during chemotherapy in breast cancer patients is definitely a high priority problem to be solved. Routine assessment of *ABCB1* or *ABCC1* genes or their proteins should be the starting point in order to detect the affected patients. Switching to new drugs which are not substrates for ABC transporter proteins or termination of chemotherapy and continuing treatment with hormonotherapy or radiotherapy as early as possible will be the strategies to be followed in drug resistant patients. In addition, research should continue seeking for new ways to overcome MDR keeping in mind that it will be cumbersome to adopt successfull strategies developed in laboratory studies to clinical practice.

REFERENCES

Allen, J.D., van Loevezijn, A., Lakhai, J.M., van der Valk, M., van Tellingen, O., Reid, G., Schellens, J.H., Koomen, G.J., and Schinkel, A.H. 2002. Potent and specific inhibition of the breast cancer resistance protein multidrug transporter in vitro and in mouse intestine by a novel analogue of fumitremorgin C. *Mol. Cancer Ther. 1*: 417–425.

Alonso, O., Delgado, L., Nunez, M., Vargas, C., Lopera, J., Andruskevicius, P., Sabini, G., Gaudiano, J., Muse, I.M., and Roca, R. 2002. Predictive value of ^{99m}Tc sestamibi scintigraphy in the evaluation of doxorubicin based chemotherapy response in patients with advanced breast cancer. *Nucl. Med. Commun. 23*: 765–771.

Ambudkar, S.V., Kimchi-Sarfaty, C., Sauna, Z.E., and Gottesman, M.M. 2003. P-glycoprotein: from genomics to mechanism. *Oncogene 22*: 7468–7485.

Arnal, M., Franco, N., Fargeot, P., Riedinger, J.M., Brunet-Lecomte, P., and Lizard-Nacol, S. 2000. Enhancement of *mdr1* gene expression in normal tissue adjacent to advanced breast cancer. *Breast Cancer Res. Treat. 61*: 13–20.

Atalay, C. 2007. Multi-drug resistance and cancer. *Expert Opin. Ther. Patents 17*: 511–520.

Atalay, C., Gurhan, I.D., Irkkan, C., and Gunduz, U. 2006. Multidrug resistance in locally advanced breast cancer. *Tumour Biol. 27*: 309–318.

Bear, H.D., Anderson, S., Brown, A., Smith, R., Mamounas, E.P., Fisher, B., Margolese, R., Theoret, H., Soran, A., Wickerham, D.L., and Wolmark, N. 2003. The effect on tumor response of adding sequential preoperative docetaxel to preoperative doxorubicin and cyclophosphamide: preliminary results from National Surgical Adjuvant Breast and Bowel Project Protocol B-27. *J. Clin. Oncol. 21*: 4165–4174.

Belpomme, D., Gauthier, S., Pujade-Lauraine, E., Facchini, T., Goudier, M.J., Krakowski, I., Netter-Pinon, G., Frenay, M., Gousset, C., Marie, F.N., Benmiloud, M., and Sturtz, F. 2000. Verapamil increases the survival of patients with anthracycline-resistant metastatic breast carcinoma. *Ann. Oncol. 11*: 1471–1476.

Botti, G., Chiappetta, G., D'Aiuto, G., de Angelis, E., de Matteis, A., Montella, M., Picone, A., and Cascione, F. 1993. PCNA/cyclin and P-glycoprotein as prognostic factors in locally advanced breast cancer. An immunohistochemical, retrospective study. *Tumori 79*: 214–218.

Burger, A.M., and Seth, A.K. 2004. The ubiquitin-mediated protein degradation pathway in cancer: Therapeutic implications. *Eur. J. Cancer 40*: 2217–2229.

Burger, H., Foekens, J.A., Look, M.P., Meijer-van Gelder, M.E., Klijn, J.G., Wiemer, E.A., Stoter, G., and Nooter, K. 2003. RNA expression of breast cancer resistance protein, lung resistance-related protein, multidrug resistance-associated proteins 1 and 2, and multidrug resistance gene 1 in breast cancer: correlation with chemotherapeutic response. *Clin. Cancer Res. 9*: 827–836.

Carlson, R.W., O'Neill, A.M., Goldstein, L.J., Sikic, B.I., Abramson, N., Stewart, J.A., Davidson, N.E., and Wood, W.C.; Eastern Cooperative Oncology Group. 2006. A pilot phase II trial of valspodar modulation of multidrug resistance to paclitaxel in the treatment of metastatic carcinoma of the breast (E1195): a trial of the Eastern Cooperative Oncology Group. *Cancer Invest. 24*: 677–681.

Chearwae, W., Wu, C.P., Chu, H.Y., Lee, T.R., Ambudkar, S.V., and Limtrakul, P. 2006. Curcuminoids purified from turmeric powder modulate the function of human multidrug resistance protein 1 (ABCC1). *Cancer Chemother. Pharmacol. 57*: 376–388.

Chevillard, S., Lebeau, J., Pouillart, P., de Toma, C., Beldjord, C., Asselain, B., Klijanienko, J., Fourquet, A., Magdelenat, H., and Vielh, P. 1997. Biological and clinical significance of concurrent *p53* gene alterations, *MDR1* gene expression, and S-phase fraction analyses in breast cancer patients treated with primary chemotherapy or radiotherapy. *Clin. Cancer Res. 3*: 2471–2478.

Chung, H.C., Rha, S.Y., Kim, J.H., Roh, J.K., Min, J.S., Lee, K.S., Kim, B.S., and Lee, K.B. 1997. P-glycoprotein: the intermediate end point of drug response to induction chemotherapy in locally advanced breast cancer. *Breast Cancer Res. Treat. 42*: 65–72.

Cole, S.P., Bhardwaj, G., Gerlach, J.H., Mackie, J.E., Grant, C.E., Almquist, K.C., Stewart, A.J., Kurz, E.U., Duncan, A.M., and Deeley, R.G. 1992. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science 258*: 1650–1654.

Dixon, A.R., Bell, J., Ellis, I.O., Elston, C.W., and Blamey, R.W. 1992. P-glycoprotein expression in locally advanced breast cancer treated by neoadjuvant chemotherapy. *Br. J. Cancer 66*: 537–541.

Doyle, L.A., Yang, W., Abruzzo, L.V., Krogmann, T., Gao, Y., Rishi, A.K., and Ross, D.D. 1998. A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc. Natl. Acad. Sci. USA 95*: 15665–15670.

Ee, P.L., He, X., Ross, D.D., and Beck, W.T. 2004. Modulation of breast cancer resistance protein (BCRP/ABCG2) gene expression using RNA interference. *Mol. Cancer Ther. 3*: 1577–1583.

Juliano, R.L., and Ling, V. 1976. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim. Biophys. Acta 455*: 152–162.

Kafka, A., Sauer, G., Jaeger, C., Grundmann, R., Kreienberg, R., Zeillinger, R., and Deissler, H. 2003. Polymorphism C3435T of the MDR-1 gene predicts response to preoperative chemotherapy in locally advanced breast cancer. *Int. J. Oncol. 22*: 1117–1121.

Kang, H., Fisher, M.H., Xu, D., Miyamoto, Y.J., Marchand, A., van Aerschot, A., Herdewijn, P., and Juliano, R.L. 2004. Inhibition of MDR1 gene expression by chimeric HNA antisense oligonucleotides. *Nucleic Acids Res. 32*: 4411–4419.

Linn, S.C., Pinedo, H.M., van Ark-Otte, J., van der Valk, P., Hoekman, K., Honkoop, A.H., Vermorken, J.B., and Giaccone, G. 1997. Expression of drug resistance proteins in breast cancer in relation to chemotherapy. *Int. J. Cancer 71*: 787–795.

Ludwig, J.A., Szakacs, G., Martin, S.E., Chu, B.F., Cardarelli, C., Sauna, Z.E., Caplen, N.J., Fales, H.M., Ambudkar, S.V., Weinstein, J.N., and Gottesman, M.M. 2006. Selective toxicity

of NSC73306 in MDR1-positive cells as a new strategy to circumvent multidrug resistance in cancer. *Cancer Res. 66*: 4808–4815.

Nagata, J., Kijima, H., Hatanaka, H., Asai, S., Miyachi, H., Abe, Y., Yamazaki, H., Nakamura, M., Watanabe, N., Mine, T., Kondo, T., Scanlon, K.J., and Ueyama, Y. 2002. Reversal of drug resistance using hammerhead ribozymes against multidrug resistance-associated protein and multidrug resistance 1 gene. *Int. J. Oncol. 21*: 1021–1026.

Pusztai, L., Wagner, P., Ibrahim, N., Rivera, E., Theriault, R., Booser, D., Symmans, F.W., Wong, F., Blumenschein, G., Fleming, D.R., Rouzier, R., Boniface, G., and Hortobagyi, G.N. 2005. Phase II study of tariquidar, a selective P-glycoprotein inhibitor, in patients with chemotherapy-resistant, advanced breast carcinoma. *Cancer 104*: 682–691.

Ro, J., Sahin, A., Ro, J.Y., Fritsche, H., Hortobagyi, G., and Blick, M. 1990. Immunohistochemical analysis of P-glycoprotien expression correlated with chemotherapy resistance in locally advanced breast cancer. *Hum. Pathol. 21*: 787–791.

Rudas, M., Filipits, M., Taucher, S., Stranzl, T., Steger, G.G., Jakesz, R., Pirker, R., and Pohl, G. 2003. Expression of MRP1, LRP and Pgp in breast carcinoma patients treated with preoperative chemotherapy. *Breast Cancer Res. Treat. 81*: 149–157.

Saeki, T., Nomizu, T., Toi, M., Ito, Y., Noguchi, S., Kobayashi, T., Asaga, T., Minami, H., Yamamoto, N., Aogi, K., Ikeda, T., Ohashi, Y., Sato, W., and Tsuruo, T. 2007. Dofequidar fumarate (MS-209) in combination with cyclophosphamide, doxorubicin, and fluorouracil for patients with advanced or recurrent breast cancer. *J. Clin. Oncol. 25*: 411–417.

Sauna, Z.E., Peng, X.H., Nandigama, K., Tekle, S., and Ambudkar, S.V. 2004. The molecular basis of the action of disulfiram as a modulator of the multidrug resistance-linked ATP binding cassette transporters MDR1 (ABCB1) and MRP1 (ABCC1). *Mol. Pharmacol. 65*: 675–684.

Schneider, J., Lucas, R., Sanchez, J., Ruibal, A., Tejerina, A., and Martin, M. 2000. Modulation of molecular marker expression by induction chemotherapy in locally advanced breast cancer: correlation with the response to therapy and the expression of MDR1 and LRP. *Anticancer Res. 20*: 4373–4378.

Sciuto, R., Pasqualoni, R., Bergomi, S., Petrilli, G., Vici, P., Belli, F., Botti, C., Mottolese, M., and Maini, C.L. 2002. Prognostic value of ^{99m}Tc-sestamibi washout in predicting response of locally advanced breast cancer to neoadjuvant chemotherapy. *J. Nucl. Med. 43*: 745–751.

Sobin, L.H., and Wittekind, Ch. 2002. International Union Against Cancer (UICC). TNM classification of malignant tumors. 6th edition. New Jersey: Wiley.

Takamura, Y., Miyoshi, Y., Taguchi, T., and Noguchi, S. 2001. Prediction of chemotherapeutic response by ^{99m}Tc-MIBI scintigraphy in breast carcinoma patients. *Cancer 92*: 232–239.

Toppmeyer, D., Seidman, A.D., Pollak, M., Russell, C., Tkaczuk, K., Verma, S., Overmoyer, B., Garg, V., Ette, E., Harding, M.W., and Demetri, G.D. 2002. Safety and efficacy of the multidrug resistance inhibitor Incel (biricodar; VX-710) in combination with paclitaxel for advanced breast cancer refractory to paclitaxel. *Clin. Cancer Res. 8*: 670–678.

Trock, B.J., Leonessa, F., and Clarke, R. 1997. Multidrug resistance in breast cancer: a meta-analysis of MDR1/gp170 expression and its possible functional significance. *J. Natl. Cancer Inst. 89*: 917–931.

Vargas-Roig, L.M., Gago, F.E., Tello, O., Martin de Civetta, M.T., and Ciocca, D.R. 1999. C-erbB 2 (HER-2/neu) protein and drug resistance in breast cancer patients treated with induction chemotherapy. *Int. J. Cancer 84*: 129–134.

Verrelle, P., Meisonnier, F., Fonck, Y., Feillel, V., Dionet, C., Kwiatkowski, F., Plagne, R., and Chassagne, J. 1991. Clinical relevance of immunohistochemical detection of multidrug resistance P-glycoprotein in breast carcinoma. *J. Natl. Cancer Inst. 83*: 111–116.

Woehlecke, H., Osada, H., Herrmann, A., and Lage, H. 2003. Reversal of breast cancer resistance protein-mediated drug resistance by tryprostatin A. *Int. J. Cancer 107*: 721–728.

Yang, J.M., Vassil, A.D., and Hait, W.N. 2001. Activation of phospholipase C induces the expression of the multidrug resistance (MDR1) gene through the Raf-MAPK pathway. *Mol. Pharmacol. 60*: 674–680.

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Breast Cancer: Diagnosis of Recurrence Using ^{18} F-Fluorodeoxyglucose-Positron Emission Tomography/Computed Tomography

Simona Ben-Haim and Lea Radan

INTRODUCTION

Breast cancer is the most common cancer and the second leading cause of cancer related death in women. The National Cancer Institute estimates 178,480 women will be diagnosed with breast cancer and 40,640 women will die of breast cancer in 2007 in the United States, most of them of progressive metastatic disease (Ries *et al*., 2007). Accurate diagnosis and staging, efficient monitoring response to therapy and early diagnosis of recurrence are essential for the selection of the most appropriate therapeutic strategy and major determinants of patient's prognosis and survival (Reddy and Mendelson, 2005; Esserman, 2005; Weir *et al*., 2005).

Recurrent breast cancer occurs in up to 35% of patients within 10 years of the initial surgical treatment of the primary tumor (Voogt *et al*., 2001). The 5-year survival of patients with disseminated disease (26.7%) is significantly shorter than that of patients with localized disease (98%) or regional disease (83.5%) (Ries *et al*., 2007). Therefore, early detection of recurrence can improve the prognosis and survival of patients. Serum tumor marker measurements are used for routine follow up. When these are progressively increasing, recurrent disease is suggested. An increase in serum tumor markers may precede the diagnosis of disease recurrence by imaging modalities by up to 2 years (Israel and Kuten, 2007). There appears to be a direct relationship between the degree of serum tumor marker elevations and the extent of active malignancy. Gallowich *et al*. (2003) demonstrated that despite negative serum tumor markers in 31 patients, 11 of them had clear evidence of malignant disease by imaging, including ^{18}F-fluoro-2-deoxyglucose (FDG)-positron emission tomography (PET). However, the tumor burden in these patients was limited. Local recurrence was diagnosed in three patients, and mediastinal lymph node metastases and bone metastases in four patients each. However, the use of serum tumor marker measurements is limited by potential false elevations that may occur in various benign conditions (Flamen *et al*., 2001). In addition, rising

serum tumor marker levels do not localize the site of recurrent tumors, nor do they differentiate between local relapse of disease or distant metastases. These are important parameters that can determine the most appropriate therapeutic regimen in patients with breast cancer.

Anatomic imaging studies, such as mammography, ultrasound, computed tomography (CT), and magnetic resonance imaging (MRI) have been routinely used for the diagnosis of recurrent breast cancer. Scar formation and radiation induced fibrosis may, however, compromise the ability of mammography and CT to diagnose local recurrences. Mammography has a sensitivity of 52% and a specificity of 84% for the detection of local recurrence following breast-conserving surgery (Kolasinska *et al.*, 2001). On CT the sequelae of previous surgery and radiation therapy are not easily differentiated from recurrent tumor, and small metastatic foci can be difficult to differentiate from benign lesions (Siggelkow *et al.*, 2004). As reported by Gilles *et al.* (1994), ultrasound is more sensitive for the detection of local complications and benign lesions, but is inadequate for the detection of recurrent disease. Recently, Belli *et al.* (2002) have assessed the ability of MRI to diagnose local recurrence in 40 breast cancer patients and reported a sensitivity, specificity, and accuracy of 100%, 89% and 95%, respectively.

The following is a summary of the diagnostic performance of metabolic imaging with ^{18}F- fluoro-2-deoxyglucose (FDG) positron emission tomography (PET) as well as PET integrated with CT (PET/CT) for assessment of breast cancer recurrence.

