Theranostic and Molecular Classification of Breast Cancer

Kristine M. Cornejo, MD; Dina Kandil, MD; Ashraf Khan, MD, FRCPath; Ediz F. Cosar, MD

Breast cancer is one of the leading causes of cancer-related death in women worldwide. During the past 2 decades, progress has been made in the management of breast cancer, including targeted therapy. Although morphology still remains the main cornerstone for diagnosis, molecular classification of breast carcinoma is used to identify subsets with significant prognostic and therapeutic implications. Ancillary studies, such as immunohistochemistry (IHC) markers for hormone receptors (HRs) such as estrogen receptor (ER), progesterone receptor (PR), and HER2, can act as prognostic or predictor indicators in breast cancer and are the most commonly used. During the past decade, great strides have been made in RNA- and DNA-based ancillary studies in identifying molecular portraits of breast carcinoma and influencing routine therapy.

Breast carcinoma is highly heterogeneous at both the clinical and molecular level. Therefore, separating breast cancer into various subsets based on its biologic behavior or therapeutic options may be helpful. In this review, we cover HR evaluation by IHC and the molecular classification of breast cancer.

HORMONE RECEPTORS

Estrogen receptor and PR are both prognostic and predictive markers for outcome and response to endocrine therapy, respectively. Estrogen receptor and PR bind hormones that exert their effects in the nucleus. Therefore, immunostaining for both receptor proteins is demonstrated in the nucleus of healthy breast tissue, which is commonly used as an internal control.

Progesterone receptors serve as an indicator of an intact ER pathway, which reflects the dependence of the ER/PR axis and predicts which patients will respond to hormone therapy because adequate estrogen levels are required to transcribe PR. Therefore, PR expression is generally reported with ER expression. Patients with PR+ tumors tend to have a significantly longer disease-free and overall survival than do patients with tumors that are PR-. Approximately 70% of breast cancers will be ER+, which is associated with tumor histology and grade.

Accurate and quantitative assessment of HR results is critical when using IHC studies to determine therapeutic targets. Several factors can dramatically affect HR IHC results, which we will touch on briefly below.

Preanalytic Factors That Affect IHC Staining With HR.—One factor that can affect HR IHC results is tissue fixation and its effect on IHC staining, which typically relates to the type of fixative used and the duration of fixation. Hormone receptor analysis is often performed on core biopsy samples. Both small (biopsies) and large (resection) specimens require time to fix. However, for the formalin to permeate the tissue effectively, larger specimens must be sectioned, ideally at 0.5-cm thickness. The optimum formalin exposure time for ER and PR is 6 to 8 hours, and the antigen can be retrieved by increasing the retrieval time for overexposed tissue. However, underfixation may lead to false-negative HR results by IHC. Delayed formalin fixation, leading to prolonged tumor ischemia, decreases HR expression. Therefore, specimens should be fixed within 1 hour of receipt. In addition, overnight storage at 4°C has caused diminished HR expression by IHC similar to delayed formalin fixation. Recently, the American Society of
Clinical Oncology (ASCO) and the College of American Pathologists (CAP) jointly introduced guidelines recommending prompt fixation of breast tissue, which includes both core biopsies and resections, for 8 to 72 hours in 10% neutral-buffered formalin for HR IHC evaluation.4,11

Analytic Factors That Affect IHC Staining With HR.—Analytic factors refer to the testing protocol, such as the reagents, equipment, controls, types of antibodies, and the competency of the staff performing IHC. Documentation of time of formalin exposure and the type of fixative used is recommended on pathology reports, which helps with interpreting ER results, particularly if they are aberrant or unexpected.4

The validated and widely available antibody clones for ER are 1D5, 6F11, and SP1.12–14 These antibodies target the ER-α isoform and require a high degree of concordance. However, there are subtle differences between them with various positive cutoff values, and control testing should be conducted for each test performed. A second ER isoform exists, called ER-β, which has an undetermined role.15,16 The role of ER-β and whether it has prognostic or predictive value is unclear, and ER-β can be found in several tissues other than breast.17–21 The SP1 rabbit monoclonal antibody appears to have more-intense nuclear staining but gives results similar to other monoclonal antibodies.1,2,2,2 Recent studies suggest that with use of these newer antibodies, the actual number of breast tumors that are ER+/PR+ is closer to zero as these tumors, which comprised approximately 10%, were likely falsely negative for ER.2,23

Postanalytic Factors That Affect IHC Staining With HR.—Postanalytic factors encompass reporting methods, quality assurance measures, criteria for interpretation or scoring schemes, and the competency of the pathologist interpreting the results. Nuclear staining for ER in greater than 1% of the cells is considered positive because tumors with any extent of HRs may benefit from hormonal therapy.4,14,24 The results should be semiquantitated with various positive cutoff values, and control testing should be conducted for each test performed. A second ER isoform exists, called ER-β, which has an undetermined role.15,16 The role of ER-β and whether it has prognostic or predictive value is unclear, and ER-β can be found in several tissues other than breast.17–21 The SP1 rabbit monoclonal antibody appears to have more-intense nuclear staining but gives results similar to other monoclonal antibodies.1,2,2,2 Recent studies suggest that with use of these newer antibodies, the actual number of breast tumors that are ER+/PR+ is closer to zero as these tumors, which comprised approximately 10%, were likely falsely negative for ER.2,23

Table 1. Estrogen Receptor (ER) and Progesterone Receptor (PR) Immunohistochemical Interpretation

<table>
<thead>
<tr>
<th>Test</th>
<th>Cells Stained, %</th>
<th>Intensity</th>
<th>Status*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER invasive carcinoma</td>
<td>1; 1–10; 11–25; 26–75; &gt;75</td>
<td>1; 2; 3</td>
<td>Negative/low positive/positive</td>
</tr>
<tr>
<td>ER in situ carcinoma</td>
<td>1; 1–10; 11–25; 26–75; &gt;75</td>
<td>1; 2; 3</td>
<td>Negative/low positive/positive</td>
</tr>
<tr>
<td>PR invasive carcinoma</td>
<td>1; 1–10; 11–25; 26–75; &gt;75</td>
<td>1; 2; 3</td>
<td>Negative/low positive/positive</td>
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<td>1; 2; 3</td>
<td>Negative/low positive/positive</td>
</tr>
</tbody>
</table>

*Criteria for ER/PR status: negative, <1% tumor cell nuclei immunoreactive; low positive, 1%–10% tumor cell nuclei immunoreactive; positive, >10% tumor cell nuclei immunoreactive.

false-negative results, particularly in small core biopsies.27 Therefore, HR IHC should be repeated on resections in which the ER and PR were negative on the biopsy. There are 2 isoforms for PR—PRA and PRB—which are both recognized by the commercially available antibodies.

