Breast cancer biomarkers and molecular medicine: part II

Jeffrey S Ross†, Gerald P Linette, James Stec, Edwin Clark, Mark Ayers, Nick Leschly, W Fraser Symmans, Gabriel N Hortobagyi and Lajos Pusztai

In this second part of the two-part review of breast cancer biomarkers and molecular medicine, the first section will consider additional breast cancer prognostic factors, including oncogenes, tumor suppressor genes, cell adhesion molecules, invasion-associated proteins and proteases, hormone receptor proteins, drug resistance proteins, apoptosis regulators, transcription factors, telomerase, DNA repair and methylation and transcriptional profiling using high-density genomic microarrays. The second section will consider the prediction of therapy response using the techniques of pharmacogenetics and pharmacogenomics.


The continuation of this two-part review of breast cancer biomarkers and molecular medicine includes additional considerations of known and emerging prognostic factors, and is focused upon the prediction of response of the disease to therapy.

Prognostic versus predictive factors

Prognostic factors

In clinical use in patients diagnosed with breast cancer, prognostic factors are designed to forecast the most likely clinical outcome of the disease without regard to the nature and intensity of the selected treatment. Factors widely used in this fashion include the tumor type (infiltrating ductal vs. lobular, ductal subtypes, such as medullary, tubular, papillary and mucinous), grade and size, lymph node status, extent of the intraductal component and presence of vascular space invasion. Ancillary tests in common use and recommended by both the College of American Pathologists (CAP) and American Society of Clinical Oncologists (ASCO) include hormone receptor status and HER-2/neu status. The key features of a clinically useful prognostic factor include: ease and reliability of the assay; confirmation that the prognostic significance is not confounded by the type of treatment used; and that the factor provides disease outcome information that is independent of the status of other classic factors.

Predictive factors

There are two types of predictive factors: factors that predict the likelihood that breast cancer will develop in a currently disease-free woman; and factors that specifically predict whether a newly diagnosed or relapsed case of breast cancer will or will not respond to a specific single or combination of therapies. As seen in Part I of this two-part review, the HER-2/neu status in newly diagnosed breast cancer can serve both as a stand-alone prognostic factor and as a predictive factor for response to trastuzumab (Herceptin®, Genentech, CA, USA) [1]. The estrogen receptor (ER) test is an example of a proven predictive factor for the response to hormonal therapy that is a much weaker general prognostic factor for forecasting therapy-independent disease outcome.

Breast cancer prognosis

Oncogenes

The c-myc proto-oncogene located on chromosome 8 encodes a 439-amino acid nuclear binding protein that directly stimulates cell division and participates in most aspects of cellular function, including replication, metabolism, differentiation and apoptosis [2]. The c-myc gene is amplified in approximately 16% of breast cancer cases and in the majority of outcome-based studies is associated with decreased disease-free patient survival [2,3]. In a...
recent study of node-negative cases only, c-myc amplification outperformed both H ER-2/neu amplification and ER status in the prediction of breast cancer disease-free survival [4]. Of the three ras signal transduction-encoding genes, the H-ras (chromosome 11p15) gene has been consistently associated with breast cancer progression [5]. Unlike K-ras (chromosome 12p12) and N-ras (chromosome 1p13), where point mutations are the most common cause of gene malfunction resulting in abnormal levels of activated ras p21 protein, in breast cancer, mutations are rarely observed and loss of heterozygosity (LOH) is a far more frequent occurrence [5,6]. Measurements of the c-fos (chromosome14q21) and c-jun (chromosome 22q13) regulators of the activating protein (AP)-1 complex and c-myc (chromosome 6q21) have successfully predicted breast cancer recurrence, response to hormonal therapy and survival [7-11]. Expression of the Jun activation domain-binding protein (JAB)-1 appears to correlate inversely with expression of the cell cycle inhibitor p27 and may be an adverse prognostic factor [12].

**Tumor suppressor genes**

**p53**

p53 is a tumor suppressor gene localized to chromosome 17p that codes for a multifunctional DNA-binding protein involved in cell cycle arrest, DNA repair, differentiation and apoptosis [13]. The p53 gene is mutated in approximately 50% of human cancers and the germline DNA of families with inherited cancer disorders, such as Li–Fraumeni syndrome [13]. The prognostic significance of p53 status in breast cancer has been impacted by the accuracy of immunohistochemistry (IHC) versus molecular methods (single-strand conformation polymorphism [SSCP], direct sequencing and the yeast colony functional assay) [13,14]. IHC evaluations with and without image analysis-assisted slide scoring using either the DO-1 or PAb1801 antibodies have yielded variable associations of p53-stabilized mutant protein nuclear staining with outcome in breast cancer. In general, the DO-1 antibody has been favored by investigators over the PAb1801 [15]; however, the high number of IHC false-positive and false-negative results (compared with gene sequencing) precludes reliable use of IHC as an indicator for p53 gene mutation in human breast cancer [16,17,48].