METHODOLOGY: FDG-PET AND PET/CT IMAGING

Positron emission tomography is a non-invasive nuclear medicine imaging technique that utilizes radiopharmaceuticals to detect metabolic alterations within cells. Conventional anatomic imaging modalities, such as CT and MRI, provide primarily structural or anatomic information, whereas PET imaging provides molecular or functional information. ^{18}F- fluoro-2-deoxyglucose is the most commonly used positron emitting tracer in routine clinical practice. It has a half-life of 110 min and can be easily shipped from a central production area to imaging centers. Similarly to glucose, FDG is transported into cells and is phosphorylated by hexokinase, but unlike glucose, FDG is not further metabolized in the cells. In addition, FDG cannot undergo dephosphorylation and cannot cross the cell membrane. The uptake of FDG is increased in tumors due to increased glycolysis and glucose avidity of malignant cells as compared with normal tissue. In cancer patients metabolic changes usually precede structural changes, and these are readily detected by PET. In addition, PET is a whole body imaging modality performed routinely head to mid-thighs, which represents an additional benefit, improving the detection rate of cancer.

In addition to malignancy, phosphorilated FDG is also "trapped" in cells of other metabolically active tissues, such as inflammation or infection. The interpretation of FDG-PET images is sometimes difficult, due to the lack of anatomical landmarks on the functional PET images. Visual correlation of PET with anatomical imaging modalities, such as CT and MRI,

is frequently needed in clinical practice. Combined assessment of these different sets of data can also be obtained by software coregistration. However, this is technically complicated and cumbersome, and therefore of limited use in routine clinical practice. Recently developed integrated PET/CT scanners enable molecular imaging by PET and anatomical imaging by CT to be acquired in a single imaging session (Beyer *et al*., 2000), combining data from functional and morphological examinations. The PET/CT system is composed of a dedicated PET scanner with full-ring bismuth germinate oxy-ortho silicate (BGO) or lutetium oxy-ortho silicate (LSO) detectors and a multislice CT. Computed tomography information is used for attenuation correction of the PET data and, in addition, dual-modality FDG-PET/CT imaging provides better localization and characterization of foci of increased tracer uptake. The integrated PET/CT systems add functional information to the CT data and therefore enable better localization of tracer uptake on PET. PET/CT imaging is offering the combined power of molecular/metabolic imaging with detailed anatomical mapping, improving the accuracy for tumor staging compared to either PET or CT alone (Bar-Shalom *et al*., 2003).

For the FDG-PET or PET/CT studies, patients are instructed to fast for 4–6 h before injection of the radiotracer, except for glucose-free oral hydration. Blood glucose is measured before tracer injection to ensure level of < 11 mmol/l. 370–550 MBq of FDG are injected. Patients are kept lying comfortably for the uptake phase and whole-body FDG-PET scanning in prone position is performed 60–90 min after FDG injection.

The imaging protocol includes an initial CT followed by PET study acquisition. Usually the slice thickness of CT is 4.25 mm, equal to that of PET. Computed tomography images are reconstructed onto 512 × 512 matrix. The emission scan duration varies with the detector technology in use. With the conventional BGO, PET is acquired by sequential fields of view, each covering 15 cm during an acquisition time of 4–6 min. The new LSO technology reduces emission scans to 2–3 min. Positron emission tomography acquisition can be performed in 2-dimensional mode using a matrix of 128 × 128, or in a 3-dimentional mode, resulting in improved spatial resolution. Positron emission tomography data are then reconstructed and the data obtained from CT acquisition are used for low-noise attenuation correction of PET emission data, and for fusion of attenuation-corrected PET images with corresponding CT images. After completion of PET acquisition, the reconstructed X-ray attenuation-corrected PET images, CT images, and fused images are reviewed in axial, coronal and sagittal planes, and in a 3-dimensional cine mode.

Image Interpretation

The presence and localization of foci of increased FDG uptake are recorded on PET or PET/CT, and each lesion is characterized as benign or malignant. A focus of increased FDG is defined as benign when related to the physiologic biodistribution of FDG or to a known non-malignant process. A focus of abnormal FDG activity of higher intensity than that of surrounding tissues, which could not be related to benign or physiologic FDG uptake, or is localized in a suspicious mass on CT, is defined as malignant.

FDG-PET AND PET/CT IMAGING FOR BREAST CANCER RECURRENCE

The role of FDG imaging in the management of breast cancer patients has been assessed.

At the time of initial diagnosis, as well as for staging, FDG imaging has been reported to be of controversial, limited diagnostic value, mainly in detecting small noninvasive primary breast tumors, in staging of the axillary region, and in the detection of osteoblastic metastases (Quon and Gambhir, 2005; Ell, 2006). While Avril *et al*. (2000) have reported a poor performance for FDG-PET in non-invasive breast cancer, Wu and Gambhir (2003) demonstrated an overall sensitivity, specificity, and accuracy of 90%, 93% and 92%, respectively, for the detection of primary invasive breast cancer. These discordant results can be explained, at least in part, by the histopathology and size of the primary breast tumors and regional nodal metastases. Among the invasive subtypes, infiltrating ductal carcinoma has higher FDG uptake, and is therefore detected at higher sensitivity when compared with the infiltrating lobular subtype (Avril *et al*., 2000). With respect to tumor size, detection of small tumors is limited by the resolution of the systems, whereas modern PET/CT devices provide a spatial resolution of 4 mm.

Axillary lymph node involvement in breast cancer patients is an important factor for planning the therapeutic strategy and also has prognostic implications. The performance of FDG imaging for axillary nodal staging in patients with breast cancer has been previously described. Wu and Gambhir (2003) report an overall sensitivity of 88%, specificity of 92%, and accuracy of 89%. In a study of 360 patients, Wahl *et al*. (2004) report a sensitivity of 61% and a specificity of 80% for axillary lymph node assessment, suggesting that FDG-PET may fail to detect axillary nodal involvement, mainly due to involvement of few small nodes or micrometastatic disease. In contrast, FDG-PET performs well and better than CT in the detection of internal mammary and mediastinal metastases with sensitivity, specificity, and accuracy of 85%, 90% and 88% for PET vs. 54%, 85%, and 73% for CT, respectively (Wu and Gambhir, 2003). FDG-PET also performs well for the detection of distant metastases, with an overall sensitivity of 84–93% and a negative predictive value > 90% (Lonneux *et al*., 2000; Wu and Gambhir, 2003).

Local or regional recurrence occurs in 7–35% of patients treated for breast cancer (Voogt *et al*., 2001; Zangheri *et al*., 2004). The most common sites of loco-regional recurrence are the chest wall and supraclavicular nodes. Involvement of internal mammary nodes has been found in ~ 25% of patients. Metastatic spread to mediastinal nodes is also common, and distant metastases are encountered mainly in the skeleton.

Unlike patients with other advanced-stage malignancies, patients with advanced and recurrent breast cancer can benefit from a variety of therapeutic options, including surgery, radiation, chemotherapy, and hormonal therapy. Choosing the most appropriate treatment strategy depends primarily on accurately defining the presence and extent of disease. Therefore, early diagnosis of recurrence, as well as defining its extent and precise localization are of utmost importance. Early detection

of recurrent breast cancer with a small tumor burden is expected to provide more treatment options and potentially increase survival rates of these patients.

Whole body FDG-PET has been used in search for metastases, mainly when suggested by clinical symptoms or by a progressive increase in biochemical tumor markers, such as CA 15-3 or CEA (Siggelkow *et al*., 2004). The accuracy of FDG-PET in restaging recurrent breast cancer has been demonstrated. In asymptomatic patients with rising serum tumor markers, metastatic disease can be diagnosed by FDG-PET with an accuracy of 87–90%, compared with an accuracy of 50–78% for conventional imaging (Gallowitsch *et al*., 2003; Zangheri *et al*., 2004; Tatsumi *et al*., 2006). Weir *et al*. (2005) have assessed the role of PET/CT in the management of breast cancer in 165 patients, with 27 of these patients evaluated for suspected recurrence. In this subgroup PET/CT was of particular value. Recurrence was confirmed in 31% of patients, with FDG-PET having a 5% false-negative rate and a 20% false-positive rate. In 30% of patients with newly diagnosed recurrence PET identified additional distant metastases, thus providing important information for management decisions (Weir *et al*., 2005) (Figure 46.1).

Because it provides functional information, FDG-PET is complementary to conventional staging or restaging modalities, such as physical examination, CT or MRI and bone scintigraphy, mainly in regions previously treated with surgery or radiation therapy, where differentiation between scar and recurrent tumor is otherwise challenging. Due to its high sensitivity for the detection of metabolically active disease, FDG-PET can help in the diagnosis as well as the definition of the extent of metabolically active recurrent disease when conventional imaging results are equivocal or negative. Isasi *et al*. (2005) performed

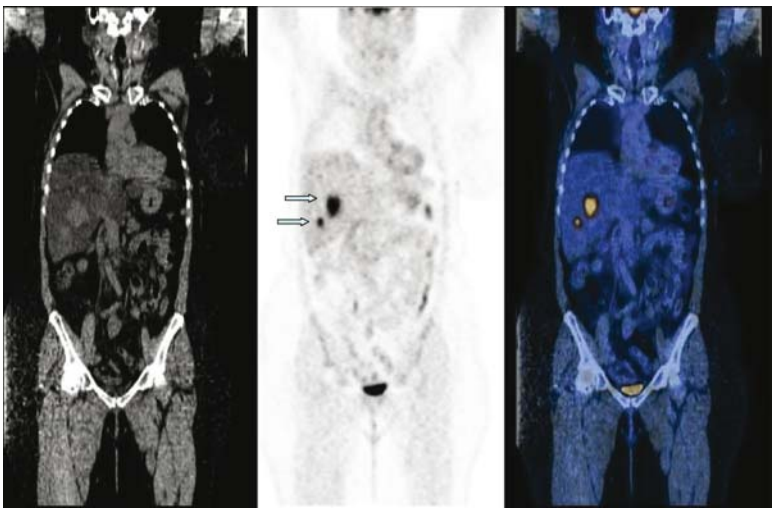

FIGURE 46.1. A 60-year-old patient, 3 years after right mastectomy and adjuvant chemo- and radiotherapy, on continued hormonal therapy due to invasive duct breast cancer. Referred with elevated serum Ca 15.3 and a small liver lesion on CT. CT (left), PET (middle) and PET/CT (right) coronal images FDG avid liver lesions in the right lobe of the liver (arrows). The patient received chemotherapy and after improvement underwent partial hepatectomy. 16 months later the patient was diagnosed with disease progression, with new FDG avid lesions in left lobe (not shown)

a meta-analysis of 16 studies including > 800 patients to evaluate the diagnostic performance of FDG-PET in the assessment of breast cancer recurrence. This analysis showed a mean sensitivity of 93% and a mean specificity of 82% for the diagnosis of recurrent breast cancer.

Prior to FDG-PET, most patients with suspected recurrent breast cancer were evaluated with a combination of tests including brain MRI, bone scintigraphy, and CT of the chest, abdomen and pelvis. However, FDG-PET performs better than conventional imaging modalities in the assessment of disease recurrence. Suárez *et al*. (2002) evaluated 45 asymptomatic breast cancer patients who had progressive elevation of tumor markers. FDG-PET detected recurrence in 24 patients, and was superior to the combination of several conventional imaging modalities (CT, MRI, ultrasound, and X-rays), that detected recurrent tumor in 21 patients. FDG-PET findings guided changes in management in these 24 patients. Yap *et al*. (2001) prospectively assessed 50 women with suspected recurrent breast cancer. FDG-PET changed patients staging in 36% and changed management in 58% of patients. Eubank *et al*. (2004) retrospectively assessed 125 patients with suspected recurrent breast cancer who performed FDG-PET and conventional imaging studies. FDG-PET changed the extent of disease in 67% and changed patient management in 32% of patients.

The specificity of FDG imaging improves significantly by using combined molecular-anatomical PET-CT imaging. Fueger *et al*. (2005) presented initial data regarding the specific role of FDG-PET/CT in the assessment of suspected recurrence of breast cancer with an accuracy of 90% for PET/CT as compared with 79% for PET. The specificity of FDG-PET was only partially improved by using PET/CT, and false-positive results were related to the FDG avidity of inflammatory lesions. Tatsumi *et al*. (2006) summarized the initial experience with FDG-PET/CT in 75 breast cancer patients, including 52 with suspected recurrence. FDG-PET/CT increased the diagnostic confidence in 60% of patients showing foci of increased tracer uptake, mainly due to better lesion localization. Radan *et al*. (2006) retrospectively assessed 47 FDG-PET/CT studies in 46 patients with suspected recurrence of breast cancer due to elevated serum tumor markers. FDG PET/CT had a sensitivity of 90%, specificity of 71%, and accuracy of 83%. In a subgroup of 37 patients FDG-PET/CT had higher performance indices than contrast-enhanced CT with a sensitivity of 85% vs. 70%, specificity of 76% vs. 47%, and accuracy of 81% vs. 59%. False-negative findings included small lesions and peritoneal metastases. False-positive findings, due mainly to inflammatory changes, resulted in the reduced specificity of 71%. FDG-PET/CT had an impact on the management of 51% of these patients with chemo- or radiation therapy started in 16 patients showing PET/CT abnormalities in previously unknown disease sites. In addition, treatment was changed in two patients following PET/CT which showed more extensive disease than previously suspected. PET/CT also guided biopsy for tissue sampling in six patients with abnormal studies, with two of them being further referred for surgery. Israel and Kuten (2007) demonstrated improved localization and lesion characterization by FDG-PET/CT in chest wall findings. A change in management of

breast cancer is most frequently noted in patients suspected of loco-regional disease recurrence who are considered for aggressive local therapy (Eubank and Mankoff, 2005; Radan *et al*., 2006) and following FDG PET/CT are diagnosed with disseminated disease (Figure 46.2).

Dedicated breast MRI is gaining a major role in the diagnosis and management of breast cancer. In 40 women with suspected recurrence of breast cancer who were assessed after conservative therapy Belli *et al*. (2002) have demonstrated that MRI identified all 22 cases of recurrence, and additional 2 false-positive cases resulting in sensitivity, specificity, and accuracy values of 100%, 89% and 95%, respectively, and a negative predictive value of 100%. The authors conclude that MRI is valuable for the differentiation of post-therapy changes from recurrent breast cancer and its high negative predictive value may have an impact on the follow up assessment of these patients (Belli *et al*., 2002). Goerres *et al*. (2003) compared MRI of the breast with FDG-PET in 32 patients with suspected local or regional breast cancer recurrence and found comparable sensitivity, specificity, and accuracy for MRI (79%, 94%, and 88%) and FDG-PET (100%, 72%, and 84%). However, PET detected metastases outside the field of view of MRI in five patients. PET/CT has the ability to detect and further characterize distant metastatic disease sites in patients with local disease or a single metastasis (Figure 46.3). Between 16% and 30% of patients with loco-regional recurrence are diagnosed with PET as having distant metastatic disease (Tafra, 2007). Van Oost *et al*. (2004) have shown that 24% of patients develop distant metastases within 18 months of confirmation of disease recurrence. Iagaru *et al*. (2007) compared

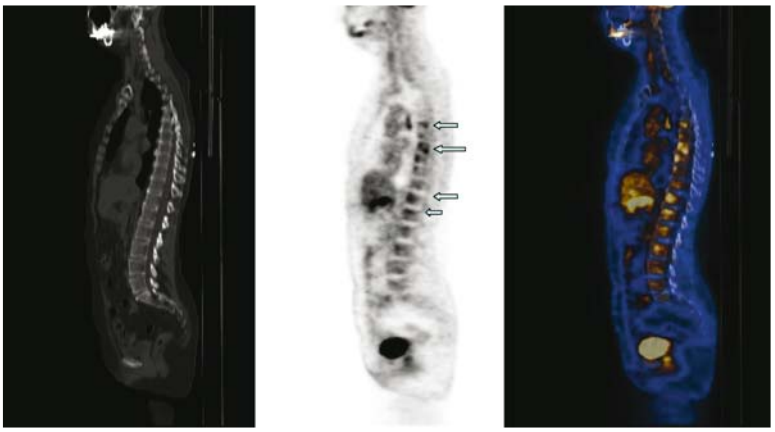

FIGURE 46.2. A 58-year-old patient, 9 years after mastectomy and adjuvant chemo- and radiotherapy for breast cancer, treated with hormonal therapy. Referred for assessment of elevated Ca 15-3 serum levels. PET (middle) and fused PET/CT sagittal images show increased FDG uptake in thoracic and lumbar spine (arrows) with no clear abnormality on the CT image (left). The hormonal therapy was stopped and chemotherapy was started. The patient died 2 years later of progressive metastatic disease

the performance of FDG PET/CT and breast MRI in a mixed patient population, including patients assessed after surgery. While MRI was more sensitive than PET/CT for the detection of local and axillary lymph node disease (86% vs. 75%), FDG imaging had a sensitivity of 100% for the detection of distant metastases.

The skeleton is the most common site for distant metastases in breast cancer. Bone scintigraphy has a high overall sensitivity for the detection of skeletal metastases; however, sensitivity is lower for the detection of purely lytic lesions or foci confined to the bone marrow. FDG-PET/CT plays a complementary role in these patients. Nakai *et al*. (2005) reported overall comparable performance indices of bone scintigraphy and FDG-PET in 55 breast cancer patients with bone metastases. While FDG-PET had a sensitivity of 100% for lytic bone metastases vs. 70% for bone scintigraphy, it was less sensitive, 56%, in sclerotic, osteoblastic, metastases, as compared to a sensitivity of 100% for bone scintigraphy. In addition, FDG-PET/CT provides structural information, regarding the presence of skeletal lesions on CT as well as their metabolic activity on FDG-PET.