HER2

The human epidermal receptor protein-2 (HER2) is an oncprotein, which belongs to the epidermal growth factor receptor (EGFR) family and is expressed at low levels in breast duct epithelium.2 Approximately 10% to 30% of breast cancers demonstrate HER2 gene amplification or protein overexpression.2,28–30 HER2 positivity is an independent prognostic marker of clinical outcome in patients with either node-negative and node-positive tumors and plays a role as a predictive factor because it can determine response to taxane and anthracycline-based therapy.2,31–34 HER2+ tumors generally show relative resistance toward selective endocrine receptor modulators, such as tamoxifen, and such resistance is less likely toward estrogen-depletion therapies, such as aromatase inhibitors.35–37 In 1998, the US Food and Drug Administration approved trastuzumab (Genentech, Inc., San Francisco, California), a humanized monoclonal antibody to HER2 for use in metastatic breast cancer. Trastuzumab improves survival, response rates, and time to progression when used alone or in combination with chemotherapy.38,39 Although approved for use in metastatic cancer, several prospective randomized clinical trials have shown therapeutic benefit in early stage breast cancers by potentially reducing the risk of mortality by one-third and of recurrence by one-half.40–43

An accurate determination of HER2 status is essential given the enormous therapeutic benefit derived from trastuzumab in HER2+ tumors. In addition, lapatinib (GlaxoSmithKline, King of Prussia, Pennsylvania), which is an inhibitor of HER2 and EGFR (HER1), was approved by the US Food and Drug Administration for the treatment of HER2+ breast cancers and has been shown to provide significant improvement in outcome.41 Because of its prognostic and predictive value, HER2 status should be determined on all newly diagnosed, invasive breast cancers, as recommended by the ASCO/CAP guidelines.44 Since publication of the 2007 guidelines, the ASCO/CAP Update Committee, after a comprehensive review of the peer-reviewed literature since 2006, identified criteria and areas requiring clarifications and recently revised the guidelines and recommendations as deemed appropriate. This article will cover the important points of the updated guidelines and recommendations. More details, including comparison with 2007 HER2 testing guidelines can be found in the recently updated 2013 HER2 testing guidelines and recommendations.32 To ensure reliable HER2 testing, the 3 quality improvement categories are reviewed.
Preanalytic Quality Improvement.—The current ASCO/CAP guidelines recommend using 10% neutral-buffered formalin with tissue fixed for 6 to 72 hours.\textsuperscript{52} HER2 testing can be compromised by both overfixation and underfixation. Overfixation may occur with alcohol, which can lead to antigen diffusion. However, that is generally not an issue with formalin. In addition, enzymatic digestion times for in situ hybridization can be altered for optimal antigen retrieval. Underfixation may alter antigen retrieval as well, as shown for ER on tissues fixed for less than 6 hours.\textsuperscript{45} Therefore, needle core biopsies fixed for less than 1 hour or resections fixed for less than 6 hours should not be tested.

Analytic Quality Improvement.—The monoclonal antibodies CB11 and 4D5 were used in the first clinical trial assay for determining HER2 status and the effect of trastuzumab on metastatic cancer. Only patients with scores of 2\textsuperscript{+} or 3\textsuperscript{+} were eligible to receive the drug in those studies. Retrospective analyses revealed therapeutic benefit with trastuzumab in cases with a 3\textsuperscript{+} score or HER2 amplification by fluorescence in situ hybridization (FISH) because only 24% of cases with a 2\textsuperscript{+} score showed amplification by FISH.\textsuperscript{39} A polyclonal antibody, HercepTest (DAKO Corporation, Carpinteria, California), was compared with CB11 using the same scoring criteria and was found to have a 79% concordance rate, which ultimately led to US Food and Drug Administration approval. However, consequent studies revealed that HercepTest had a slightly higher false-positive rate than did the other monoclonal antibodies when compared with FISH.\textsuperscript{40–49} Therefore, all 2\textsuperscript{+} cases by IHC are sent for reflex FISH testing, which, in most cases, will resolve any HER2 status dilemma. A rabbit monoclonal antibody 4B5 (Ventana Medical Systems, Inc, Tucson, Arizona) has recently become available and has been found to be more reliable with excellent inter-laboratory reproducibility and cleaner staining.\textsuperscript{50}