The p53 mutation rate is lower in breast than in other epithelial cancers and has been associated with more aggressive disease and worse overall survival [18]. A series of 29 studies involving 9793 patients testing the association of p53 gene and protein status as a marker of prognosis in breast cancer is provided in Table 1 [19-47]. Seven (24%) of the studies including 1910 (20%) patients found no association of p53 status with prognosis. Six (21%) of the studies that featured multivariate data analysis including 2822 (30%) of the patients found a significant prognostic impact on univariate but not multivariate analyses. Furthermore, 15 (54%) of these studies involving 4814 (50%) of the patients found prognostic significance on both univariate and multivariate analyses. Of the seven negative outcome studies in Table 1, six (86%) used an IHC approach for determining p53 status.

p53 status & predicting response to therapy

In general, breast carcinomas with p53 mutations are consistently associated with high histologic grade, high mitotic index, high cell proliferation rate, aneuploid DNA content, negative assays for ER and progesterone receptor (PR) [40-51] and variable association with amplification of oncogenes, such as H ER-2/neu, c-myc, ras and int-2 [23,26]. A number of studies of metastatic disease have implicated p53 mutations with resistance to hormonal, adjuvant, neoadjuvant and combination chemotherapy, encompassing a variety of agents including anthracyclines and taxanes [38,40,41,52-55]. However, other reports have failed to link p53 status with therapy response [56-58]. Currently, whether tissue-based or serum-based, assessments of p53 status are not included as a part of the standard practice for the management of breast cancer.

Other tumor suppressor genes

The MDM2 gene encodes a protein that binds to p53, reducing its cell cycle progression inhibitory role [59]. MDM2 amplification appears to be a rare event in breast cancer [60,61]. Multiple reports have linked MDM2 overexpression to adverse outcome in patients with node-negative and -positive breast cancer [62-64]; however, one report found no correlation [65]. Abnormal expression of the retinoblastoma (Rb) tumor suppressor gene and protein occurs in 10-20% of primary breast cancer tumors [66,67]. Rb gene alterations have been associated with smaller node-negative tumors, but have not been predictive of relapse-free or overall survival [68]. Localized to chromosome 17q, NM23 belongs to a large family of structurally and functionally conserved proteins that exhibit nucleoside diphosphate kinase (NDPK) activity and bind DNA [69]. Although the exact mechanism of metastasis suppression associated with NM23 expression is not completely understood, this gene is believed to function by regulating downstream signal transduction associated with an as yet unconfirmed receptor. NM23 expression has predicted a favorable outcome in some studies of breast cancer patients [70-72], but not in others [73,74]. The p16INK4A tumor suppressor gene cyclin-dependent kinase inhibitor inhibits cell growth at the G1/S checkpoint of the cell cycle in concert with Rb, p14 and p15 [75-77]. p16 expression may be lost by a process of mutation (less common) or CpG island hypermethylation of the gene promoter (more common) [75-77]. Studies of the p16 gene, mRNA and protein in breast cancer have been conflicting, with some studies linking overexpression with adverse outcome, some studies linking loss of expression with adverse outcome and others finding no association with outcome [78-82]. Germline mutations in the PTEN tumor suppressor gene are causal of Cowden’s breast cancer predisposition syndrome and PTEN is frequently mutated in sporadic breast cancers [83]. The PTEN tumor suppressor protein inhibits activation of Akt and this restricts MDM 2 to the cytoplasm, promoting p53 function and sustaining the sensitivity of cancer cells to chemotherapy [84]. Loss of PTEN expression has recently been linked to adverse outcome in breast cancer [85]. M aspin is a novel serine protease inhibitor related to the serpin 170

family with a tumor-suppressing function possibly associated with poor prognosis in breast cancer [86–88]. Maspin mRNA detection by reverse transcriptase (RT)-PCR has also been used to detect micrometastases and minimal residual disease in breast cancer [88]. Maspin expression has been correlated with breast cancer prognosis with maspin nuclear staining significantly associated with good prognostic factors and cytoplasmic staining associated with poor prognostic markers [89]. Truncations and other alterations of BRCA proteins have not, to date, been linked to disease outcome in sporadic breast cancer [90]. A study showed that 29% of sporadic tumors (35 of 122) contain methylated and therefore silenced BRCA1 promoter region [90]. The impact of the apparently frequent silencing of the BRCA1 gene on clinical outcome has not been systematically studied.

**Cell adhesion molecules**

Cell adhesion molecule expression has been extensively studied in breast cancer as a biomarker of tumor development, differentiation, progression and metastasis [91,92].