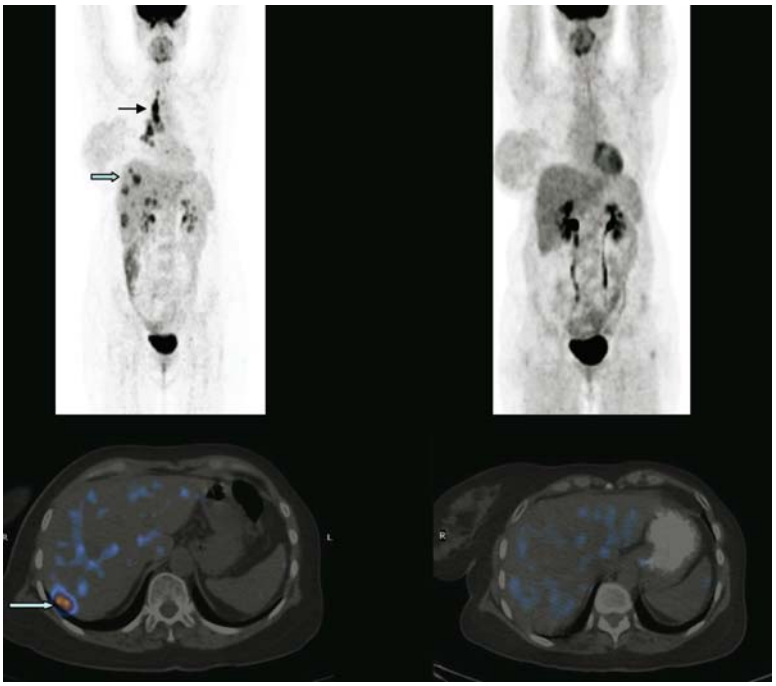

FIGURE 46.3. A 50-year-old patient, 6 years after left mastectomy and adjuvant chemo- and radiotherapy due to breast carcinoma, referred with elevated serum Ca 15.3 and negative CT of the chest, abdomen and pelvis. Maximum-intensity-projection PET (upper left) image and a PET/CT transaxial image of the liver (lower left) show FDG avid disease in the mediastinum (left arrow) and liver (blue arrow).
On follow up study post three cycles of palliative therapy with Docetaxel the maximum-intensity-projection PET (upper right) and a PET/CT transaxial image of the liver (lower right) show excellent response to therapy

Recently, Mion *et al*. (2006) have assessed whether PET/CT may be used as the first diagnostic procedure in the detection of recurrent breast cancer. In a retrospective study of 32 PET/CT studies performed in 22 patients, these preliminary data suggest that whole body PET/CT may prove to be more useful than other imaging modalities in this patient population, many of which may have widespread disease.

In conclusion, present data indicate that while PET/CT appears to be of limited value in the diagnosis of breast cancer, it is superior to conventional imaging modalities and has an important role in the assessment of recurrent breast tumor and in identifying distant disease sites. FDG-PET/CT is superior to PET alone. FDG imaging and PET/CT in particular is of value in asymptomatic patients with suspected recurrence of breast cancer due to elevated serum tumor markers. Although FDG-PET/CT cannot exclude microscopic disease, it provides assessment of the extent of disease in a single imaging session, with impact on patient management decisions and prognosis. Preliminary studies indicate a potential role for FDG-PET/CT as a first diagnostic modality in the initial assessment of patients with suspected recurrent breast cancer.

Acknowledgments. The authors thank Professor Ora Israel for her useful suggestions. This work was undertaken at UCLH/UCL who received a proportion of funding from the Department of Health's NIHR Biomedical Research Centres funding scheme.

REFERENCES

Avril, N., Rose C.A., Schelling, M., Dose, J., Kuhn, W., Bense, S., Weber, W., Ziegler, S., Graeff, H., and Schwaiger, M. 2000. Breast imaging positron emission tomography and flour 18 fluorodeoxyglucose: use and limitations. *J. Clin. Oncol. 18*: 3495–3502.

Bar-Shalom, R., Yefremov, N., Guralnik, L., Gaitini, D., Frenkel, A., Kuten, A., Altman, H., Keidar, Z., and Israel, O. 2003. Clinical performance of PET/CT in evaluation of cancer: additional value for diagnostic imaging and patient management. *J. Nucl. Med. 44*: 1200–1209.

Belli, P., Constantini, M., Romani, M., Marano, P., and Pastore, G. 2002. Magnetic resonance imaging in breast cancer recurrence. *Breast Cancer Res. Treat. 73*: 223–235.

Beyer, T., Townsend, D.W., Brun, T., Kinahan, P.E., Charron, M., Roddy, R., Jerin, J., Young, J., Byars, L. and Nutt, R. 2000. A combined PET-CT scanner for clinical oncology. *J. Nucl. Med. 41*: 1369–1379.

Ell, P.J. 2006. The contribution of PET/CT to improved patient management. *Br. J. Radiol. 79*: 32–36.

Esserman, L. 2005. Integration of imaging in the management of breast cancer. *J. Clin. Oncol. 23*: 1601–1602.

Eubank, W.B., and Mankoff, D.A. 2005. Evolving role of positron emission tomography in breast cancer imaging. *Semin. Nucl. Med. 35*: 84–99.

Eubank, W.B., Mankoff, D.A., Bhattacharya, M., Gralow, J., Linden, H., Ellis, G., Lindsley, S., Austin-Seumour, M., and Livingston, R. 2004. Impact of [F-18]-Fluorodeoxyglucose PET on defining the extent of disease and management of patients with recurrent or metastatic breast cancer. *Am. J. Roentgenol. 183*: 479–486.

Flamen, P., Hoekstra, O.S., Homans, F., Van Custem, E., Maes, A., Stroobants, S., Peeters, M., Penninckx, F., Filez, L., Bleichrodt, R.P., and Mortelmans, L. 2001. Unexplained rising carcinoembryonic antigen (CEA) in the post-operative surveillance of colorectal cancer: the utility of positron emission tomography (PET). *Eur. J. Cancer 37*: 862–869.

Fueger, B.J., Weber, W.A., Quon, A., Crawford, T.L., Allen-Auerbach, M.S., Halpern, B.S.,

Ratio, O., Phelps, M.E., and Czernin, J. 2005. Performance of 2-deoxy-2-[F-18]fluoro-d-glucose positron emission tomography and integrated PET/CT in restaged breast cancer patients. *Mol. Imag. Biol. 7*: 369–376.

Gallowitch, H.J., Kresnik, E., Gasser J., Kumnig, G., Igerc, I., Mikosch, P., and Lind, P. 2003. F-18 fluorodeoxyglucose positron-emission tomography in the diagnosis of tumor recurrence and metastases in the follow-up of patients with breast carcinoma. A comparison to conventional imaging. *Invest. Radiol. 38*: 250–256.

Gilles, R., Guinebretiere, J.M., Lucidarme, O., Cluzel, P. Janaud, G., Finet, J.F., Tardivon, A., Masselot, J., and Vanel, D. 1994. Nonpalpable breast tumors: diagnosis with contrast-enhanced subtraction dynamic MR imaging. *Radiology 191*: 625–631.

Goerres, G.W., Michel, S.C.A., Fehr, M.K., Kaim, A.H., Steinert, H.C., Seifert, B., von Schulthess, G.K., and Kubik-Hoch, R.A. 2003. Follow-up of women with breast cancer: comparison between MRI and FDG PET. *Eur. Radiol. 13*: 1635–1644.

Iagaru, A., Masamed, R., Keesara, H., and Conti, P.S. 2007. Breast MRI and ^{18}F FDG PET/CT in the management of breast cancer. *Ann. Nucl. Med. 21*: 33–38.

Isasi, C.R., Moadel, R.M., and Blaufox, M.D. 2005. A meta-analysis of FDG-PET for the evaluation of breast cancer recurrence and metastases. *Breast Cancer Res. Treat. 90*: 105–112.

Israel, O., and Kuten, A. 2007. Early detection of cancer recurrence: ^{18}F-FDG PET/CT can make a difference in diagnosis and patient care. *J. Nucl. Med. 48*: 28S–35S.

Kolasinska, A.D., Buscombe, J.R., Cwilka, J.B., Holloway, B., Parbhoo, S.P, Davidson, T., and Hilson, A.J. 2001. The role of scintimammography and mammography in recurrent breast cancer. Evaluation of their accuracy using ROC curves. *Nucl. Med. Rev. Cent. East. Eur. 4*: 77–81.

Lonneux, M., Borbath, I., Berliere, M., Kirkore, C., and Panwels, S. 2000. The place of whole body PET FDG for the diagnosis of distant recurrence of breast cancer. *Clin. Positron Imaging 3*: 45–49.

Mion, M., Chierichetti, F., Liessi, G., Bissoli, S., Milan, E., Oniga, F., Sartor, L., Bortolin, M., Vicario, G., Sgarbossa, G., and Manente, P. 2006. Diagnosis of recurrent breast cancer: 18 F-FDG PET/CTA as first choice. *J. Clin. Oncol. 24, June 20 (Suppl.)*: 18 S.

Nakai, T., Okuyama, C., Kubota, T., Yamada, K., Ushijima, Y., Taniike, K., Suzuki, T., and Nishimura, T. 2005. Pitfalls of FDG-PET for the diagnosis of osteoblastic bone metastases in patients with breast cancer. *Eur. J. Nucl. Med. Mol. Imaging 32*: 1253–1258.

Quon, A., and Gambhir, S.S. 2005. FDG-PET and beyond: molecular breast cancer imaging. *J. Clin. Oncol. 23*: 1664–1673.

Radan, L., Ben-Haim, S., Bar-Shalom, R., Guralnik, L., and Israel, O. 2006. The role of FDG-PET/CT in suspected recurrence of breast cancer. *Cancer 107*: 2545–2551.

Reddy, D.H., and Mendelson, E.B. 2005. Incorporating new imaging models in breast cancer management. *Curr. Treat. Options Oncol. 6*: 135–145.

Ries, L.A.G., Melbert, D., Krapacho, M., Mariotto, A., Miller, B.A., Feuer, E.J., Clegg, L., Horner, M.J., Howlader, N., Eisner, M.P., Reichman, M., and Edwards, B.K. (eds). SEER Cancer Statistics Review, 1975–2004, National Cancer Institute. Bethesda, MD, http://seer.cancer.gov/csr/1975,2004/, posted to the SEER web site 2007.

Siggelkow, W., Rath, W., Buell, U., and Zimny, M. 2004. FDG PET and tumour markers in the diagnosis of recurrent and metastatic breast cancer. *Eur. J. Nucl. Med. Mol. Imaging 31 (Suppl. 1)*: S118–S124.

Suárez, M., Pérez-Castejón, M.J., Jiménez, A., Romper, M., Ruiz, G., Montz, R., and Carreras, J.L. 2002. Early diagnosis of recurrent breast cancer with FDG-PET in patients with progressive elevation of serum tumor markers. *Q. J. Nucl. Med. 46*: 113–121.

Tafra, L. 2007. Positron emission tomography (PET) and mammography (PEM) for breast cancer: importance to surgeons. *Ann. Surg. Oncol. 14*: 3–13.

Tatsumi, M., Cohade, C., Mourtzikos, K.A., Fishman, E.K., and Wahl, R.L. 2006. Initial experience with FDG-PET/CT in the evaluation of breast cancer. *Eur. J. Nucl. Med. Mol. Imaging 33*: 254–262.

Van Oost, F.J., van der Hoeven, J.J., Hoekstra, O.S., Voogt, A.C., Coebergh, J.W.W., and van de Poll-Franse, L.V. 2004. Staging in patients with locoregionally recurrent breast cancer: current

practice and prospects for positron emission tomography. *Eur. J. Cancer 40*: 1545–1553.

Voogt, A.C, Nielsen, M., Peterse, J.L., Blichert-Toft, M., Bartelink, H., Overgaards, M., van Tienhoven, G., Andersen, K.W., Sylvester, R.J., van Dongen, J.A., and Danish Breast Cancer Cooperative Group. 2001. Breast cancer cooperative group of the European Organization for Research and Treatment of Cancer. Differences in risk factors for local and distant recurrence after breast-conserving therapy or mastectomy for stage I and II breast cancer: pooled results of two large European randomized trials. *J. Clin. Oncol. 19*: 1688–1697.

Wahl, R.L., Siegel, B.A., Coleman, R., and Gatsonis, C.G. 2004. A prospective multicentre study of axillary nodal staging by positron emission tomography in breast cancer: a report of the staging breast cancer with PET study group. *J. Clin. Oncol. 22*: 277–285.

Weir, L., Worsley, D., and Bernstein, V. 2005. The value of FDG positron emission tomography in the management of patients with breast cancer. *Breast J. 11*: 204–209.

Wu, D., and Gambhir, S.S. 2003. Positron emission tomography in diagnosis and management of invasive breast cancer: current status and future perspectives. *Clin. Breast Cancer 4 (Suppl. 1)*: S55–S63.

Yap, C.S., Seltzer M.A., Schieppers, C., Gambhir, S.S., Rao, J., Phelps, M.E., Valk, P.E., and Czernin, J. 2001. Impact of whole-body ^{18}FDG PET on staging and managing patients with breast cancer: the referring physician's perspective. *J. Nucl. Med. 42*: 1334–1337.

Zangheri, B., Messa, C., Picchio, M., Gianolli, L., Landoni, C., and Fazio, F. 2004. PET/CT and breast cancer. *Eur. J. Nucl. Med. Mol. Imaging 31 (Suppl. 1)*: S135–S142.

47
Role of Sentinel Lymph Node Biopsy in Ductal Carcinoma *In Situ*: Diagnosis and Methodology

Amit Goyal

INTRODUCTION

During the past decade, sentinel lymph node (SLN) biopsy has become well established as a new standard for axillary node staging in early stage invasive breast cancer. Since the initial reports of SLN mapping by Krag *et al*. (1993) (using radioisotope), Giuliano *et al*. (1994) (using blue dye), and Albertini *et al*. (1996) (using a combination of isotope and dye), four randomized trials (Veronesi *et al*., 2003; Mansel *et al*., 2006; Purushotham *et al*., 2005; Krag *et al*., 2007) and 69 observational studies (Kim *et al*., 2006) of SLN biopsy validated by a completion axillary dissection (ALND) have established that SLN biopsy is feasible, accurate and safe.

Ductal carcinoma in situ (DCIS) currently accounts for ~ 20% of all screen detected breast cancers (UK NHSBSP 2005–2006). By definition, DCIS is not an invasive malignancy and does not have the ability to metastasize to regional lymph nodes. Axillary dissection is contraindicated in DCIS because of the low (1%) incidence of lymph node metastasis using conventional hematoxylin and eosin (H&E) staining (Silverstein *et al*., 1994; Yiangou *et al*., 1999). The 5 year relative survival for DCIS is higher than 100%. Moreover, the lower limits of the 95% confidence intervals for the 5 year relative survival of women with DCIS are > 100%. This indicates that their chance of survival is no worse than that of the general female population as a whole (UK NHSBSP 2005–2006). Given this background, SLN biopsy in all patients with DCIS cannot be justified. As we have previously argued (Goyal *et al*., 2006), the justification for SLN biopsy in DCIS is diagnostic uncertainty. Core needle biopsy has an inherent sampling error leading to histologic underestimation of invasive disease. United States based series have a lower underestimation rate because of higher open biopsy rates compared with European series. UK 2005/06 NHS Breast Screening Programme audit data combined with that of many others supports an emerging consensus that in ~ 20% of patients with a B5a (non-invasive) non-operative diagnosis, invasive disease is found at surgery (UK NHSBSP 2005–2006).

SLN biopsy following lumpectomy is associated with increased failed localisation and false-negative rate (Julian *et al*., 2004) and is impossible after a mastectomy. Therefore, if SLN biopsy is not performed at the time of the definitive operative procedure, a significant number of patients found to have an invasive cancer, will require a second operative procedure and, in all likelihood, axillary lymph node dissection. The majority of these patients will be found to have a negative axilla, yet miss out on the advantages of SLN biopsy if not performed initially. Therefore, the dilemma about SLN biopsy in patients initially diagnosed with DCIS resides in determining the predictors of invasive disease in these patients.

PATIENT SELECTION

Historically, risk factors reportedly associated with invasive disease have included large tumors, high-grade tumors, tumors with comedo-type necrosis, and presence of palpable mass or mass that is appreciated by imaging studies (Table 47.1). It is difficult to compare different series as the biopsy techniques, grading system for DCIS and patient populations have varied. Furthermore in most series, numbers are

TABLE 47.1. Predictors of invasive breast cancer on final pathology in patients with a pre-operative diagnosis of DCIS.