Postanalytic Quality Improvement.—A HER2 IHC score of 2\textsuperscript{+} appeared to be problematic, causing reflex testing with FISH for HER2 gene copy number assessment. Coupling a 2\textsuperscript{+} IHC score with FISH avoids any adverse clinical outcomes. However, a false-positive 3\textsuperscript{+} IHC score can result in initiation of inappropriate therapy, which may be expensive, ineffective, and potentially harmful.\textsuperscript{38,51–53} Therefore, the pathologist interpreting HER2 IHC results, especially when the staining intensity is a weak 3\textsuperscript{+} or strong 2\textsuperscript{+}, should exercise caution. To reduce misinterpretation of HER2 IHC, the 2007 ASCO/CAP guidelines modified the criteria for a 3\textsuperscript{+} score from 10% to 30% of positive cells.\textsuperscript{44} HER2 staining heterogeneity is more common in 1\textsuperscript{+} to 2\textsuperscript{+} weakly positive cases. In addition, nonneoplastic breast epithelium expresses low levels of HER2 protein, which can produce a 1\textsuperscript{+} or even 2\textsuperscript{+} staining, which may cause false-positive results.\textsuperscript{54} Therefore, the criteria aids in reducing the number of 3\textsuperscript{+} false-positive results, and those results that score as 2\textsuperscript{+} (11%–30%) or equivocal are reflexed to FISH for confirmation. However, based on the recent literature and clinical experience concerning the possibility of false-negative HER2 test results, the Update Committee in 2013 decided to revert back to the previously used IHC criterion of more than 10% stained cells that was used for patient entry into the clinical trials.\textsuperscript{52} To achieve consistent interpretation, image analysis systems can be used. However, they require regular maintenance and calibration. To effectively communicate the results of the HER2 IHC to clinicians, a scoring template with a description of the staining can be used (Table 2).

Quality assurance measures should be produced for HER2 testing and should include a comparison of HER2 IHC positive case results and amplification by FISH. Correlation of those results is essential for quality control, and the ASCO/CAP guidelines require validation that IHC samples disagree with a validated assay in only 5% or fewer tests. A rigorous clinical validation is required to achieve such a high level of concordance. The US Food and Drug Administration approved a scoring system for HER2 IHC by obtaining a normalized IHC score for breast cancer, which significantly decreases the false-positive results.\textsuperscript{54} The HER2 IHC score is obtained by subtracting the score representing the level of immunostaining on the nonneoplastic breast epithelium from the score representing the level of immunostaining on the tumor. This technique can yield high concordance rates between the FISH results and IHC testing.

**HER2 FISH**

Fluorescence in situ hybridization is a molecular cytogenetic technique that detects specific DNA sequences on the chromosome using fluorescent probes. A HER2 probe is used to identify HER2 gene amplification in HER2 FISH testing. The HER2 gene resides on chromosome 17. Probes for HER2 may include a single-color or dual-color probe with one sequence labeled for the HER2 gene and another for the centromere of chromosome 17 (CEP17). To determine amplification, an absolute HER2 gene copy number or a ratio of HER2 to CEP 17 is used for single- and dual-color probes, respectively.

The HER2 FISH assay is a better determinant for predicting response to trastuzumab than HER2 IHC, which may be because of tissue fixation, the type of antibody used, the criteria for determining positivity, or the interpretation of the IHC test.\textsuperscript{30} The ASCO/CAP guidelines require 95% concordance of both negative and unequivocal results with FISH results, which, in theory, is reasonable, given HER2 gene amplification almost always results in HER2 protein overexpression.\textsuperscript{54,55} However, in actuality, such concordance is quite difficult, with many studies demonstrating concordance rates closer to 80% to 90%, which may reflect the
wide variation in instrumentation, methodology, and experience of the laboratories performing the test.\textsuperscript{46,56–58} 

HER2-positive threshold by FISH was revised by the 2007 ASCO/CAP panel from \( \text{HER2}/\text{CEP17} \) ratio of 2.0 to 2.2.\textsuperscript{44} This raised the concern that many patients with HER2 FISH status falling between ratio 2 and 2.2 would not be eligible for the initial trastuzumab adjuvant trial. After careful consideration the 2013 Update Committee has decided to revert the HER2-positive FISH criteria to \( \text{HER2}/\text{CEP17} \) ratio to 2 and above.\textsuperscript{182} Algorithm for the evaluation of HER2 protein expression by IHC and \( \text{HER2} \) gene amplification by single-probe or dual-probe in situ hybridization (ISH) is presented in Figure 1.

A subpopulation of breast cancers with \( \text{HER2} \) gene amplification by FISH may reveal genetic heterogeneity, which can give rise to discrepant results between IHC and FISH.\textsuperscript{39} Genetic heterogeneity exists in 5% to 30% of tumors and was defined by the 2007 ASCO/CAP guidelines as having 5% to 50% of infiltrating tumor cells with a \( \text{HER2} \) to CEP17 ratio greater than 2.2.\textsuperscript{60} The 2013 guidelines recommend scanning the entire FISH slide prior to counting \( \text{HER2} \) signals and/or using \( \text{HER2} \) IHC to identify areas of amplified cells. If aggregates of amplified cells comprise >10% of the total invasive tumor cell population, the number of CEP17 and \( \text{HER2} \) signals should be counted in a minimum of 20 non-overlapping and contiguous invasive cancer cell nuclei in at least 2 tumor areas of each population (amplified and non-amplified) and reported separately.\textsuperscript{182}

The standards of care use \( \text{HER2} \) FISH to determine gene amplification when a \( \text{HER2} \) IHC result is equivocal (2\textsuperscript{+}), which occurs in up to 25% of all breast cancers.\textsuperscript{61} Although FISH is useful in clarifying gene amplification, cases with 2\textsuperscript{+} staining by \( \text{HER2} \) IHC may be biologically different than cases of 3\textsuperscript{+} staining because 3% to 15% of breast cancers that show protein overexpression do not have concomitant gene amplification.\textsuperscript{50} Some IHC cases with 2\textsuperscript{+} scores in which \( \text{HER2} \) is not amplified may be due to polyploidy/aneuploidy or polysomy for chromosome 17.\textsuperscript{52–66} Therefore, it has been proposed that probes targeting other genes on chromosome 17, such as \( \text{TP53} \), instead of CEP17, may better determine true \( \text{HER2} \) amplification status.\textsuperscript{67} In addition, a \( \text{HER2} \)-equivocal FISH results may be due to the specimen type on which the test was performed. \( \text{HER2} \) status determining by FISH assay on a larger tumor sample obtained from a resection affects patient management if the result is equivocal on the biopsy, indicating genetic heterogeneity in tumors with low-level \( \text{HER2} \) gene copy numbers.\textsuperscript{68} Overall, equivocal IHC and FISH cases are extremely difficult to interpret, even by highly experienced laboratories, emphasizing the importance of standardization.\textsuperscript{69}

Although FISH testing is useful, there are limitations to FISH assays, such as the lack of morphologic details, the use of dark-field fluorescence microscopy, and the longer...
evaluation time for interpretation. Since the publication of the 2007 guidelines, new diagnostic strategies including detection of HER2 gene amplification by bright-fieldISH have been introduced. Because of high concordance with FISH, reproducibility and demonstrable clinical utility, the current ASCO/CAP guidelines endorses FDA-approved bright-fieldISH methods for the detection of HER2 gene amplification. Among these methods, chromogenic in situ hybridization uses diaminobenzidine as a chromogen, which causes brown signals that can be visualized by light microscopy with better preservation of the morphologic details. However, the signals may be difficult to interpret because they may not be discrete on light microscopy. Silver in situ hybridization uses an enzyme-linked probe to deposit silver ions from the solution on the target site, making interpretation easier, resulting in a dense, high-resolution, black stain that is readily identifiable. These new in situ hybridization assays will become more widely available and possibly automated. Furthermore, FISH evaluation is performed under an oil immersion lens (100X), limiting the amount of tumor analyzed. These new in situ hybridization assays will allow greater tumor analysis and better examination of the tumor for heterogeneity with respect to HER2 gene copy number.

HER2 Reverse Transcription-Polymerase Chain Reaction

Reverse transcription-polymerase chain reaction (RT-PCR) is a comparatively newer approach to detect HER2 gene amplification in breast cancer. Studies have shown a concordance rate of 92% to 94% between FISH and RT-PCR. Reverse transcription PCR may represent a better methodology than FISH, especially for equivocal samples by IHC. HER2 gene amplification is highly associated with mRNA overexpression and determination of mRNA by RT-PCR is highly matched with DNA amplification by FISH. Therefore, RT-PCR offers quantitative values, compared with FISH, which offers semiquantitative values. The advantages of RT-PCR is that it is not as time consuming as FISH. The pitfalls of RT-PCR are that it requires tissue dissection to ensure only invasive tumor cells are evaluated because ductal carcinoma in situ may cause false results. In addition, other tumor-cell components can lead to dilution and could cause an underestimation of HER2 messenger RNA (mRNA) levels. Studies have found a high level of false-negative results for HER2 with RT-PCR, requiring further investigation.

Molecular Classification of Breast Carcinoma

Perou et al proposed the first molecular classification of breast cancer using gene expression analysis on DNA microarrays. The intrinsic gene set, or the genes in which the expression patterns were characteristic of a specific tumor, were analyzed in 115 breast carcinoma cases. Based on the expression of various gene sets, the researchers categorized breast cancers into 5 major subsets with prognostic significance, such as the basal-like, HER2 overexpressing, luminal A, luminal B, and normal breastlike subtypes. Those tumors can further be separated into the ER+ and ER- branches. Several independent groups have reproduced those findings, noting that the various molecular subtypes have distinct clinical outcomes and responses to therapy. For instance, the luminal A subtype has the best prognosis, basal-like and HER2 subtypes have the worst prognosis, and the luminal B subtype has an intermediate prognosis. In addition, IHC markers can be used to routinely identify molecular subtypes (Table 3).

<table>
<thead>
<tr>
<th>Molecular Subtype</th>
<th>ER/PR/HER2</th>
<th>Other IHC</th>
<th>Molecular Pathways</th>
<th>Histologic Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal A</td>
<td>ER++, PR++, HER2+</td>
<td>CK8+, CK18+, GATA3+</td>
<td>ER-responsive genes</td>
<td>Low</td>
</tr>
<tr>
<td>Luminal B</td>
<td>ER+, PR-/-, HER2-/-</td>
<td>CK8+, CK18+, GATA3+</td>
<td>ER-responsive genes, TP53 mutations</td>
<td>Intermediate</td>
</tr>
<tr>
<td>ERBB2</td>
<td>ER-, PR-, HER2+</td>
<td>CK5+, CK14+, CK17+, EGFR+, c-KIT+, CD44+, nestin+, caveolin 1+, caveolin 2+, P-cadherin+</td>
<td>HER2 genes, TP53 mutations</td>
<td>High</td>
</tr>
<tr>
<td>Basal-like</td>
<td>ER-, PR-, HER2-</td>
<td>CK8/18, CK18, TP53 mutations</td>
<td>TP53 mutations, BRCA1 pathway</td>
<td>High</td>
</tr>
<tr>
<td>Breast-like</td>
<td>ER++, PR-/-, HER2-</td>
<td>CK8/18, CK18, TP53 mutations</td>
<td>Low</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CK, cytokeratin; ER, estrogen receptor; GATA3, GATA binding protein 3; HER2, human epidermal receptor protein-2; IHC, immunohistochemistry; PR, progesterone receptor.
high response rate to chemotherapy.\textsuperscript{85,102,103} The immunohistochemical and morphologic features of basal-like tumors are similar to those arising in women with BRCA1 germline mutations because of abnormalities in the BRCA1 pathway.\textsuperscript{95,101,104}

**Breastlike Subtypes.**—These tumors often express adipose tissue and other nonepithelial cell genes, including basal cell genes, and typically cluster with healthy breast and fibroadenomas.\textsuperscript{88,105} The clinical significance of this group has yet to be determined.\textsuperscript{80,81,88,106} However, some researchers have proposed that this subtype was misrepresented because of poor tissue sampling, creating a false category.\textsuperscript{87,105,107}

**Gene Expression Profiling of Breast Cancer**

There are several other prognostic or predictive gene sets in breast cancer, such as MammaPrint (Agendia, Amsterdam, the Netherlands), Oncotype Dx (Genomic Health Inc, Redwood City, California), the Rotterdam Signature (Erasmus MC-Daniel den Hoed Cancer Center, Rotterdam, Netherlands, in conjunction with Veridex, LLC.), and the HOXB13/IL17RB Ratio (AviaraDx Inc, Carlsbad, California), which are now widely used in clinical practice. Algorithms have been created using these modalities for therapy decision-making, and they provide excellent treatment of tumors with specific clinicopathologic features (Figure 2).