Author	n	No. with DCIS upstaged	Predictors of invasive breast cancer
Goyal *et al*. (2006)	587	220(38%)	Clinically palpable mass Mammographic mass
Wilkie *et al*. (2005)	675	66(10%)	High grade DCIS Mammographic mass Microinvasion
Mittendorf *et al*. (2005)	85	7(20%)	Diagnosis by core-needle biopsy
Yen *et al*. (2005)	398	80(20%)	Age ≤ 55 years Diagnosis by core-needle biopsy Mammographic DCIS size ≥ 4 cm High grade DCIS
Hoorntje *et al*. (2003)	255	41(16%)	High grade DCIS Periductal inflammation in core biopsies
Renshaw (2002)	91	17(19%)	Comedo DCIS with cribriform/papillary pattern DCIS > 4 mm with lobular extension
Cox *et al*. (2001)	240	30(13%)	None
Jackman *et al*. (2001)	1,326	183(14%)	Diagnosis by core-needle biopsy Mammographic mass ≤ 10 specimens per lesion
Lee *et al*. (2000)	59	17(29%)	None

not large enough to provide the power to detect significance. Our own data show that presence of a breast mass (clinical, mammographic) is the only independent predictor of finding an invasive component at excision (Goyal *et al*., 2006). We recommend that SLN biopsy should be performed at the time of the initial procedure in this selective group of patients to avoid a second operative procedure for axillary nodal staging. In addition, SLN biopsy should be performed in patients undergoing mastectomy and/or reconstruction because both preclude SLN biopsy if invasive disease is subsequently discovered.

METHODOLOGY – SENTINEL LYMPH NODE BIOPSY

Our data combined with that of many others show that a combined technique maximizes both the success and accuracy of SLN biopsy (Cody *et al*., 2001; Goyal *et al*., 2006; Lyman *et al*., 2005). Data from MSKCC also show that isotope identifies the SLN more often than blue dye, and that with increasing experience the "marginal benefit" of blue dye (the proportion of procedures in which the SLN is identified *by dye alone*) is quite small, ~ 2–3% (Derossis *et al*., 2001).

CHOICE OF RADIOISOTOPE

All lymphatic mapping protocols rely on ^{99m}technetium complexed to a carrier molecule, and radiocolloid uptake in lymph nodes represents active phagocytosis of the particulate material by macrophages. Radiocolloid preparations with a large particle size tend to remain at the injection site with little or no migration to regional nodes; conversely, those with a small particle size rapidly migrate to SLN and beyond, causing diffuse nodal uptake and requiring the removal of excessive additional nodes. The goal is to use an isotope preparation with an optimal and consistent particle size which will identify 1–3 SLN in most patients.

Three preparations are in widespread use and all appear to work well. ^{99m}Tc-sulfur colloid, used in the United States and Canada, is characterized by a wide variation in particle size (50–2,000 nm, median 300 nm), and therefore some day-to-day variation in performance; filtering of the radiocolloid to remove those particles > 200 nm (0.2 μ) allows more rapid visualization of the afferent lymphatic vessels and of the SLN. ^{99m}Tc-albumin nanocolloid, used by us in the UK and in most European centers, appears to have a more consistent particle size (50–200 nm, median 40 nm) and less day-to-day variation in performance. ^{99m}Tc-antimony trisul fide is widely used for sentinel lymph node procedures in Australia and may have the most consistent particle size of all (3–30 nm, median 20).

DOSE OF RADIOISOTOPE

The literature reports good results for SLN biopsy with radioisotope doses ranging from 0.1 to 10.0 mCi (3.7–370 MBq). A typical dose for most centers is ~ 1.0 mCi, and there are few comparative data to suggest than any one dosage is optimal. Our standard radioisotope dosage for SLN biopsy is 0.3–0.5 mCi. Licensure for the acquisition and use of radioactive material varies in different countries, as do the protocols for

handling and disposal. It is worth noting that the dose of radioisotope used in SLN biopsy is trivially small (by comparison, a conventional bone scan requires about 25 mCi (925 MBq)), and therefore does not require any special precautions.

SITE AND VOLUME OF INJECTION

In the early reports of SLN biopsy for breast cancer, isotope was given as a deep peritumoral injection into the breast parenchyma at four sites, requiring a total volume of 4–8 cc. Since then, many investigators have reported equally good results with the intradermal, subdermal or subareolar routes of injection, and a comparably low rate of false-negative procedures (Klimberg *et al.*, 1999; Povoski *et al.*, 2006; McMasters *et al.*, 2001). It appears from these studies that most of the breast tissue and its overlying skin drains to the same few axillary SLN regardless of injection site. Intradermal and subdermal injections are given as a single dose into the breast skin directly over the tumor or just cephalad to a previous biopsy scar. Subareolar injections are given into the subareolar lymphatic plexus independently of the tumor site. Superficial injection techniques require a much lower-volume of injection than the peritumoral method, and leave a much smaller "hot spot" in the breast. This facilitates the identification of SLN, especially for patients with tumors high in the axillary tail of the breast.

Our standard method is to inject the isotope intradermally at a single periareolar site in the index quadrant (or cephalad to the previous biopsy scar), in a volume of 0.05 cc of normal saline. Blue dye injection is always given in the operating room just prior to surgery. We inject the blue dye subdermally at a single periareolar site in the index quadrant.

TIMING OF INJECTION

Radioisotope may be injected the morning of, or the day before, surgery (as noted above, blue dye is always injected in the OR). Same-day injection and day-before mapping have equivalent results (McCarter *et al.*, 2001). Because ^{99m}Tc has a half life of 6 h, we allow for decay by giving a larger isotope dose for day-before than for same-day mapping (0.5 mCi versus 0.3 mCi), and with this protocol have observed comparable isotope counts and number of SLN for each method. Day-before injection has the advantage of allowing patients to proceed directly to surgery on the following day, thereby minimizing operating room delays.

LYMPHOSCINTIGRAPHY

Post-injection lymphoscintigraphy (LSG) with a large field-of-view gamma camera is a routine part of SLN mapping at many centers. The advantages of LSG are: (1) the identification of non-axillary SLN and (2) estimating the number of axillary SLN (the surgeon should identify at least that many SLN at operation). The disadvantages are: (1) added cost, and (2) added time. Because most patients with a negative LSG will still have "hot" SLN identified intraoperatively by the hand-held gamma probe (a much more sensitive instrument than the gamma camera)

(Goyal *et al*., 2005b), many centers have abandoned routine LSG.

OPERATIVE TECHNIQUE

We inject 1–5 cc of Patent Blue V dye subdermally, as noted above, at the start of the procedure. We always perform the SLN biopsy prior to the breast operation, using the hand-held gamma probe to identify the hot spot in the axilla, marking the site on the skin, and making a small transverse axillary skin line incision appropriately placed to allow an axillary lymph node dissection if needed.

The incision is deepened through the clavipectoral fascia, maintaining two- to three-point countertraction with retractors, and taking care to preserve any blue-stained lymphatics and the intercostal sensory nerves (T2-4). Using direct inspection and the hand-held gamma probe, all blue and/or "hot" sentinel nodes are carefully identified and removed. In ~ 80% of cases the SLN are found predictably in the mid-portion of level I; the remaining 20% are found variably in the axillary tail of the breast, high up along the axillary vein, in levels II–III, or in the interpectoral (Rotter's) space. The goal is to remove a median of 1–2 SLN per patient.

While a blue node is defined as any node which is visibly blue (or contiguous with a blue lymphatic vessel), there is no universal definition of a "hot" SLN. The most popular are the "10% rule" (which defines a SLN as any node with isotope counts at least 10% those of the hottest SLN) (McMasters *et al*., 2000), and those which employ various threshold ratios of SLN counts to axillary background counts (4-1, 5-1, 10-1). None are perfect but all work well. Some patients will have isotope uptake by many nodes presenting as a "diffusely hot" axilla. In these cases, it is particularly important to remove the *hottest SLN* (this node is positive in 80% of node-positive patients) (Martin *et al*., 2001b), but it is rarely necessary to remove *> 4 SLN* (we have shown that 98.5% of positive SLN are identified within the first 3, and 99.6% within the first 4 SLN removed) (Goyal *et al*., 2005a).

The final step in SLN biopsy (after removal of all blue and/or hot SLN) is careful palpation of the axilla, and the removal of any *palpably suspicious* nodes. Axillary palpation reduces the false-negative rate for SLN biopsy (Martin *et al*., 2001a). This concept makes sense intuitively; nodes replaced by tumor may be dysfunctional and cannot be expected to take up dye or isotope normally.

REFERENCES

UK NHS Breast Screening Programme Statistics 2005–2006.http://www.cancerscreening.nhs.uk/breastscreen/statistics.html.

Albertini, J.J., Lyman, G.H., Cox, C., Yeatman, T., Balducci, L., Ku, N., Shivers, S., Berman, C., Wells, K., Rapaport, D., Shons, A., Horton, J., Greenberg, H., Nicosia, S., Clark, R., Cantor, A., and Reintgen, D.S. 1996. Lymphatic mapping and sentinel node biopsy in the patient with breast cancer. *JAMA 276*: 1818–1822.

Cody, H.S., III, Fey, J., Akhurst, T., Fazzari, M., Mazumdar, M., Yeung, H., Yeh, S.D., and Borgen, P.I. 2001. Complementarity of blue dye and isotope in sentinel node localization for breast cancer: univariate and multivariate analysis of 966 procedures. *Ann. Surg. Oncol. 8*: 13–19.

Derossis, A. M., Fey, J., Yeung, H., Yeh, S. D. J., Heerdt, A. S., Petrek, J., VanZee, K. J., Montgomery, L. L., Borgen, P. I, and Cody, H. S. 2001. A trend analysis of the relative value of blue dye and isotope localization in 2000 consecutive cases of sentinel node biopsy for breast cancer. *J. Am. Coll. Surg. 193*: 473–478.

Giuliano, A.E., Kirgan, D.M., Guenther, J.M., and Morton, D.L. 1994. Lymphatic mapping and sentinel lymphadenectomy for breast cancer. *Ann. Surg. 220*: 391–398.

Goyal, A., Newcombe, R.G., and Mansel, R.E. 2005a. Clinical relevance of multiple sentinel nodes in patients with breast cancer. *Br. J. Surg. 92*: 438–442.

Goyal, A., Newcombe, R.G., Mansel, R.E., Chetty, U., Ell, P., Fallowfield, L., Kissin, M., and Sibbering, M. 2005b. Role of routine preoperative lymphoscintigraphy in sentinel node biopsy for breast cancer. *Eur. J. Cancer 41*: 238–243.

Goyal, A., Douglas-Jones, A., Monypenny, I., Sweetland, H., Stevens, G., and Mansel, R.E. 2006. Is there a role of sentinel lymph node biopsy in ductal carcinoma in situ?: analysis of 587 cases. *Breast Cancer Res. Treat. 98*: 311–314.

Julian, T.B., Krag, D.N., Brown, A., Anderson, S., Harlow, S., Ashikaga, T., Weaver, D.L., and Miller, M.B. 2004. Preliminary technical results of NSABP B-32, a randomized phase III clinical trial to compare sentinel node resection to conventional axillary dissection in clinically node-negative breast cancer patients. *Breast Cancer Res. Treat. 11*: 208S–210S.

Kim, T., Giuliano, A.E., and Lyman, G.H. 2006. Lymphatic mapping and sentinel lymph node biopsy in early-stage breast carcinoma. *Cancer 106*: 4–16.

Klimberg, V.S., Rubio, I.T., Henry, R., Cowan, C., Colvert, M., and Korourian, S. 1999. Subareolar versus peritumoral injection for location of the sentinel lymph node. *Ann. Surg. 229*: 860–864.

Krag, D.N., Weaver, D.L., Alex, J.C., and Fairbank, J.T. 1993. Surgical resection and radiolocalization of the sentinel lymph node in breast cancer using a gamma probe. *Surg. Oncol. 2*: 335–339.

Krag, D.N., Anderson, S.J., Julian, T.B., Brown, A.M., Harlow, S.P., Ashikaga, T., Weaver, D.L., Miller, B.J., Jalovec, L.M., Frazier, T.G., Noyes, R.D., Robidoux, A., Scarth, H.M., Mammolito, D.M., McCready, D.R., Mamounas, E.P., Costantino, J.P., and Wolmark, N. 2007. Technical outcomes of sentinel-lymph-node resection and conventional axillary-lymph-node dissection in patients with clinically node-negative breast cancer: results from the NSABP B-32 randomised phase III trial. *Lancet Oncol*. 8: 881–888.

Lyman, G.H., Giuliano, A.E., Somerfield, M.R., Benson, A.B., III, Bodurka, D.C., Burstein, H.J., Cochran, A.J., Cody, H.S., III, Edge, S.B., Galper, S., Hayman, J.A., Kim, T.Y., Perkins, C.L., Podoloff, D.A., Sivasubramaniam, V.H., Turner, R.R., Wahl, R., Weaver, D.L., Wolff, A.C., and Winer, E.P. 2005. American Society of Clinical Oncology guideline recommendations for sentinel lymph node biopsy in early-stage breast cancer. *J. Clin. Oncol. 23*: 7703–7720.

Mansel, R.E., Fallowfield, L., Kissin, M., Goyal, A., Newcombe, R.G., Dixon, J.M., Yiangou, C., Horgan, K., Bundred, N., Monypenny, I., England, D., Sibbering, M., Abdullah, T.I., Barr, L., Chetty, U., Sinnett, D.H., Fleissig, A., Clarke, D., and Ell, P.J. 2006. Randomized multicenter trial of sentinel node biopsy versus standard axillary treatment in operable breast cancer: the ALMANAC Trial. *JNCI Cancer Spectrum 98*: 599–609.

Martin, R.C.G., Derossis, A., Fey, J., Yeung, H., Yeh, S.D.J., Akhurst, T., Heerdt, A.S., Petrek, J., VanZee, K.J., Montgomery, L.L., Borgen, P.I., and Cody, H.S. 2001a. Intradermal isotope injection is superior to intramammary in sentinel node biopsy for breast cancer. *Surgery 130*: 432–438.

Martin, R.C.G., Fey, J., Yeung, H., Borgen, P.I., and Cody, H.S. 2001b. Highest isotope count does not predict sentinel node positivity in all breast cancer patients. *Ann. Surg. Oncol. 8*: 592–597.

McCarter, M.D., Yeung, H., Yeh, S.D.J., Fey, J., Borgen, P.I., and Cody, H.S. 2001. Localization of the sentinel node in breast cancer: identical results with same-day and day-before isotope injection. *Ann. Surg. Oncol. 8*: 682–686.

McMasters, K.M., Tuttle, T.M., Carlson, D.J., Brown, C.M., Noyes, R.D., Glaser, R.L., Vennekotter, D.J., Turk, P.S., Tate, P.S., Sardi, A., Cerrito, P.B., and Edwards, M.J. 2000. Sentinel lymph node biopsy for breast cancer: a suitable alternative to routine axillary dissection in multi-institutional practice when optimal technique is used. *J. Clin. Oncol. 18*: 2560–2566.

McMasters, K.M., Wong, S.L., Martin, R.C., Chao, C., Tuttle, T.M., Noyes, R.D., Carlson, D.J., Laidley, A.L., McGlothin, T.Q., Ley, P.B., Brown, C.M., Glaser, R.L., Pennington, R.E., Turk, P.S., Simpson, D., Cerrito, P.B., and Edwards, M.J. 2001. Dermal injection of radioactive colloid is superior to peritumoral injection

for breast cancer sentinel lymph node biopsy: results of a multiinstitutional study. *Ann. Surg. 233*: 676–687.

Povoski, S.P., Olsen, J.O., Young, D.C., Clarke, J., Burak, W.E., Walker, M.J., Carson, W.E., Yee, L.D., Agnese, D.M., Pozderac, R.V., Hall, N.C., and Farrar, W.B. 2006. Prospective randomized clinical trial comparing intradermal, intraparenchymal, and subareolar injection routes for sentinel lymph node mapping and biopsy in breast cancer. *Ann. Surg. Oncol. 13*: 1412–1421.

Purushotham, A.D., Upponi, S., Klevesath, M.B., Bobrow, L., Millar, K., Myles, J.P., and Duffy, S.W. 2005. Morbidity after sentinel lymph node biopsy in primary breast cancer: results from a randomized controlled trial. *J. Clin. Oncol. 23*: 4312–4321.

Silverstein, M.J., Gierson, E.D., Waisman, J.R., Senofsky, G.M., Colburn, W.J., and Gamagami, P. 1994. Axillary lymph node dissection for T1a breast carcinoma. Is it indicated? *Cancer 73*: 664–667.

Veronesi, U., Paganelli, G., Viale, G., Luini, A., Zurrida, S., Galimberti, V., Intra, M., Veronesi, P., Robertson, C., Maisonneuve, P., Renne, G., De Cicco, C., De Lucia, F., and Gennari, R. 2003. A randomized comparison of sentinel-node biopsy with routine axillary dissection in breast cancer. *New Eng. J. Med. 349*: 546–553.