**MammaPrint.**—MammaPrint is the first prognostic gene set available in clinical practice and was developed in Amsterdam by van ‘t Veer et al\textsuperscript{79} and van de Vijer et al\textsuperscript{108} based on a 70-gene set profile using an oligonucleotide array. The complementary DNA (cDNA) microarray-based test, which was initially performed on freshly frozen tissue has now been revised to work on formalin-fixed, paraffin-embedded tissue with RT-PCR, helps in deciding whether the patient should receive adjuvant chemotherapy.\textsuperscript{109} The test is offered for patients with tumors less than 5 cm, with lymph node–negative disease and low clinical stage (stage I or II), and separates breast cancers by gene signatures into those with good versus poor prognoses. If the gene signature shows a good prognosis, the patient may decide not to receive chemotherapy, opting instead for hormone therapy, if their tumor is ER\textsuperscript{+} with a minimal risk of recurrence.\textsuperscript{88} The poor prognosis signature consists of genes regulating cell cycle, invasion, angiogenesis, and metastasis.\textsuperscript{110} Patients with a poor prognostic signature receive chemotherapy and hormone therapy if the tumor is ER\textsuperscript{+}.\textsuperscript{88}

The Microarray in Node-Negative Disease May Avoid Chemotherapy (MINDACT) trial is a large, multicentric, prospective randomized controlled study aimed at investigating the clinical utility of MammaPrint in 6000 patients with node-negative breast cancer. The patients have their risk assessed by MammaPrint and their clinicopathologic factors analyzed using Adjuvant! Online (www.adjuvantonline.com, accessed February 13, 2013). If both methods classify the patient as having a high risk of relapse, chemotherapy is offered. If both methods classify the patient as having a low risk of relapse, chemotherapy is withheld. If the methods are discordant, they are randomly assigned for treatment decision-making. The results of the MINDACT trial will help to assess the feasibility of its clinical application and to determine whether it can accurately identify women who can be spared the morbidity of adjuvant chemotherapy without a negative effect on their survival.\textsuperscript{111}

The advantage of MammaPrint is that the test can be performed on both ER\textsuperscript{+} and ER\textsuperscript{−} tumors. However, the clinical utility of MammaPrint with ER\textsuperscript{−} breast cancers is limited because only 0% to 4% of patients with ER\textsuperscript{−} tumors are considered to have a good prognosis by the gene signature.\textsuperscript{108,112–116}

**Oncotype Dx.**—Oncotype Dx is a commercially available, RT-PCR–based assay, which provides a recurrence score

Figure 2. Algorithm for commercially available cancer prognostic signatures. Abbreviations: ER, estrogen receptor; FFPE, formalin-fixed, paraffin-embedded tissue; LN, lymph node; PR, progesterone receptor; RS, recurrence score.
(RS) based on a 21-gene panel associated with cancer prognosis and pathology formed from a prospectively selected set of 250 candidate genes. The test has been shown to provide predictive and prognostic information in ER\(^+\), lymph node–negative tumors by providing an RS value, which varies from 0 to 100 and predicts the risk of 10-year recurrence. Patients are divided into 3 risk groups by their RS: low, less than 18; intermediate, 18 to 31; and high, 31 or greater. Patients with a low RS have a low risk of recurrence and gain little benefit from chemotherapy versus those with a high RS, who have a high risk of recurrence and benefit from chemotherapy. Patients with an intermediate risk of recurrence appear to gain little benefit from chemotherapy, based on the preliminary results from the National Surgical Adjuvant Breast and Bowel Project B20 study.

Oncotype Dx has been included in the ASCO/CAP guidelines on the use of tumor markers in breast cancer as a predictor of recurrence for patients with ER\(^+\), lymph node–negative breast cancer. The Trial Assessing Individualized Options for Treatment for Breast Cancer (TAILORx) for breast cancer is a randomized study designed to evaluate whether RS can predict the outcome of adjuvant chemotherapy in patients with node-negative, HR\(^+\) breast cancers. In addition, it will help to decide whether chemotherapy is beneficial in patients with an intermediate risk of recurrence. Goldstein et al\(^{119}\) evaluated the prognostic utility of this assay on patients with either lymph node–negative or lymph node–positive, ER\(^+\) tumors treated with doxorubicin–containing chemotherapy and found that the RS was a highly significant predictor of recurrence in either subset of patients. Another study examined the correlation of the RS and the likelihood of distant relapse after 5 years of tamoxifen therapy and found that it was predictive of relapse as well as overall survival. Furthermore, there is a high degree of concordance between FISH and RT-PCR using Oncotype Dx for HER2 status. However, if breast cancers are already found to be HER2\(^+\), it is not recommended to send the tumor for Oncotype Dx testing because the patient should not have trastuzumab therapy withheld. Recently, Oncotype Dx was approved for testing in ductal carcinoma in situ (DCIS) based on the results of a validation study using the ECOG E5194 data set, which found significant correlation between the RS and ipsilateral carcinoma (DCIS or invasive). The RS may be useful in identifying patients that can benefit from radiation therapy following lumpectomy for DCIS. In our practice, we use Oncotype Dx testing on invasive carcinomas and have not yet adopted its use for DCIS.

The difficulty with Oncotype Dx is that the assay does not apply to ER\(^+\) tumors. However, the advantage of the test is that it can be performed on formalin-fixed, paraffin-embedded tissue.