Yiangou, C., Shousha, S., and Sinnett, H.D. 1999. Primary tumour characteristics and axillary lymph node status in breast cancer. *Br. J. Cancer 80*: 1974–1978.

48
Breast Conservation Treatment of Early Stage Breast Carcinoma: Risk of Cardiac Mortality

Eleanor E.R. Harris

INTRODUCTION

Breast conservation treatment for early stage breast cancer is defined as the surgical removal of gross or radiographically detected disease with a partial mastectomy (or lumpectomy), usually followed by radiation therapy to the breast. This treatment approach has been demonstrated to be equivalent to mastectomy in a large number of randomized trials (Clarke *et al*., 2005). Breast conservation treatment has become the standard of care and preferred treatment for the majority of breast cancer patients with stage 0, I, and II breast cancer. The earliest trials validating the use of breast conservation treatment date to the 1960s, and numerous randomized trials comparing mastectomy to breast conserving surgery, with or without radiation, have been conducted through the contemporary era. The majority of these trials demonstrated a significant improvement in local control with the use of whole breast irradiation after breast conserving surgery, and a survival benefit associated with the addition of radiation on the order of 5%.

These trials also raised concerns about the risks of whole breast or chest wall irradiation. A number of earlier studies did not show an improvement in overall survival despite large reductions in local recurrence rates. The decrease in breast cancer deaths associated with post-operative irradiation was offset by an increase in cardiovascular deaths in some studies. In particular, radiation techniques that incorporated large volumes of the heart in order to encompass the chest wall, breast and nodal targets appeared to be detrimental to overall survival outcomes (Krueger *et al*., 2004). Refinements in radiation techniques were developed with the goal of reducing the radiation exposure of the intrathoracic structures, especially the heart and lungs. Subsequent studies have examined the impact of evolving radiation technique on the risk of cardiac morbidity and mortality. Cardiac toxicity of breast irradiation is a late event, only manifesting clinically 10 or more years after breast cancer treatment. Data regarding the interaction of left breast irradiation and cardiotoxic systemic therapies, such as doxorubicin and trastuzumab, are

limited, as these agents have only been in use more recently and long term outcome data are lacking.

Radiation therapy planning techniques utilize anatomical landmarks and target volumes to delineate the organs at risk and localize the borders of the irradiation fields. In breast cancer treatment, the tissues targeted for full-dose irradiation include the whole breast and underlying chest wall. Whole breast irradiation has traditionally been used in order to eradicate microscopic deposits of cancer cells that may be distributed throughout the breast parenchyma. Whole breast irradiation reduces the risk of in-breast recurrence by approximately two-thirds compared to partial mastectomy alone. The most commonly used current technique to treat the whole breast is called a tangent beam (Figure 48.1). This field arrangement is designed to minimize exposure of the intrathoracic structures, the heart and lung in particular, to radiation. However, due to the curvature of the breast and chest wall, some volume of lung and, in left-sided breast cancers the heart, is included in the radiation beam.

The goal of radiation treatment in breast conservation therapy is to eradicate areas of residual microscopic disease in order to reduce local and regional recurrence and potentially improve overall survival, while minimizing the risk of acute and late treatment-related toxicities. The focus of contemporary studies is to modify the technical delivery of left breast radiation to minimize or eliminate cardiac toxicity, and to better understand the risk of radiation-induced cardiac sequelae for the benefit of the many breast cancer survivors who may be at risk for cardiac toxicity.

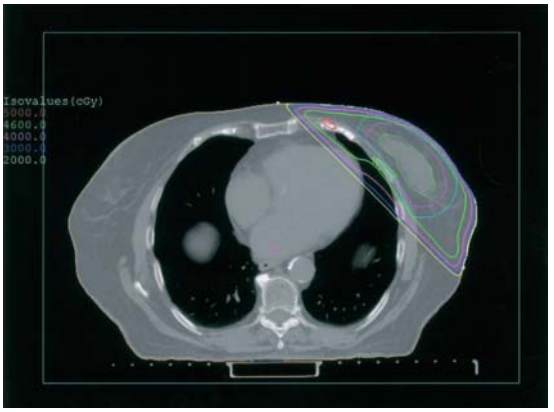

FIGURE 48.1 shows an axial slice from a computed tomography (CT) simulation treatment plan for a left sided breast cancer being treated with tangential fields. The isovalues on the left hand side correspond to the thicker colored isodose lines that overlay the breast volume. A typical prescription dose to the whole breast is 4,600–5,000 cGy. The thinner colored lines demonstrate the target volumes including the tumor bed (thin green line), clinical target volume (CTV; thin red line) and planning target volume (PTV; thin blue line). This isodose distribution demonstrates a relatively homogeneous plan with good coverage of the PTV, the volume to which the dose is typically evaluated. Also demonstrated is a segment of heart that is included in the radiation fields. Despite the use of tangent beam arrangements, in left sided patients it is usually not possible to completely exclude the heart from the treatment field, due to the curvature of the chest wall and the need to cover the breast tissue both anterior and lateral to the heart in the chest cavity.

CARDIAC MORTALITY

Many early studies of breast cancer treatment, either post-mastectomy or post-lumpectomy, randomized women to no further local treatment versus chest wall or breast irradiation. A meta-analysis by the Early Breast Cancer Trialists' Collaborative Group including 78 randomized trials of breast or chest wall irradiation

after surgery included over 23,000 women randomized to receive radiation or not. (Clarke *et al*., 2005) This meta-analysis examined the risk of death from breast cancer and non-breast cancer causes. In this analysis, there was an excess of non-breast cancer deaths among women who received radiation, mainly due to heart disease and lung cancer and mainly in older trials. The excess mortality was seen only 5 years or more after treatment, and was not age dependent. The studies included were conducted between 1961 and 1995, and the older trials used outdated radiation techniques that exposed more volume of heart and lung to larger doses of irradiation than current standard tangential beams (Krueger *et al*., 2004) accounting for the excess cardiovascular mortality.

Data from randomized trials of surgery alone versus surgery with breast or chest wall irradiation provide comparative survival outcomes and mortality risks attributable to irradiation. A number of these trials have reported increased rates of cardiac death in irradiated groups. These studies are primarily in the post-mastectomy setting, or include both breast conservation and mastectomy patients, but they provide valuable information on the long term cardiac risks of irradiation, which is applicable to the breast conservation setting. The earliest randomized trials of post-mastectomy radiation are mainly of historical interest. These studies were the first to highlight the risk of cardiac sequelae of chest wall irradiation for breast cancer, which is largely attributable to the large volume of heart irradiated in these older studies. One of the oldest studies was conducted by the Christie Hospital from 1949 to 1955, in which 1461 patients were randomized after radical mastectomy to post-operative radiation or observation. Jones and Riberio (1990) reported long-term follow up over 34 years and found that at 15 years there was no difference in survival between the two groups. After 15 years, however, there was a significant increase in mortality in the irradiated group ($p = 0.003$). The excess mortality in all irradiated patients compared to those observed was attributable to deaths from cardiovascular disease, although no differences were seen between right and left sided irradiated patients. The Oslo study reported by Host *et al*. (1986) was conducted from 1964 to 1972, and randomized women with operable breast cancer after radical mastectomy to receive radiation or no further treatment. In the early years of the study, radiation was delivered using 200 Kv X-rays, and then using cobalt-60 in the later years. A large volume including the chest wall, axilla, and internal mammary nodes was targeted. Interestingly, this study showed a survival benefit for stage II, but not stage I, patients compared to unirradiated controls ($p = 0.15$). In addition, with a minimum follow-up of 11 years, the study noted a significant increase in deaths from acute myocardial infarction (MI) among the irradiated group ($p = 0.004$), and seven out of these ten deaths occurred in left sided patients. Haybittle *et al*. (1989) reported follow-up on non-breast cancer causes of death from the Cancer Research Campaign early breast cancer trial conducted from 1970 to 1975, which randomized women with clinical stage I and II breast cancer post-mastectomy to radiation or no further treatment. Some women in the observation arm received radiation at the time of local

or regional recurrence. At 18 years follow-up, there was a slight excess overall mortality in the irradiated group, with a relative risk (RR) of 1.04, while the risk of death from breast cancer had a RR of 0.97. This was offset by an increased RR of 1.37 of death from other causes, and this increase was mainly seen in left sided patients (RR = 1.61) and those treated with orthovoltage (RR =1.85). The relative risk of death from cardiac causes in these groups more than 5 years after treatment was 2.67. Cuzick *et al*. (1994) took available information from eight randomized trials of surgery alone versus surgery and radiation conducted prior to 1975, and reported 10 year mortality outcomes. An excess of cardiac deaths was seen in trials from both early and later in the study period ($p < 0.001$), but was offset by a reduction in the rate of breast cancer deaths in irradiated groups. Gyenes *et al*. (1998) reported long-term outcomes for the Stockholm Southern Hospital randomized trial of 960 women with operable breast cancer. Those allocated to surgery alone were compared to either pre-operative or post-operative radiation between 1971 and 1976. There was no significant difference in the incidence of MI among the three groups. The investigators calculated cardiac dose-volumes in irradiated patients and divided the patients into three dose-volume groups. They found that patients in the highest dose-volume group had a hazard for MI of 1.3 compared to surgical controls, and a hazard for death from ischemic heart disease of 2.5.

Still other randomized and institutional series have found no impact of left sided irradiation on cardiac mortality. These studies generally represent a more contemporary cohort, with the use of megavoltage tangential treatment technique predominating, although they largely still precede the use of 3D treatment planning. Hojris *et al*. (1999) reported on mortality from ischemic heart disease among patients who participated in the Danish Breast Cancer Cooperative Group (DBCG) 82b and 82c randomized trials for high risk post-mastectomy patients. High risk was defined as one or more positive axillary lymph nodes, tumor size greater than 5 cm or skin or pectoral fascia invasion. Between 1982 and 1990, 3,083 women were randomized after mastectomy and chemotherapy to receive chest wall and regional nodal irradiation versus no further treatment. The nodal regions, which lie well superior to the heart, were treated with anterior photon fields. The chest wall was treated with two anterior electron fields, including the internal mammary nodes, and used custom blocks to shield the heart and lung caudal to the first rib. All diagnoses of morbidities and mortality from ischemic heart disease were recorded in follow-up, excluding 37 women with pre-existing ischemic heart disease. At a median follow-up of 10 years, overall survival was significantly better in the radiotherapy group (46% vs. 36%, $p < 0.0001$), while similar proportions in each group died of ischemic heart disease (0.8% all radiotherapy patients; 0.9% no radiotherapy patients; 0.7% left sided radiotherapy patients; 0.9% right sided radiotherapy patients). Similar rates were seen for death from acute myocardial infarction. When patients were censored at the time of local or distant cancer recurrence, deaths from ischemic heart disease appeared to be associated with a higher relative hazard in left sided radiotherapy patients, but the overall rates were quite low (relative hazards:

left sided radiotherapy = 0.7%, left sided no radiotherapy = 0.3%, relative hazard 2.18).

Rutqvist *et al.* (1998) of the Karolinska Hospital in Stockholm included 684 women with early stage breast cancer treated between 1976 and 1987 with breast conservation therapy using tangential fields. This study compared a contemporaneous group of 4,996 post-mastectomy patients who did not undergo irradiation. At median follow-up of 9 years, 0.7% of the irradiated group had died of MI, with a relative hazard for acute MI in the irradiated group versus the mastectomy group of 0.6. In addition, the incidence of MI was similar in the right and left-sided patients. While finding no evidence of increased MI, the authors did not rule out a possible effect in left-sided subgroups with an anteriorly placed heart. Nixon *et al.* (1998) from the Joint Center for Radiation Therapy, reported on 745 stage I and II patients with breast conservation therapy using contemporary tangential technique and with at least 12 years of follow-up, comparing right and left sided patients. The authors found an equivalent incidence of death from non-breast cancer causes in both groups (11%), including 2% in each group who died from cardiac causes. In addition, they did not find an increase in cardiac death over time.

Vallis *et al.* (2002) performed a retrospective cohort linkage study of breast cancer patients treated with post-lumpectomy radiation at Princess Margaret Hospital in Toronto between 1982 and 1988. Outcomes were obtained from two province-wide health databases for hospitalizations and mortality. In addition to comparing right to left-sided patients, the incidence of MI was also compared to the general population. The 2,128 patients in the study cohort had a median follow-up of 10 years. There were equal numbers of fatal MI in the right and left-sided patients ($p = 0.66$), and there was no difference in the incidence of MI between the irradiated study cohort and the general population. Harris *et al.* (2006) reported long term outcomes for 961 stage I and II breast conservation patients treated with contemporary tangential technique at the University of Pennsylvania between 1977 and 1994. The median follow-up was 12 years. This study found no significant difference in overall mortality from any cardiac cause between right and left-sided patients up to 20 years (2% vs. 3.5%). However, in a cumulative risk analysis that looked at the risk of cardiac mortality over time, an increase in cardiac deaths among left-sided patients compared to right was noted in the second decade after treatment. Examination of different treatment eras over the study period showed no effect of era of treatment on risk of cardiac death, likely reflecting a relatively uniform contemporary treatment technique over time in this single institution series.

Several studies of cardiac effects of irradiation in breast cancer patients using datasets from either the National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER) or other cancer registry databases have been conducted. These cancer registries have the advantage of large numbers of patients and long term follow-up, allowing for the potential identification of statistical trends that may be masked in smaller cohorts. However, the number of data points available for study is limited, particularly with respect to radiation dose and technique. Radiation use is only reported up to 4 months after diagnosis and has been underreported

in the SEER database when compared with Medicare records (Hankey *et al*., 1999). Paszat, *et al*. (1998) first used the SEER database to study the registry of 206,523 women with locoregional breast cancer diagnosed from 1973 to 1992, to compare the time to fatal MI in right and left-sided patients with or without radiation among different age groups (less 60 years or 60 years or more) and treatment eras (1973–1982 and 1983–1992). At a median follow-up of 6 years, they found a greater likelihood of fatal MI in left-sided irradiated patients compared to right-sided (RR = 1.17), but no difference by laterality among non-irradiated women (RR = 1.04). The increased risk of fatal MI was only seen in the age group 60 years or younger at diagnosis (RR = 1.98), and especially among those with regional stage disease (RR = 2.24), as opposed to local stage disease. The risk was significantly greater for this younger age group at longer follow up times of 10–15 years, while not before 10 years. There also appeared to be a higher relative risk for women treated in the older era, though there were insufficient data to examine the younger age group in the more recent era.

Giordano *et al*. (2005) also used the SEER 12-registry 1973–2000 dataset, including 27,283 women treated with radiation for breast cancer between 1973 and 1989. In order to assess whether presumed changes in radiation technique over time had any impact on the incidence of cardiac mortality, the cohort was divide into treatment eras of 1973–1979, 1980–1984, and 1985–1989. The investigators found that women with left-sided cancers treated in the 1973–1979 era had a higher 15 year mortality from ischemic heart disease than right-sided patients (13% vs. 10%, p = 0.02). No such difference was seen in the other 2 eras, although median follow-up was necessarily shorter in the more recent eras. Darby *et al*. (2005) also utilized the SEER cancer registry for 1973–2001 to examine the risk of cardiac mortality over time, including 308,861 women with early breast cancer, 37% of whom received radiation. For women diagnosed and treated prior to 1983, the cardiac mortality ratio for left versus right-sided patients was 1.20 under 10 years follow-up, 1.42 at 10–14 years, and 1.58 at 15 years or more. For women diagnosed and treated between 1983 and 1992, cardiac mortality ratio was 1.04 under 10 years and 1.27 at 10 or more years. The final era group, 1993–2001, only had follow-up data at under 10 years, with a cardiac mortality ratio of 0.96. While the reduction of cardiac mortality risk for women treated more recently may be related to improvements in radiation technique, they may also be related to improvements in screening and treatment of ischemic heart disease during the study period.

Other registries have been analyzed for cardiac outcomes in breast cancer survivors. Paszat *et al*. (1999) compared the risk of death from MI between right and left-sided patients radiated post-lumpectomy between 1982 and 1987 (N = 3006) by linking records from the Ontario Cancer Registry to hospital discharge records. At median follow-up of 9 years, there was a higher risk of death from MI in the leftsided group (2%) compared to the rightsided group (1%; p = 0.02; relative risk = 2.1). In this cohort, age stratified analysis showed that the increase risk of MI was only seen after age 60, while no difference was seen for patients under 60 years at the time of treatment. The mean

dose per fraction in this cohort was about 2.4 Gy, somewhat higher than in series from the United States population, and 77% received treatment on cobalt-60 units. The study found that such radiation treatment quality factors were associated with a higher relative risk of MI in left sided patients. The authors postulate that differences in available data from the SEER versus the more detailed Ontario dataset, in particular with regard to staging and radiation technique, that may account for the different outcomes seen in their different population-based studies. Paszat *et al*. (2007) conducted another population-based case control series of 6,680 women treated with post-lumpectomy or mastectomy radiation between 1982 and 1988 in Ontario, Canada. For this study, the investigators identified verified cases of acute MI both validated by enzyme criteria or ECG findings, and those identified as acute MI on a death certificate. When adjusting for a history of smoking and prior MI, the risk of an acute MI among women 60 or older with left-sided versus right-sided breast cancers was associated with a hazard ratio of 1.96. Treatment of the internal mammary nodal chain was associated with a hazard ratio of 1.90. Examination of other technical factors, however, did not show treatment of an anterior left breast boost to significantly increase the risk of acute MI (HR = 1.02).

Rutqvist and Johansson (1990) reported mortality rates by laterality among 54,617 breast cancer patients from the Swedish Cancer Registry for patients treated from 1970 to 1985. The study found no difference at a median of 9 years between right and left-sided patients for total mortality, total intercurrent deaths or total cardiovascular mortality. However, the rate of deaths from MI were higher in the left-sided patients with a relative risk of 1.09. This risk became more pronounced with longer follow up, such that relative risk for left-sided versus right-sided was 1.06 at year 0–5, 1.13 at year 5–10 and 1.20 at 10–17 years (p = 0.22). Roychoudhuri *et al*. (2007) were interested in long term cardiac mortality in women treated in an older era, and used the Thames Cancer Registry of 20,871 women with any breast cancer diagnosed between 1971 and 1988 to identify deaths from ischemic heart disease and any cardiovascular disease, using right-sided patients who did or did not receive any radiation as controls against left-sided irradiated patients. The hazard ratio of death from ischemic heart disease at 15 years or more after treatment for left-sided patients compared to unirradiated controls was 1.59, and for any cardiovascular death was 1.27. Hazard ratios for left-sided patients compared to irradiated right-sided controls were 1.23 and 1.25, respectively. Table 48.1 summarizes the findings from the studies described in this section as reported by the type of surgery.

One major limitation of most of the studies of cardiac toxicity is the lack of long term data in patients who have also received the cardiotoxic systemic therapies that are commonly used in contemporary patients, especially doxorubicin and trastuzumab. Woodward *et al*. (2003) reported outcomes in 470 patients who were treated with a series of prospective institutional protocols at MD Anderson Cancer Center; they received doxorubicin-based chemotherapy and radiation, and compared toxicity rates to 1,031 women with the same systemic therapy who did not receive radiation. While this study includes only post-mastectomy patients,

Table 1 Long Term Studies of Cardiac Mortality by Laterality in Breast Cancer Patients With and Without Radiation

Author Treatment	Data Source	Number	Follow-up (Years)	Cardiac Death (L vs. R)
Post-Mastectomy Studies				
Hojris (1999) PM	Danish BCCGT 1982–1990	3083 (+/– RT; MV)	10	HR = 0.86 RT vs. no RT
Gyenes (1998) PM	Swedish Registry 1971–1976	960 (+/– RT; Co-60, Electrons)	20	No difference (MI only) HR=2.5 for highest cardiac dose-volumes No difference lower cardiac dose-volume groups
Rutqvist (1990) PM	Swedish Registry 1970–1985	54,617 (+/– RT)	9	Yr 0-5: RR = 1.01 (MI) Yr 5-10: RR = 1.13 Yr 10-17: RR = 1.20 Yr 0-17: RR = 1.09
Haybittle (1989) PM	MRC Trial 1970–1975	2800 (+/– RT; Orthovoltage)	13–18	HR = 2.67 (“cardiac diseases”)
Host (1986) PM	Oslo Trial 1964–1972	1115 (+ RT; KV, C0-60)	11–20	Increased (MI) Stage I only
Post-Mastectomy Studies and Breast Conservation Studies				
Darby (2005) BCT&PM	SEER 1973–2001	115,165 (+ RT)	—	1973–1982: Increased > 10 yrs 1983–1992: Increased > 10 yrs 1993–2001: No difference < 10 yrs
Giordano (2005) BCT&PM	SEER 1973–1989	27,283 (+ RT)	9	1973–1979: Increased at 15 Yrs 13% L vs. 10% R 1980–1984: No difference 1985–1989: No difference
Clarke (2005) BCT&PM	Meta-analysis	23,500 (+/– RT)	–	HR = 1.27 (“Heart Disease”) RT vs. no RT
Paszat (1998) BCT&PM	SEER 1973–1992	46,596 (+/– RT)	6	Overall, HR = 1.17 < 60 years, HR= 5.28, > 10 yrs

				> 60 years, no difference
Breast Conservation Studies				
Vallis (2002)	PMH	2128	10.2	No difference (MI)
BCT	1982–1988	(+ RT)		
Paszat (1999)	Ontario Cancer	3006	8.8	Increased (MI)
BCT	Registry	(+ RT)		2% L vs. 1% R (p=0.02)
	1982–1987			HR=2.10
Harris (2006)	Penn	961	12	No difference
BCT	1977–1994	(+ RT)		Cumulative hazard: 6.4% L vs. 3.6% R at 20 years
Rutqvist (1998)	Karolinska	684	9	No difference (MI)
BCT	1976–1987	(+ RT)		
Nixon (1998)	JCRT	745	12	2% R & L
BCT	1968–1986	(+ RT)		No increase over time

BCT = breast conservation therapy; PM = post-mastectomy; RT = radiation therapy; MI = myocardial infarction; RR = relative risk; HR = hazard ratio; MV = megavoltage; KV = kilovoltage; C0-60 = cobalt-60; R = right; L = left; Yr = year

it is the only currently published series with long term follow-up in patients who received a doxorubicin-based chemotherapy regimen. The investigators looked at chronic toxicities with and without chest wall radiation, including the rates of cardiac related deaths, primarily from MI. While the 10 year rate of death from MI was higher in the irradiated group (2.4% vs. 0.5%, $p = 0.057$), two of eight patients died of MI during systemic therapy, and only two patients who died of MI had left-sided cancers. This argues against a direct relationship between death from MI and radiation. Consistent with other series not including cardiotoxic chemotherapy, the median time to MI was 9 years after treatment and the overall rate of MI was low. The NSABP B-31 trial randomized women with node positive and HER-2 positive breast cancer to doxorubicin, cylcophosphamide and paclitaxel with or without trastuzumab (Tan-Chiu *et al*., 2005). Post-lumpectomy radiation was required, while post-mastectomy radiation was optional, and all patients in the trastuzumab arm received the drug concurrently with radiation when given. The cumulative incidence of cardiac events in the trastuzuzmab arm was 4.1% compared to 0.8% in the chemotherapy alone arm, and most of the cardiac events were congestive heart failure. There are currently no published studies of any possible interactive effects of trastuzumab and radiation; thus, concurrent therapy, as conducted in the randomized trials, is considered acceptable.

Two major themes have emerged from the accumulated data on the risk of cardiac injury after breast or chest wall irradiation. The first is that the greater the volume of heart included in the radiation fields, the higher the likelihood of cardiac injury leading to ischemic heart disease and cardiac mortality. This is evident from the higher rate of cardiac deaths in the older trials, which seems to dissipate in more contemporary studies. The second theme is that radiation-induced cardiac injury is a very late event. In most studies, cardiac deaths are not seen to increase among irradiated patients compared to controls, or among left-sided patients compared to right-sided, until 10 or more years after irradiation.

IMAGING STUDIES ASSESSING CARDIAC INJURY

In addition to studying the post-treatment outcomes in irradiated patients for incidence of cardiac related deaths and morbidities, several investigators have conducted studies using various forms of cardiac imaging techniques to directly assess the injury to the heart from radiation. Myocardial perfusion scintigraphy (MPS) is the most commonly employed technique. MPS employs a radiotracer, most commonly thallium-201, which is taken up in the myocardium and used to assess myocardial perfusion, blood flow and cell viability for diagnoses of ischemia and coronary artery disease. Using this approach, some investigators have conducted prospective studies pre- and post-treatment, while others have performed perfusion testing in the follow-up period after treatment.

In a series of 37 asymptomatic patients who had been previously irradiated either pre- or post-operatively for early breast cancer, Gyenes *et al*. (1994) conducted echocardiograms and technetium-99 MPS, including 20 left-sided patients and 17

right-sided or non-irradiated controls. Patients were treated with either cobalt-60 or electrons. Scintigraphic defects consistent with ischemia were seen in 25% of left-sided patients while none of the controls had any defects ($p < 0.05$). However, no left ventricular dysfunction was documented on echocardiogram. The South Sweden Breast Cancer Trial included an arm of women who were premenopausal at diagnosis, and who were randomized to post-mastectomy radiation and chemotherapy. As reported by Gustavsson *et al.* (1999), 90 women from this group underwent MPS and echocardiography 10–17 years (median 13 years) after irradiation; 34 had left-sided cancer and 33 had right-sided cancer. Radiation was delivered with a combination of roentgen, electron beam, and megavoltage photons to the chest wall and regional nodes, with daily fraction size of 1.9–2.4 Gy to a total dose of 38–48 Gy. While multiple abnormal findings were recorded on testing, there was only a trend of abnormal findings among irradiated patients. Abnormal test results included perfusion defects in 7%, ECG abnormalities in 16%, and valvular problems in 22%, while all subjects had normal systolic function. In this series, therefore, there was no evidence of significant radiation related cardiac injury despite the use of outdated techniques. Hojris *et al.* (1999) conducted a similar study with 17 participants from the Danish Breast Cancer Group trials with left-sided cancer who did or did not receive post-mastectomy radiation to the chest wall and regional nodes. The chest wall and internal mammary nodes were treated with electron beam to a median dose of 50 Gy in 25 fractions. At a median follow-up of 8 years, these subjects underwent ECG and sestamibi SPECT (single photon emission computerized tomography) myocardial perfusion imaging. These investigators also did not find any difference in scintigraphic findings between the two groups because four subjects in both the irradiated and nonirradiated group had defects. Seddon *et al.* (2002) studied 24 left-sided subjects and 12 right-sided controls who had radiation with contemporary technique administered 5 years or more prior to undergoing SPECT MPS testing. Left-sided subjects were required to have at least 1 cm of heart in the radiation field to be eligible. Perfusion defects were seen in 70% of left-sided and 17% of right-sided subjects ($p = 0.002$). In left-sided patients, the defects were located in the apex in 16 of 17. All patients studied had a normal left ventricular ejection fraction and none were clinically significant or required any follow up testing or treatment. All of these studies were conducted on a relatively small number of subjects, so it is difficult to draw firm conclusions regarding the long term rate of cardiac perfusion defects after post-mastectomy irradiation. None show any clinically relevant cardiac disease in otherwise asymptomatic patients.

Two prospective series of myocardial perfusion imaging in breast cancer patients receiving radiation have been conducted. Gyenes *et al.* (1996) at the Stockholm Southern Hospital initially recruited 12 left-sided patients with CT-based radiation planning post-lumpectomy or mastectomy for quantification of heart dose showing at least 85% total dose to part of the left ventricle. Radiation was given with tangentially opposed photon beams in intact breast patients and with electrons in post-mastectomy patients. Pre- and post-treatment echocardiograms and MPS were

performed at a median of 13 months after radiation. Half of the patients exhibited new fixed scintigraphic defects consistent with regional hypoperfusion, and defects were localized to the irradiated left ventricle. However, no systolic or diastolic or left ventricular dysfunction was detected.

In an elegant ongoing prospective study, investigators from Duke University have followed patients with serial SPECT MPS at various intervals after undergoing radiation with or without doxorubicin-based chemotherapy. Hardenbergh *et al*. (2001) have reported on three dimensional radiation planning used to correlate volumes of irradiated myocardium to perfusion test findings. All patients received megavoltage tangent beam whole breast irradiation to a total dose of 46–50 Gy. In this initial report, 60% of patient had new perfusion defects, and regional perfusion decreased 20% at doses of over 41 Gy compared to minimally at less than 10 Gy, suggesting a dose dependent effect. One patient had a decrease in left ventricular ejection fraction. Lind *et al*. (2003) updated this series which included 69 patients with SPECT at 6, 12, and 18 months post-treatment, further quantifying the distribution of coronary artery involvement on SPECT abnormalities. For the left anterior descending (LAD) artery distribution specifically, which is most likely to be located in the irradiate portion of the left ventricle, perfusion defects were significantly increased, while no such increase was seen in the distribution of the left circumflex or right coronary arteries. The percent of perfusion defects did not change over the 12 and 18 month time periods. LAD perfusion changes correlated with the percent of irradiated left ventricle, as well as other cardiac risk factors including hormonal therapy and hypercholesterolemia. A second study, update by Marks *et al*. (2005), reported on 114 patients with serial post-treatment SPECT MPS testing up to 24 months post-treatment. This update showed that new perfusion defects continued to appear as time from treatment increased, occurring in 27% at 6 months and in 42% at 24 months. The likelihood of new perfusion defects was correlated with percent irradiated left ventricle, which occurred in up to 20% of patient with $< 5\%$ irradiated left ventricle and in up to 60% with $> 5\%$ irradiated left ventricle. Wall motion abnormalities were also correlated with the presence of new perfusion defects. Left ventricular volume irradiated was the primary technical factor associated with the rate of new perfusion defects, however, patient body mass index (BMI) was also correlated. Evans *et al*., (2006) reported that the tendency for daily radiation field set up to lie "deep", exposing more left ventricle than intended to radiation dose, was seen more often in patients with a BMI $> 25\,kg/m^2$. This important observation highlights the necessity not only of performing 3D CT-based treatment planning in order to customize heart blocks or other avoidance techniques, but that daily set up accuracy is also a critical factor in preventing long term cardiac injury in breast cancer patients. This appears to be especially true for larger patients whose daily set up is inherently more inaccurate when relying on external measurements of unstable soft tissue structures.

The clinical significance of imaging findings post-irradiation is unclear, so the Duke investigators assessed the likelihood of symptoms related to perfusion abnormalities in 83 evaluable patients enrolled in their prospective series at a median follow

up of 16 months (Yu *et al*., 2003). At that time point, 12% of patients had episodes of transient chest pain, which was more likely to occur in those with new post-treatment perfusion defects than in those without ($p = 0.0004$), suggesting that these detected perfusion defects may in fact be clinically relevant. Patients were however aware of whether they had post-treatment perfusion changes. Segregation based on chemotherapy use showed similar results. While there were a few instances of pericarditis, there were no instances of MI or congestive heart failure. Longer follow-up is anticipated from this landmark prospective series that may quantify the clinical impact of various degrees of left ventricular irradiation and lead to the development of recommendations for screening assessments and interventions in breast cancer survivors.

TECHNIQUES FOR REDUCING CARDIAC TOXICITY

Since it has long been recognized that radiation has the potential to induce long term toxicity to exposed myocardium, many investigators have examined techniques for improving upon the technical delivery of breast or chest wall irradiation in order to reduce the dose and volume of exposed heart and thereby presumably decrease the risk of toxicity. Such studies have been facilitated by the development of new technologies for radiation delivery and for evaluating the accuracy of daily positioning, or for dealing with patient anatomical constraints. Considerable interest has been paid to how the volume of irradiated heart may vary during a 5 week course of daily treatments due to breathing motion, cardiac motion, daily set up variations and the field arrangements themselves.

Daily Positioning

One of the simplest yet most overlooked aspects of radiation treatment delivery is the daily patient set up, or positioning. Substantial effort is typically expended to achieve treatment plans that optimize target coverage while minimizing normal tissue doses, but these carefully constructed plans may not reflect the reality of dose delivered. One technical innovation that has made daily positioning assessment easier to perform is the electronic portal imaging device (EPID), which allows online portal imaging of treatment fields for more frequent and potentially more accurate daily positioning at a faster throughout than X-ray film technology. EPID technology may be used to observe the movement of organs within the treatment fields in real time by rapidly obtaining multiple intrafraction images. Initial studies used the EPID to assess the accuracy of daily positioning for breast tangent irradiation. Louwe *et al*. (2007) calculated a method for EPID images to reconstruct the actual heart dose during left breast treatment, correlating the portal imaging data with 3D data from planning CT simulation. The EPID dosimetry was found to agree with 3D DVH calculations within 1.5% for the heart dose, suggesting a method to incorporate actual daily dose into normal tissue complication probability (NTCP) calculations for late cardiac mortality.