Rotterdam Signature.—A test developed in Rotterdam, the Netherlands, in collaboration with Veridex, LLC, comprises a 76-gene microarray assay that was developed to predict metastatic disease comprises a 76-gene microarray assay that was developed in collaboration with Veridex, LLC, in the Netherlands, to predict metastatic disease. The Rotterdam Signature assay is a highly significant predictor of recurrence in either subset of lymph node–positive, ER\(^+\) breast cancers who have received treatment with tamoxifen. The assay analyzes the ratio of homeobox B13 (HOXB13) and interleukin 17 receptor (IL17RB) gene expression using RT-PCR. Ma et al\(^{120}\) initially identified those 2 genes in a cohort of 60 patients treated with tamoxifen as being highly associated with clinical outcome. They later analyzed 852 breast cancers from both untreated (n = 566; 66%) and tamoxifen-treated (n = 286; 34%) tumors using real-time quantitative RT-PCR and found that the expression of HOXB13 was associated with recurrence, and expression of IL17RB was associated with nonrecurrence. The HOXB13/IL17RB (H/I) index predicted clinical outcome independent of treatment in ER\(^+\), lymph node–negative patients.

To improve the assay, a numerical score called the molecular grade index (MGI) was created by selecting 5 cell-cycle–related genes (BUB1B, CENPA, NEK2, RACGAP1, and RRM2) to be used concurrently with the H/I index to improve risk stratification. The combined MGI and H/I index stratifies patients into 3 risk groups: low risk, low for MGI and low or high H/I index; intermediate risk, high MGI and low H/I index; and high risk, high for both MGI and H/I index. A high MGI and H/I index together predicted poor outcome for the high-risk group. The estimated 10-year metastasis-free, survival probability was 98%, 87%, and 60% for the low, intermediate, and high-risk groups, respectively. The MGI and H/I index are complementary because a high proliferation rate (high MGI) and decreased cell death (high HOXB13/IL17RB) promote aggressive tumor growth when in combination.

The HOXB13/IL17RB Ratio is commercially available. The advantage of the H/I index test is that it can be performed on formalin-fixed, paraffin-embedded tissue. In addition, the assay can be applied to patients with lymph node–negative, ER\(^+\) breast cancers who have received treatment with tamoxifen.

Comparison of Prognostic Signatures

In comparing prognostic signatures, Oncotype Dx and the H/I index are done on formalin-fixed, paraffin-embedded tumor tissues and use RT-PCR. The Rotterdam Signature is performed on freshly frozen tumor tissues for DNA microarrays. MammaPrint has now been revised to work on both freshly frozen and formalin-fixed, paraffin-embedded tumor tissue. Oncotype Dx and MammaPrint are the 2 best-validated models. When comparing these 2 assays using the Theranostic and Molecular Classification—Cornejo et al
same cohort of 295 patients, they were found to have an agreement of 81%. Despite minimal gene overlap, both were found to be accurate prognostic indicators. In a more recent study, Oncotype Dx, MammaPrint, and the Rotterdam Signature were found to be prognostic indicators in patients with ER+, lymph node–negative tumors. Furthermore, Oncotype Dx and MammaPrint were found to be independent predictors of relapse.

OTHER BREAST TUMOR MARKERS

**Ki-67**

Many studies have evaluated the expression of proliferating cell nuclear antigen or Ki67 by IHC as a method of monitoring tumor proliferation and its role as a prognostic marker. However, different cutoff points have been used to determine the labeling index, making it difficult to compare the studies because there is a lack of standardization in scoring. Therefore, the 2007 ASCO/CAP guidelines do not recommend using proliferative markers in routine clinical practice. However, the International Ki67 Breast Cancer Working Group recognized the utility of Ki67 and its role as a possible pharmacodynamic marker for neoadjuvant hormone therapy. Tumor recurrence rates were reflected by the differences in Ki67 suppression by various treatment arms using endocrine therapy regimens. The early absence of Ki67 suppression by hormone therapy was predictive of therapeutic failure. Therefore, the Ki67 index may be used to stratify patients into those who may benefit from neoadjuvant chemotherapy versus endocrine therapy, and it is currently being evaluated in the Z1031 trial. In addition, the Ki67 index, as a natural log-transformed interval in the posttreatment specimens of patients in the P024 trial, which compared tamoxifen and letrozole, showed it was significantly associated with relapse–free survival and breast cancer–specific survival. Therefore, an elevated Ki67 index despite treatment with an aromatase–inhibitor infers a poor prognosis. Although positive staining of Ki67 index despite treatment with an aromatase–inhibitor infers a poor prognosis. Although positive staining of Ki67 index despite treatment with an aromatase–inhibitor infers a poor prognosis. Although positive staining of Ki67 index despite treatment with an aromatase–inhibitor infers a poor prognosis.

**p53**

TP53 is a tumor suppressor gene that is commonly mutated in cancer and is routinely assessed by IHC looking for p53 overexpression. The DO-7 antibody clone is commonly used and recognizes both the mutant p53 and wild-type proteins, making it difficult to interpret the stain. Overall, there is more intense and diffuse staining with the mutant protein because it has a longer half-life than does the wild-type protein. Therefore, a negative result should be rendered with weak or patchy staining. p53 staining has been associated with a poor prognosis in breast cancer as there are certain tumors that demonstrate strong p53 immunoreactivity or contain p53 mutations, such as the basal-like tumors. Olivier et al showed that TP53 gene abnormalities were associated with a worse prognosis. Furthermore, TP53 deletions or mutations were prognostic in ER+, lymph node–negative patients. However, per the 2007 ASCO/CAP guidelines for testing in breast cancer, there is currently insufficient data to provide recommendations on the use of p53.

**Epidermal Growth Factor Receptor**

EGFR is a transmembrane growth factor receptor protein that has been found to be overexpressed in less than 10% of all breast cancers. Similar to HER2 IHC, the best correlation of increased gene copy number of EGFR is with an IHC score of 3+. Breast cancers that overexpress EGFR tend to be negative for HR and are predominantly basal-like tumors. The mutations demonstrated in lung cancers that are responsive to tyrosine kinase inhibitors have not been identified in breast cancers. Further studies need to be performed to assess the role of EGFR as both a diagnostic and therapeutic tool.