Lirette *et al*. (1995) performed EPID imaging on 20 early stage breast cancer patients to evaluate the influence of respiration on daily set up positioning and the concordance with originally simu-

lated fields. Random and systematic errors from 2,120 images were analyzed, and the intrafraction variation was about 3 mm. Variation between set up and simulation was about 4.3 mm, although rarely large deviations up to a maximum of 23 mm were noted. Magee *et al*. (1997), audited EPID images in 169 left sided breast cancer patients treated with tangential fields, and noted that the cardiac apex was included in the radiation field in 9% of images. Smith *et al*. (2005) evaluated 1,709 EPID images taken during beam delivery and quantified the heart area within the irradiated field throughout treatment. The maximum intrafraction deviation in central lung distance was 25 mm. The effect of intrafraction variation on heart area ranged between 0.1 to 3.6 cm^2. The interfraction variation in central lung distance ranged from 5.9 to 29 mm, with a resultant interfraction effect on heart area ranging from 3.9 to 38.5 cm^2, emphasizing the importance of accurate daily set up verification and the relative unimportance of breathing motion during an individual treatment with respect to variations of the dose delivery. These studies reveal that it is critical to achieve precise daily positioning so that calculated cardiac doses are similar to those actually delivered, and that interfraction variations are significantly more varied than intrafraction motion attributable to breathing motion. Other methods for improving accuracy of daily set up, including various types of localization and verification devices, are currently under development and study, and will be valuable tools for improving positioning for breast cancer treatment so that the ideal dose achieved in the treatment planning process can be translated to the daily dose delivery with greater precision.

Intensity Modulated Radiation Therapy

The standard tangent field technique for treatment of the breast or chest wall customarily employed wedge compensation in fixed wedge angles in order to improve homogeneity across an essentially triangle shaped volume. Once MLC became available, custom compensation using field-in-field MLC shaping has come increasingly into use. In this technique, the MLC is used to shape smaller fields that correspond to the areas of high dose inhomogeneity, covering the high dose regions with custom shaped fields for small portions of the total dose in order to achieve improved homogeneity. IMRT typically employs 3D planning using dose-volume constraints for target tissues and normal organs with either forward or inverse planned dose calculations. It is debated whether the field-in-field method may accurately be referred to as IMRT, which some have argued should include either multiple beam angles, inverse planning or a higher number of beamlets (Potters, 2003), while others have argued that the term IMRT may encompass a variety of technical approaches, and that the treatment of breast cancer should be subject to the advantages of technological improvements as many other disease sites have (Vicini *et al*., 2004). The definition of IMRT continues to evolve and has both clinical and economic components. It is most important to note that while studies and publications may refer to a particular technique as IMRT and others may not, ultimately what is critical for evaluation of a technique is the dose coverage of the target tissues and normal tissues, and the dose homogeneity.

IMRT for breast cancer treatment is a relatively new technique and several methods for IMRT delivery have been described. These techniques all attempt to achieve better homogeneity across the target volumes while reducing normal tissue doses compared to 3D conformal techniques. Kestin *et al*. (2000) published the first breast IMRT technique, which utilized MLC segments (6–8 segments per patient) to conform to the individual anatomy based on 3D isodose surfaces. The IMRT technique was planned in 10 patients and compared to 2 other techniques: tangents with wedge compensation with and without homogeneity corrections. The IMRT technique achieved better homogeneity across the breast tissue with lower maximum doses while maintaining similar target doses coverage, although cardiac doses were not reported. Landau *et al*. (2001) reported on the ability of IMRT to achieve cardiac avoidance compared to conventional 3D conformal tangents. Plans were compared in ten left-sided patients with at least 1 cm of heart in the tangent fields. IMRT using 2, 4, and 6 field arrangements were planned. The study showed that despite techniques used, irradiated cardiac volumes could be reduced by the use of partial cardiac shielding with some compromise of target volume coverage, and that each of the IMRT techniques was able to reduce irradiated cardiac volumes. The average percent of heart receiving > 60% prescribed dose was 4.4% for conventional tangents, 1.5% with partial shielding, 2.3% for 2-field IMRT and 2.2% for 4-field IMRT. While partial shielding achieved the greatest reduction in irradiated cardiac volume, it was associated with a mean of 4% of the target volume receiving < 95% prescribed dose, while the IMRT techniques increased the contralateral breast dose. Hurkmans *et al*. (2002) examined the ability of IMRT to reduce heart and lung complication probabilities compared to conformal techniques. Using the planning datasets form 17 left-sided patients, they compared treatment plans with optimized wedges with and without blocks with IMRT. Coverage of the breast planning target volume (PTV) was slightly better with the conformal techniques than with IMRT. The calculated NTCP for late cardiac mortality was 6% for fields without blocks, 4% for fields with blocks, and 2% with IMRT. The cardiac NTCP was highly correlated with the maximum heart distance, and the authors suggest that this parameter could be used for screening patients who may benefit from the use of IMRT technique to reduce the cardiac toxicity risk. Cuttino *et al*. (2005) compared dose distributions of breast and regional nodal coverage with IMRT plans with 2, 4, 6 and 9 fields to 3D planning in five left-sided patient datasets. This planning study showed improved target coverage with all IMRT techniques versus conventional plans, and reduction in the mean doses to heart, lungs and contralateral breast with 2, 4 and 6-field IMRT, with the 4–field arrangement providing the best overall plans.

Several other investigators have described IMRT techniques for the treatment of breast cancer. Olivotto *et al*. (2005) described a multi-field, inverse planned IMRT arrangement administered to 24 random patients with internal mammary nodes included in the target volume. Compared to best standard plans, the IMRT plans showed improved homogeneity, while reducing heart V30 from a mean of 6.6% to 0.5%, and the lung V20. Cho *et al*. (2004)

described a "simplified" IMRT technique using pre-defined segments, which was compared to conventional conformal technique and to full fluence IMRT plans for nine left-sided patients with a maximum heart distance of at least 2 cm. While the full IMRT and simplified IMRT techniques demonstrated heart sparing over conformal plans, with late excess cardiac mortality probabilities of 0.2% or less, this was at the expense of somewhat poorer doses to the target volumes.

Keall *et al*. (2001) developed an automated IMRT technique to improve the efficiency of planning and delivery. The technique used parallel opposed beams with dynamic MLC delivery, constraining the heart and lung doses to 0 Gy, and planning target volume (PTV) coverage to 47 Gy. The IMRT technique showed reduced doses to heart, lung and contralateral breast over 3D conformal, but the magnitude was highly dependent on patient anatomy. These investigators also reported on an important aspect of IMRT delivery that the earlier technique studies did not address: the impact of motion on the dose distribution. Since IMRT relies heavily on small beamlets and segmental fields that are designed to precisely cover particular volumes of tissue, daily positioning and motion are much more critical than for 2D or even 3D conformal techniques. As a result, the margins around the target volumes need to account for motion, and if the patient motion is not synchronized with the MLC motion, then the targeted tissues may not be properly aligned with the intended segments. George *et al*. (2003) studied the impact of intrafraction motion during IMRT delivery by generating virtual plans with eight different motion magnitudes on seven patient datasets. This planning study demonstrated that both target dose heterogeneity and heart and lung doses increased with respiratory motion. Due to the lack of coordination between respiratory motion and the MLC motion, the planned dose distributions differed from the actual distributions, with differences increasing with respiratory motion. These differences could obviously be eliminated by techniques that regulate breathing, as discussed in the next section.

To date, there has been one randomized trial published comparing standard 2D radiation to IMRT in women with early stage breast cancer. In this trial, 306 women who were candidates for whole breast irradiation after lumpectomy were randomly assigned to 3D IMRT or 2D radiation treatment with wedge compensation. CT-based planning was not available at the start of the trial, so an alternate method of 3D planning based on EPID was devised. IMRT was delivered either with physical compensators or with a 3 field step-and-shoot MLC method, and was primarily designed to reduce inhomogeneity. A total dose of 50 Gy was delivered in 25 fractions over 5 weeks, followed by a tumor bed electron boost. The primary endpoint of this study was appearance of the breast. Patients treated with IMRT were less likely to have a change in the appearance of the breast and had less palpable induration than the 2D group. This study has many limitations, especially given that the comparison was between 2D and IMRT, rather than 3D conformal technique, which was in standard usage during the study period of 1997 to 2000. Also, the IMRT technique used was simplistic and not based on 3D anatomy in all patients. There was no attempt to compare normal tissue doses

between the two groups. Therefore, additional randomized or institutional studies will be important to further define the situations in which breast IMRT will be optimally employed.

Woo *et al*. (2006) evaluated the whole body radiation exposure during breast radiation treatment with 50 Gy using IMRT with MLC or wedge compensation. Whole body dose has been correlated with the risk of secondary leukemia. In a prospective group of 120 women, radiation measurements were taken at four places on the body during treatment. Abdominal organs received an average of 0.45 Gy (range (0.06–1.55 Gy), and was three times higher with wedge compensation than with IMRT. Thus, IMRT resulted in a significantly lower scatter dose to the whole body, at least with the technique used in this study.

These studies highlight the challenges of implementing highly complex treatment techniques, requiring an assessment of all aspects of treatment delivery and not relying overly on virtual data. In addition, the potential drawbacks and toxicity profiles of newer techniques must always be carefully assessed. It is likely that IMRT will provide lower doses to the heart and lungs in some patients, but may not provide significantly improved dose distributions in others; therefore, more study is required to define the patients in whom this complex technique will be most beneficial.

Breath Hold Techniques

Several investigators have studied the use of breath hold techniques to reduce cardiac volumes during breast or chest wall irradiation. A deep inspiration breath hold can reposition the heart deeper in the chest, further away from the lung-rib interface, thereby potentially reducing or eliminating heart from the radiation fields. Such techniques can potentially increase the volume of lung irradiated, however. Breath hold has been accomplished by instructing patients to maintain a breath hold during the beam-on time, and by using devices that limit assist the patient in holding a breath at the proper breathing phase. Chen *et al*. (1997) conducted a study among 16 healthy women volunteers in which cardiac volume was assessed by cardiac magnetic resonance imaging (MRI) during breath holding at baseline, deep inspiration and forced expiration. Mock radiation portals were created, and 92% had inclusion of some cardiac volume in the mock portals at baseline, with a median volume of 21 cm^3. Deep inspiration decreased the cardiac volume by a median of 10.7 cm^3, while expiration increased the cardiac volume by a mean of 4 cm^3. Lu *et al*. (2000) then evaluated the heart volume changes with respiratory maneuvers during CT simulation of 15 patients with breast cancer. Patients were responsible to maintain a deep inspiration breath hold, and all were reportedly able to do so for 20 s without difficulty. CTs were obtained in this position as well as during normal breathing. Radiation fields using the same borders for each position were compared. Cardiac volume was reduced by 86% during deep inspiration, and for 47% (N = 7) the heart was moved completely out of the fields, while lung volume was increased, although the fractional lung volume was not significantly larger. This group as reported by Chen *et al*. (2002) performed a similar study among 17 women who had been previously treated for left-sided breast cancer and who volunteered to

return for cardiac MRI with and without breath hold and virtual reconstruction of their radiation portals. The mean reduction in heart volume in the radiation portal was 18 cm^3 (–49%), and in 21% the heart was completely displaced form the portals. Breath hold does require the use of minifractions, as an overall beam on time is longer than the ability to breath hold. Problematically for a voluntary breath hold technique, patients will differ in their ability to hold the breath and for variable amounts of time, making it difficult to precisely coordinate the beam-on cycle with the breath hold.

Devices to assist in breath hold are commercially available. These devices coordinate the breath hold with the treatment machine beam-on time using a method called active breathing control (ABC). Sixel *et al*. (2001) described this technique to maintain a deep inspiration breath hold (DIBH) during left-sided breast irradiation with a pilot study of five patients. Serial planning CTs were obtained with and without breath hold for DVH comparison. The results of ABC were varied, with three patients showing reduced cardiac volumes in-field with DIBH, and two others with little effect, demonstrating that the success of the technique will vary depending upon anatomy and lung capacity. Remouchamps *et al*. (2003b) at William Beaumont Hospital published a pair of studies using moderate DIBH with ABC in left-sided patients treated for either early stage breast cancer with tangent fields, or requiring deep tangents for coverage of the internal mammary nodes (IMNs). In the early stage series, five patients with > 2% heart receiving > 30 Gy (V30) were treated with the ABC device (Remouchamps *et al*., 2003a). Treatment was delivered to tangent beams using step-and-shoot IMRT, while open beams during certain segments were imaged using EPID to analyze inter- and intrafraction variations, and virtual simulation was used to compare DIBH to free breathing dose distributions. Breath hold durations ranged from 18 to 26 s (median 22 s), and the average number of required breath holds per beam was 2.5, or 4 to 6 per treatment, for a median treatment time of 18 min, longer than without the device. ABC treatment resulted in a mean absolute reduction of 3.6% heart V30, with an associated NTCP of 1.5%. As seen in other studies, the intrafraction variation was less than 2 mm, while the interfraction variation was greater. In a second series of patients requiring IMN coverage, deep tangents with DIBH using ABC was compared to a standard technique using electrons to cover the IMNs in nine left-sided patients. (Remouchamps *et al*., 2003b). Without DIBH, the heart V30 was lower for the electron technique (7%) than the deep tangents with wedge compensation (19%) or IMRT (16%). With DIBH, the heart V30 for the deep tangent IMRT technique was reduced by 81% to a mean of 3.1%. For six of nine patients, this technique resulted in a better heart V30 than the electron technique without DIBH (electron technique with DIBH was not compared). The mean lung dose and NTCP were also reduced with IMRT DIBH technique compared to electron technique. This was at a cost of increased dose to the contralateral breast. These investigators, as reported by Krauss *et al*. (2005), studied 15 left-sided patients treated with tangents to 45 Gy using DIBH with ABC, and obtained ECG-gated cardiac MRIs in order to better quantify the in-field heart volumes

during the cardiac cycle. The percent of left ventricle receiving > 50% prescribed dose (V22.5) was reduced with ABC compared to free breathing by 85% during late diastole, by 92% during mid-diastole, and by 95% during systole. Although CT based cardiac volumes are somewhat less accurate, and provide less ability to distinguish left ventricle from whole heart, correlations between MRI and CT–defined volumes of whole heart in this study were adequate, suggesting that CT-based whole heart volumes may be used for estimates of cardiac volumes for planning purposes.

A potential drawback of DIBH using ABC lies in its complexity and requirement for patient cooperation, coaching and training. Some patients are not able to tolerate the device or the breath hold. It requires special equipment for both simulation and for daily treatment and increases overall treatment times. Such limitations may be acceptable for a significant reduction in irradiated myocardium. Korreman *et al*. (2005) have described a free breathing gating technique, compared to breath hold technique. This technique utilizes breathing adapted radiotherapy (BART), in which the patient breathes freely and the CT scanner and treatment machine are gated to the patients' free breathing pattern, using the Real-time Position Management (RPM) system (Varian). For this study, 17 patients were scanned during free breathing, end-inspiration gating, end-expiration gating, voluntary deep inspiration breath hold and end expiration breath hold. The breathing cycle was incorporated into the optimized 3 field wide tangent plans. End inspiration gating (IG) and DIBH reduced the median heart volume receiving > than 50% prescribed dose compared to free breathing: 19% compared to 3% for IG and 2% for DIBH. The median left anterior descending coronary artery dose was reduced from 89% with free breathing to 22% for IG and 4% for DIBH. Both techniques also reduced the median lung volume irradiated compared to free breathing. The authors concluded that free breathing gated treatment compared favorably to DIBH. These investigators conducted NTCP calculations for the heart comparing the voluntary DIBH and IG techniques for a prescription dose of 48 Gy to breast, internal mammary and periclavicular nodes (Korreman *et al*., 2006). These calculations used clinical data from 33 patients, and indicated that the risk of cardiac mortality could be reduced from 5% for free breathing to 0.5% for IG and to 0.1% for DIBH. Sidhu *et al*. (2006) compared the effect of breathing motion on dose distributions using different treatment techniques, using a film dosimeter and static dosimetry date for comparisons on moving breast phantoms at three respiratory velocities. The dose uniformity was improved with gating, but with step-and-shoot IMRT, gating compromised the breast coverage when the timing interval of the gate was too large.

The majority of the studies for cardiac avoidance using new and innovative treatment planning and delivery techniques show that improved dose distributions can be achieved using a variety of methods and combinations of newer technologies. However, the majority of these studies are planning exercises conducted on patient datasets with virtual comparisons, andthere are few clinical trials comparing the feasibility of accurate dose delivery in a large group of patients, nor have outcomes been reported to date. These will be the next generation of studies. Treatment planning

studies are essential to the development of new techniques in order to ensure safe implementation, but ultimately it is in the realm of clinical practice that these techniques will be refined. Most likely different techniques will benefit different patients, given the large variation in anatomy and other risk factors among individuals. After several decades without significant innovation, many new possibilities now exist for the potential improvement in quality of life, through the reduction of late toxicity, for early stage breast cancer patients requiring radiation.

REFERENCES

Chen, M.H., Chuang, M.L., Bornstein, B.A., Gelman, R., Harris, J.R., and Manning, W.J. 1997. Impact of respiratory maneuvers on cardiac volume within left-breast radiation portals. *Circulation 96*: 3269–3272.

Chen, M.H., Cash, E.P., Danias, P.G., Kissinger, K.V., Bornstein, B.A., Rhodes, L.M., Gelman, R., Harris, J.R., and Manning, W.J. 2002. Respiratory maneuvers decrease irradiated cardiac volume in patients with left-sided breast cancer. *J Cardiovasc. Magn. Reson. 4*: 265–271.

Cho, B.C., Schwartz, M., Mijnheer, B.J., and Bartelink, H. 2004. Simplified intensity-modulated radiotherapy using pre-defined segments to reduce cardiac complications in left-sided breast cancer. *Radiother. Oncol. 70*: 231–241.

Clarke, M., Collins, R., Darby, S., Davies, C., Elphinstone, P., Evans, E., Goodwin, J., Gray, R., Hicks, C., James, S., MacKinnon, E., McGale, P., McHugh, T., Peto, R., Taylor, C. and Wang, Y. 2005. Early Breast Cancer Trialists' Collaborative Group. Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15-year survival: an overview of randomized trials. *Lancet 366*: 2087–2106.

Cuttino, L.W., Dogan, N., Bump, E.A., and Arthur, D.W. 2005. Optimized dose coverage of regional lymph nodes in breast cancer: the role of intensity modulated radiotherapy (IMRT). *Int. J. Radiat. Oncol. Biol. Phys. 63*: S243–S244.

Cuzick, J., Stewart, H., Rutqvist, L., Houghton, J., Edwards, R., Redmond, C., Peto, R., Baum, M., Fisher, B., and Host, H. 1994. Cause-specific mortality in long-term survivors of breast cancer who participated in trials of radiotherapy. *J. Clin. Oncol. 12*: 447–453.

Darby, S.C., McGale, P., Taylor, C.W., and Peto, R. 2005. Long-term mortality from heart disease and lung cancer after radiotherapy for early breast cancer: prospective cohort study of about 300,000 women in US SEER cancer registries. *Lancet Oncol. 6*: 557–565.

Evans, E.S., Prosnitz, R.G., Yu,. X, Zhou, S.M., Hollis, D.R., Wong, T.Z., Light, K.L., Hardenbergh, P.H., Blazing, M.A., and Marks, L.B. 2006. Impact of patient-specific factors, irradiated left ventricular volume, and treatment set-up errors on the development of myocardial perfusion defects after radiation therapy for left-sided breast cancer. *Int. J. Radiat. Oncol. Biol. Phys. 66*: 1125–1134.

George, R., Keall, P.J., Kini, V.R., Vedam, S.S., Siebers, J.V., Wu, Q., Lauderbach, M.H. Arthur, D.W., and Mohan, R. 2003. Quantifying the effects of intrafraction motion during breast IMRT planning and dose delivery. *Med. Phys. 30*: 552–562.

Giordano, S.H., Kuo, Y.F., Freeman, J.L., Buchholz, T.A., Hortobagyi, G.N., and Goodwin, J.S. 2005. Risk of cardiac death after adjuvant radiotherapy for breast cancer. *J. Natl. Cancer Inst. 97*: 419–424.

Gustavsson, A., Bendahl, P.O., Cwikiel, M., Eskilsson, J., Thapper, K.L., and Pahlm, O. 1999. No serious late cardiac effects after adjuvant radiotherapy following mastectomy in premenopausal women with early breast cancer. *Int. J. Radiat. Oncol. Biol. Phys. 43*: 745–54.

Gyenes, G., Fornander, T., Carlens, P., and Rutqvist, L.E. 1994. Morbidity of ischemic heart disease in early breast cancer 15-20 years after adjuvant radiotherapy. *Int. J. Radiat. Oncol. Biol. Phys. 28*: 1235–1241.

Gyenes, G., Fornander, T., Carlens, P., Glas, U., and Rutqvist, L.E. 1996. Myocardial damage in breast cancer patients treated with adjuvant radiotherapy: a prospective study. *Int. J. Radiat. Oncol. Biol. Phys. 36*: 899–905.

Gyenes, G., Rutqvist, L.E., Liedberg, A., and Fornander, T. 1998. Long-term cardiac morbidity and mortality in a randomized trial of pre- and post-operative radiation therapy versus surgery alone in primary breast cancer. *Radiother. Oncol. 48*: 185–190.

Hankey, B.F., Ries, L.A., and Edwards, B.K. 1999. The surveillance, epidemiology, and end results program: a national resource. *Cancer Epidemiol. Biomarkers Prev. 8*: 1117–1121.

Hardenbergh, P.H., Munley, M.T., Bentel, G.C., Kedem, R., Borges-Neto, S., Hollis, D., Prosnitz, L.R., and Marks, L.B.. 2001 Cardiac perfusion changes in patients treated for breast cancer with radiation therapy and doxorubicin: preliminary results. *Int. J. Radiat. Oncol. Biol. Phys. 49*: 1023–1028.

Harris, E.E., Correa, C., Hwang, W.T., Liao, J., Litt, H.I., Ferrari, V.A., and Solin, L.J. 2006. Late cardiac mortality and morbidity in early-stage breast cancer patients after breast-conservation treatment. *J. Clin. Oncol. 24*: 4100–4106.

Haybittle, J.L., Brinkley, D., Houghton, J., A'Hern, R.P., and Baum, M. 1989. Postoperative radiotherapy and late mortality: evidence from the Cancer Research Campaign trial for early breast cancer. *Brit. Med. J. 298*: 1611–1614.

Hojris, I., Overgaard, M., Christensen, J.J., and Overgaard, J. 1999. Radiotherapy Committee of the Danish Breast Cancer Cooperative Group. Morbidity and mortality of ischaemic heart disease in high-risk breast-cancer patients after adjuvant postmastectomy systemic treatment with or without radiotherapy: analysis of DBCG 82b and 82c randomized trials. *Lancet 354*: 1425–1430.

Host, H., Brennhovd, I.O., and Loeb, M. 1986. Postoperative radiotherapy in breast cancer – long-term results from the Oslo study. *Int. J. Radiat. Oncol. Biol. Phys*. 12: 727–732.

Hurkmans, C.W., Cho, B.C., Damen, E., Zijp, L., and Mijnheer, B.J. 2002. Reduction of cardiac and lung complication probabilities after breast irradiation using conformal radiotherapy with or without intensity modulation. *Radiother. Oncol. 62*: 163–171.

Jones, J.M., and Ribeiro, G.G. 1990. Mortality patterns over 34 years of breast cancer patients in a clinical trial of post-operative radiotherapy. *Clin. Radiol. 41*: 144–145.

Keall, P.J., Arnfield, M.R., Arthur, D.W., Lloyd, R., Lauterbach, M.H., Siebers, J.V., Wu, Q., and Mohan, R. 2001. An IMRT technique to reduce heart and lung dose for early stage breast cancer. *Int. J. Radiat. Oncol. Biol. Phys. 51*: 247–248.

Kestin, L.L., Sharpe, M.B., Frazier, R.C., Vicini, F.A., Yan, D., Matter, R.C., Martinez, A.A., and Wong, J.W. 2000. Intensity modulation to improve dose uniformity with tangential breast radiotherapy: initial clinical experience. *Int. J. Radiat. Oncol. Biol. Phys. 48*: 1559–1568.

Korreman, S.S., Pedersen, A.N., Nottrup, T.J., Specht, L., and Mystrom, H. 2005. Breathing adapted radiotherapy for breast cancer: comparison of free breathing gating with the breath-hold technique. *Radiother. Oncol. 76*: 311–318.

Korreman, S.S., Pedersen, A.N., Aarup, L., Nottrup, T.J., Specht, L., and Nystrom, H. 2006. Reduction of cardiac and pulmonary complication probabilities after breathing adapted radiotherapy for breast cancer. *Int. J. Radiat. Oncol. Biol. Phys. 65*: 1375–1380.

Krauss, D.J., Kestin, L.L., Raff, G., Yan, D., Wong, J., Gentry, R., Letts, N., Vargas, C.E., Martinez, A.A., and Vicini, F.A. 2005. MRI-based volumetric assessment of cardiac anatomy and dose reduction via active breathing control during irradiation for left-sided breast cancer. *Int. J. Radiat. Oncol. Biol. Phys. 61*: 1243–1250.

Krueger, E.A., Schipper, M.J., Koelling, T., Marsh, R.B., Butler, J.B., and Pierce, L.J. 2004. Cardiac chamber and coronary artery doses associated with postmastectomy radiotherapy techniques to the chest wall and regional nodes. *Int. J. Radiat. Oncol. Biol. Phys. 60*: 1195–1203.

Landau, D., Adams, E.J., Webb, S., and Ross, G. 2001. Cardiac avoidance in breast. radiotherapy: a comparison of simple shielding techniques with intensity-modulated radiotherapy. *Radiother. Oncol. 60*: 247–255.

Lind, P.A., Pagnanelli, R., Marks, L.B., Borges-Neto, S., Hu, C., Zhou, S.M., Light, K., and Hardenbergh, P.H. 2003. Myocardial perfusion changes in patients irradiated for left-sided breast cancer and correlation with coronary artery distribution. *Int. J. Radiat. Oncol. Biol. Phys. 55*: 914–920.

Lirette, A., Pouliot, J., Aubin, M., and Larochelle, M. 1995. The role of electronic portal imaging in

tangential breast irradiation: a prospective study. *Radiother. Oncol. 37*: 241–245.

Louwe, R.J., Wendling, M., van Herk, M.B., and Mijnheer, B.J., 2007. Three-dimensional heart dose reconstruction to estimate normal tissue complication probability after breast irradiation using portal dosimetry. *Med. Phys. 34*: 2354–1363.

Lu, H.M., Cash, E., Chen, M.H., Chin, L., Manning, W.J., Harris, J., and Bornstein, B. 2000. Reduction of cardiac volume in left-breast treatment fields by respiratory maneuvers: a CT study. *Int. J. Radiat. Oncol. Biol. Phys. 47*: 895–904.

Magee, B., Coyle, C., Kirby, M.C., Kane, B., and Williams, P.C. 1997. Use of electronic portal imaging to assess cardiac irradiation in breast radiotherapy. *Clin. Oncol. 9*: 259–261.

Marks, L.B., Yu, X., Prosnitz, R.G., Zhou, S.M., Hardenbergh, P.H., Blazing, M., Hollis, D., Lind, P., Tisch, A., Wong, T.Z., and Borges-Neto, S. 2005. The incidence and functional consequences of RT-associated cardiac perfusion defects. *Int. J. Radiat. Oncol. Biol. Phys. 63*: 214–223.

Nixon, A.J., Manola, J., Gelman, R., Bornstein, B., Abner, A., Hetelekidis, S., Recht, A., and Harris, J.R. 1998. No long-term increase in cardiac-related mortality after breast-conserving surgery and radiation therapy using modern techniques. *J. Clin. Oncol. 16*: 1374–1379.

Olivotto, I.A., Beckham, W., Poescu, C., Patenaude, V., and Wai, E.S. 2005. Inverse-planned, dynamic multi-field intensity modulated radiation therapy (IMRT) for left sided breast cancer: comparison to best standard technique. *Int. J. Radiat. Oncol. Biol. Phys. 63*: S184–S185.

Paszat, L.F., Mackillop, W.J., Groome, P.A., Boyd, C., Schulze, K., and Holowaty, E., 1998. Mortality from myocardial infarction after adjuvant radiotherapy for breast cancer in the surveillance, epidemiology, and end-results cancer registries. *J. Clin. Oncol. 16*: 2625–2631.

Paszat, L.F., Mackillop, W.J., Groome, P.A., Boyd, C., Schulze, K., and Holowaty, E. 1999. Mortality from myocardial infarction following postlumpectomy radiotherapy for breast cancer: a population-based study in Ontario, Canada. *Int. J. Rad. Oncol. Biol. Phys. 43*: 755–762.

Paszat, L.F., Vallis, K.A., Benk, V.M., Groome, P.A., Mackillop, W.J., and Wielgosz, A. 2007. A population-based case-cohort study of the risk of myocardial infarction following radiotherapy for breast cancer. *Radiother. Oncol. 82*: 294–300.

Potters, L. 2003. How one defines intensity-modulated radiation therapy. *Int. J. Radiat. Oncol. Biol. Phys. 56*: 609–610.

Remouchamps, V.M., Letts, N., Vicini, F.A., Sharpe, M.B., Kestin, L.L., Chen, P.Y., Martinez, A.A., and Wong, J.W. 2003a. Initial clinical experience with moderate deep-inspiration breath hold using an active breathing control device in the treatment of patients with left-sided breast cancer using eternal beam radiation therapy. *Int. J. Radiat. Oncol. Biol. Phys. 56*: 704–715.

Remouchamps, V.M., Vicini, F.A., Sharpe, M.B., Kestin, L.L., Martinez, A.A., and Wong, J.W. 2003b. Significant reductions in heart and lung doses using deep inspiration breath hold with active breathing control and intensity-modulated radiation therapy for patients treated with locoregional breast irradiation. *Int. J. Radiat. Oncol. Biol. Phys. 55*: 392–406.

Roychoudhari, R., Robinson, D., Putcha, V., Cuzick, J., Darby, S., and Moller, H. 2007. Increased cardiovascular mortality more than fifteen years after radiotherapy for breast cancer: a population-based study. *BMC Cancer. 7*: 9.

Rutqvist, L.E., and Johansson, H. 1990. Mortality by laterality of the primary tumor among 55,000 breast cancer patients from the Swedish Cancer Registry. *Brit. J. Cancer 61*: 866–868.

Rutqvist, L.E., Liedberg, A., Hammar, N., and Dalberg, K. 1998. Myocardial infarction among women with early-stage breast cancer treated with conservative surgery and breast irradiation. *Int. J. Radiat. Oncol. Biol. Phys. 40*: 359–363.

Seddon, B., Cook, A., Gothard, L., Salmon, E., Latus, K., Underwood, S.R., and Yarnold, J. 2002. Detection of defects in myocardial perfusion imaging in patients with early breast cancer treated with radiotherapy. *Radiother. Oncol. 64*: 53–63.

Sidhu, S., Sidhu, N.P., Lapointe, C., and Gryschuk, G. 2006. The effects of intrafraction motion on dose homogeneity in a breast phantom with physical wedges, enhanced dynamic wedges, and ssIMRT. *Int. J. Radiat. Oncol. Biol. Phys. 66*: 64–75.

Sixel, K.E., Aznar, M.C., and Ung, Y.C. 2001. Deep inspiration breath hold to reduce irradiated heart volume in breast cancer patients. *Int. J. Radat. Oncol. Biol. Phys. 49*: 199–204.

Smith, R.P., Bloch, P., Harris, E.E., McDonough, J., Sarker, A., Kassaee, A., Avery, S., and Solin, L. J. 2005. Analysis of interfraction and intrafraction variation during tangential breast irradiation with an electronic portal imaging device. *Int. J. Radiat. Oncol. Biol. Phys. 62*: 373–378.

Tan-Chiu, E., Yothers, G., Romond, E., Geyer, C.E., Ewer, M., Keefe, D., Shannon, R.P., Swain, S.M., Brown, A., Fehrenbacher, L., Vogel, V.G., Seay, T.E., Rastogi, P., Mamounas, E.P., Wolmark, N., and Bryant, J. 2005. Assessment of cardiac dysfunction in a randomized trial comparing doxorubicin and cyclophophamide followed by paclitaxel, with or without trastuzumab as adjuvant therapy in node-positive, human epidermal growth factor receptor 2 – overexpressing breast cancer. NSABP B-31. *J. Clin. Oncol. 23*: 7811–7819.

Vallis, K.A., Pintilie, M., Chong, N., Holowaty, E., Douglas, P.S., Kirkbride, P., and Wielgosz, A. 2002. Assessment of coronary heart disease morbidity and mortality after radiation therapy for early breast cancer. *J. Clin. Oncol. 20*: 1036–1042.

Vicini, F., Sharpe, M., Kestin, L., Wong, J., Remouchamps, V., and Martinez, A. 2004. The use of intensity modulated radiation therapy in the treatment of breast cancer: evolving definition, misdirected criticism, and untoward effects. *Int. J. Radiat. Oncol. Biol. Phys. 58*: 1642–1644.

Woo, T.C., Pignol, J.P., Rakovitch, E., Vu, T., Hicks, D., O'Brien, P., and Pritchard, K. 2006. Body radiation exposure in breast cancer radiotherapy: impact of breast IMRT and virtual wedge compensation techniques. *Int. J. Radiat. Oncol. Biol. Phys. 65*: 52–58.

Woodward, W.A., Strom, E.A., McNeese, M.D., Perkins, G.H., Outlaw, E.L., Hortobagyi, G.N., Buzdar, A.U., and Bucholz, T.A. 2003. Cardiovascular death and second non-breast cancer malignancy after postmastectomy radiation and doxorubicin-based chemotherapy. *Int. J. Radiat. Oncol. Biol. Phys. 57*: 327–335.

Yu, X., Prosnitz, R.R., Zhou, S., Hardenberg, P.H., Tisch, A., Blazing, M.A., Borges-Neto, S., Hollis, D., Wong, T., and Marks, L.B. 2003. Symptomatic cardiac events following radiation therapy for left-sided breast cancer: possible association with radiation therapy-induced changes in regional perfusion. *Clin. Breast Cancer 3*: 193–197.

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