**PROMISING ASSAYS IN THE FUTURE**

**MicroRNAs**

MicroRNAs (miRNAs) are small, nonprotein–coding RNAs that modulate gene expression and may act as tumor suppressor genes or oncogenes. Levels of specific miRNAs differ between normal and malignant breast tissue and among tumors of varying histologic grade, molecular subtype, lymph node status, and HR status. In addition, miRNAs have been linked to breast cancer invasion, proliferation, and metastasis. More recently, 4 miRNAs (miR-7, miR-128a, miR-21, and miR-516-3p) correlate with the aggressiveness of estrogen–positive, lymph node–negative breast cancer. MicroRNAs are an emerging and promising set of assays that may, with further study, have the potential for clinical evaluation of breast cancers. Furthermore, intrinsic molecular subtypes based on miRNA expression may have specific coexpression of miRNAs because certain miRNA signatures are coexpressed with certain molecular subtypes.

**One-Step Nucleic Acid Amplification**

One-step nucleic acid amplification (OSNA) is a novel method for evaluating lymph nodes for staging of breast cancer, and it has been tested in several studies. The OSNA is an automated molecular assay developed by Sysmex (Kobe, Japan) based on the semiquantification of cytokeratin 19 mRNA for the detection of clinically relevant nodal metastases. The assay has a 96% concordance rate with detailed histopathology complemented with IHC when using both methods on the same lymph node with a low false-negative and a low false–positive rate, making it suitable for intraoperative, sentinel lymph node evaluation. Most discordant cases are likely due to low tumor volume (micrometastases), which may be present for one test but not the other. The sensitivity of histology is inferior to OSNA, possibly because of sampling errors or small tumor volumes that can be missed. The OSNA detected more micrometastases than did detailed histology but detected similar or fewer macrometastases. However, it is uncertain whether the advantage of OSNA in detecting micrometastases is clinically more important than the exclusion of metastases detectable by complete sampling of a lymph node for histology. Although previous studies suggest micrometastases are of prognostic importance, the clinical effect in current management is unclear.

The National Surgical Adjuvant Breast and Bowel Project B32 trial analyzed the survival effect of micrometastases in sentinel lymph nodes and found it to be negligible. Therefore, it seems that, for micrometastases, current practice of intraoperative frozen sections with permanent
histology provides acceptable sensitivity. Future studies, including those with follow-up of patients who have undergone evaluation with the OSNA assay, will be helpful in clarifying the prognostic implications of this test, which may help to establish new breast cancer staging as well as adding to the arsenal of personalized medicine.

**Next-Generation Sequencing**

Important advances in the molecular analysis of tissues has led to next-generation sequencing revealing a spectrum of diverse genomic anomalies through whole genome sequencing. Next-generation sequencing, also known as *massively parallel sequencing*, is changing the molecular characterization of breast cancer at the genomic, transcriptomic, and epigenetic levels by evaluating at the nucleic acid, RNA, and DNA levels. These data, retrieved from whole-genome sequencing, provide a global view of individual tumor pathology by integrating a variety of information. Curtis et al. defined 45 regions of sequence alterations that are involved in the development of breast cancer through gene deregulation by analyzing single-nucleotide polymorphisms, copy number variants, and acquired somatic copy number aberrations of 1992 primary breast tumors. Stephens et al. examined the genomes of 100 breast tumors for somatic mutations and copy number changes and identified driver mutations in several new cancer genes, emphasizing the genetic diversity in breast cancer.

The ability to distinguish between passenger and driver mutations is helpful in determining which genes are inconsequential in tumor development but a key driver of therapeutic resistance. The identification of dynamic response markers may also be useful because the post-translational protein modifications or gene-expression changes that occur in response to therapy may be informative about the efficacy of the therapy. Additionally, markers of DNA damage, such as H2AFX, and impaired homologous recombination, such as RAD51 foci formation, may be predictive of therapeutic response to certain DNA-damaging chemotherapy agents and poly (adenosine diphosphate-ribose) polymerase inhibitors, respectively. Next-generation sequencing has identified genetic heterogeneity within breast cancers, which contain a wide spectrum of mutations, and has recently revealed that higher rates of mutations correlate with response to therapy.

Breast cancer can be divided into subtypes based on their gene expression pattern, proteomic analysis, and clinical features (Table 4). The common somatically mutated genes in breast cancer are TP53, PIK3CA, MAPK, AKT, and GATA3. In addition, recurrent mutations in the CBF transcription factor gene and deletions of RUNX1 have also been identified. The TP53, PTEN, and PIK3CA genes may be involved in the early developmental stages of triple-negative breast cancer with more genetic variation in the nonbasal subtype than there is in the nonbasal subtype. A higher rate of mutations correlate with response to therapy. Breast cancer can be divided into subtypes based on their gene expression pattern, proteomic analysis, and clinical features (Table 4). The common somatically mutated genes in breast cancer are TP53, PIK3CA, MAPK, AKT, and GATA3. In addition, recurrent mutations in the CBF transcription factor gene and deletions of RUNX1 have also been identified. The TP53, PTEN, and PIK3CA genes may be involved in the early developmental stages of triple-negative breast cancer with more genetic variation in the nonbasal subtype than there is in the nonbasal subtype. A higher rate of mutations correlate with response to therapy. Breast cancer can be divided into subtypes based on their gene expression pattern, proteomic analysis, and clinical features (Table 4). The common somatically mutated genes in breast cancer are TP53, PIK3CA, MAPK, AKT, and GATA3. In addition, recurrent mutations in the CBF transcription factor gene and deletions of RUNX1 have also been identified. The TP53, PTEN, and PIK3CA genes may be involved in the early developmental stages of triple-negative breast cancer with more genetic variation in the nonbasal subtype than there is in the nonbasal subtype. A higher rate of mutations correlate with response to therapy. Breast cancer can be divided into subtypes based on their gene expression pattern, proteomic analysis, and clinical features (Table 4). The common somatically mutated genes in breast cancer are TP53, PIK3CA, MAPK, AKT, and GATA3. In addition, recurrent mutations in the CBF transcription factor gene and deletions of RUNX1 have also been identified. The TP53, PTEN, and PIK3CA genes may be involved in the early developmental stages of triple-negative breast cancer with more genetic variation in the nonbasal subtype than there is in the nonbasal subtype. A higher rate of mutations correlate with response to therapy.

![Image](https://via.placeholder.com/150)

**Table 4. Highlights of Genomic, Clinical, and Proteomic Features of Breast Cancer Subtypes**

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Luminal A</th>
<th>Luminal B</th>
<th>Basal-like</th>
<th>HER2E</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER+/HER2− (%)</td>
<td>87</td>
<td>15</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>HER2+ (%)</td>
<td>7</td>
<td></td>
<td>80</td>
<td>68</td>
</tr>
<tr>
<td>TNBCs (%)</td>
<td>2</td>
<td></td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>TP53 pathway</td>
<td>TP53 mut (12); gain of MDM2 (14)</td>
<td>TP53 mut (32); gain of MDM2 (31)</td>
<td>TP53 mut (84); gain of MDM2 (14)</td>
<td>TP53 mut (75); gain of MDM2 (30)</td>
</tr>
<tr>
<td>PIK3CA/PTEN pathway</td>
<td>PIK3CA mut (49); PTEN mut/loss (13); INPP4B loss (9)</td>
<td>PIK3CA mut (32); PTEN mut/loss (24); INPP4B loss (16)</td>
<td>PIK3CA mut (7); PTEN mut/loss (35); INPP4B loss (30)</td>
<td>PIK3CA mut (42); PTEN mut/loss (19); INPP4B loss (30)</td>
</tr>
<tr>
<td>RB1 pathway</td>
<td>Cyclin D1 amp (29); CDK4 gain (14); low expression of CDKN2C; high expression of RB1</td>
<td>Cyclin D1 amp (58); CDK4 gain (25)</td>
<td>RB1 mut/loss (20); cyclin E1 amp (9); high expression of CDKN2A; low expression of RB1</td>
<td>Cyclin D1 amp (38); CDK4 gain (24)</td>
</tr>
<tr>
<td>mRNA expression</td>
<td>High ER cluster; low proliferation</td>
<td>Lower ER cluster; high proliferation</td>
<td>Basal signature; high proliferation</td>
<td>HER2 amplification signature; high proliferation</td>
</tr>
<tr>
<td>Copy number</td>
<td>Most diploid; many with quiet genomes; 1q, 8q, 8p11 gain; 8p, 16q loss; 11q13.3 amp (24)</td>
<td>Most aneuploid; many with focal amp; 1q, 8q, 8p11 gain; 8p, 16q loss; 11q13.3 amp (51); 8p11.23 amp (28)</td>
<td>Most aneuploid; high genomic instability; 1q, 10p gain; 8q, 5q loss; MYC focal gain (40)</td>
<td>Most aneuploid; high genomic instability; 1q, 8q gain; 8p, 16q loss; 17q12 focal ERBB2 amp (71)</td>
</tr>
<tr>
<td>DNA mutations</td>
<td>PIK3CA (49); TP53 (12); GATA3 (14); MAP3K1 (14)</td>
<td>TP53 (32); PIK3CA (32); MAP3K1 (5)</td>
<td>TP53 (84); PIK3CA (7)</td>
<td>TP53 (75); PIK3CA (42); PIK3R1 (8)</td>
</tr>
<tr>
<td>DNA methylation</td>
<td>Hypermethylated phenotype for subset</td>
<td>Hypermethylated</td>
<td>High expression of DNA repair proteins, PTEN and INPP4B loss signature (pAkt)</td>
<td>High protein and phosphoprotein expression of EGFR and HER2</td>
</tr>
<tr>
<td>Protein expression</td>
<td>High estrogen signaling; high MYB; RPPA reactive subtypes</td>
<td>Less estrogen signaling; high FOXM1 and MYC; RPPA reactive subtypes</td>
<td>High expression of DNA repair proteins, PTEN and INPP4B loss signature (pAkt)</td>
<td>—</td>
</tr>
</tbody>
</table>


Abbreviations: Amp, amplification; EGFR, epidermal-growth factor receptor; ER, estrogen receptor; GATA3, GATA binding protein 3; HER2, human epidermal receptor protein-2; HER2E, HER2-enriched; mut, mutation; PR, progesterone receptor; TNBC, triple-negative breast cancer.
from neoadjuvant aromatase inhibitors. Luminal B subtype tumors are associated with PIK3CA and TP53 mutations. The HER2 subtype cancers frequently contain HER2 amplification and TP53 and PIK3CA mutations.

Next-generation sequencing can identify specific mutations resulting in the development of a targeted approach using routine PCR assays. Some of these mutations may possibly serve as early, noninvasive indicators of disease and provide tools for monitoring therapy and disease progression. Multiplatform molecular predictors, dynamic response markers, and the identification of the vulnerabilities of individual cancers are the new promising avenues that, in composite, will be even more informative. The molecular characteristics of an individual’s tumor will give rise to tailored therapies based on identifying tumor-specific mutated genes.

CONCLUSIONS

Immunohistochemistry is a critical theranostic and diagnostic tool in analyzing breast cancers, as seen with ER, PR, and HER2. Evaluations of breast cancer using microarrays have influenced our approach in evaluating these tumors because gene expression profiling can provide prognostic and predictive information, which aids in clinical management. With the era of individualized medicine approaching, the ability to determine therapy effectiveness and the risk of relapse is important. Integration of recent advances in next-generation sequencing using new technologies that allow faster high-throughput data generation will emphasize the role of a much broader team that includes translational researchers and bioinformaticians, who will help in separating meaningful data from noise and, with new clinical trials, could potentially reshape our multidisciplinary approach to the management of breast and other cancers.

References


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