**Cadherin/catenin complex**

The cytoplasmic accumulation of catenin molecules associated with interaction of the cell surface receptor E-cadherin is now considered to be a major gene expression regulator via the Wnt signaling pathway [93-95]. The E-cadherin/catenin complex has been related to disease outcome in a variety of malignant diseases including breast cancer [96]. The majority of published studies have linked loss of expression of E-cadherin with adverse outcome in breast cancer [97-99], although there have been reports of retained expression indicating disease progression [100]. E-cadherin expression loss has consistently been attributed to gene silencing via hypermethylation of CpG islands in the promoter complex and loss of mRNA production [101]. The most consistent observation concerning the loss of E-cadherin expression in breast cancer has been the association with the infiltrating lobular pattern versus infiltrating ductal pattern of invasive carcinoma [102,103]. Increased E-cadherin expression has been described as a feature of inflammatory breast cancer [104]. E-cadherin status has not been widely used to predict the response of breast cancer to therapy.

**CD44**

The CD44 cell adhesion molecule is a polymorphic integral membrane glycoprotein associated with cell matrix adhesion, lymphocyte activation, recirculation and homing [91,92]. Featuring complex patterns of post-translational splice modification in which one or more of 12 variant exons are expressed leading to addition of amino acids to the standard CD44 glycoprotein, this molecule has been implicated to play a major role in the development of invasion and metastasis in a variety of solid tumors and hematopoietic neoplasms [91,92]. CD44 expression has been associated with the development and progression of breast cancer [105]. Abnormal expression of the standard form of CD44 has been linked to prognosis [106-109]. Overexpression of the CD44 splice variant v6 has been linked to adverse outcome in several studies [108-110], but not in others [111]. Serum-based CD44 studies of both standard form [112] and the v6 splice variant [113] have been performed, however, the results have been inconclusive as to whether this approach could achieve routine clinical use.

**Integrins**

The integrin and laminin receptor groups have been widely studied in breast cancer [114]. Laminin receptor expression has been independently associated with disease outcome in some studies [115,116], but not in others [117]. Altered expression of integrins αv [118] and α6 [119,120] has been linked to breast cancer prognosis.

**Other adhesion molecules**

The most widely studied additional adhesion molecule in breast cancer is the epithelial cell adhesion molecule (EpCAM), which has been linked to survival [121], used as a reagent for micrometastasis detection in peripheral blood and bone marrow specimens [122] and as a target of therapy [123].

**Invasion-associated proteases & proteins**

**Cathepsin D**

Cathepsin D is an estrogen-regulated lysosomal aspartyl protease localized to chromosome 11 and believed to facilitate cancer cell migration and promote stromal invasion by the digestion of basement membrane, matrix and connective tissue [124]. Numerous studies in the early 1990s using an immunoassay approach on fresh breast tumor cytosolic preparations have shown that elevated cathepsin D levels are an independent predictor of survival in breast cancer [125-127]. Attempts to convert the assay to an IHC-based format have not been successful [128,129]. Thus, given the limitations of the current assay format, interest in using cathepsin D assessment as a prognostic factor and to guide therapy in breast cancer has all but disappeared in the USA, although it is still performed in Europe.

**Serine proteases**

A variety of proteolytic enzymes have been implicated in the digestion and turnover of extracellular matrix (ECM) as a means of promoting invasion and metastasis of human malignancies. The two major groups of enzymes considered have been the serine proteases and the matrix metalloproteases (MMPs). The serine proteases studied in breast cancer invasion have focused on urokinase plasminogen activator (uPA) and its receptor (uPAR) and plasminogen activator inhibitor (PAI)-1. uPA acts on plasminogen to produce plasmin, degrades the ECM and is inhibited by PAI-1 via direct binding. The independent prognostic value of protease uPA and its inhibitor PAI-1 for survival in breast cancer patients is firmly established [130]. When evaluated on fresh tissue extracts and tumor cytosol, high uPA and PAI-1 levels have been consistently associated with disease recurrence and overall patient survival in breast cancer [131-134]. Plasminogen protease levels have also been successfully used as predictors of chemotherapy response [135]. Translation of the uPA/PAI-1 immunoassay to an on-slide IHC format has not, to date, been successful, which has limited
Table 1. Summary of selected studies on the correlation of the p53 tumor suppressor gene with prognosis in breast cancer.

<table>
<thead>
<tr>
<th>Year of study</th>
<th>Number of cases</th>
<th>Specimen type</th>
<th>Method of analysis</th>
<th>Univariate significance</th>
<th>Multivariate significance</th>
<th>Comment</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1992</td>
<td>304</td>
<td>Paraffin</td>
<td>IHC</td>
<td>Yes</td>
<td>Yes</td>
<td>p53 status was an independent predictor more in sporadic than familial breast cancer</td>
<td>[19]</td>
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<tr>
<td>1994</td>
<td>247</td>
<td>Paraffin</td>
<td>IHC</td>
<td>Yes</td>
<td></td>
<td>DO-1 antibody 16% positive. Significant prognosis only for DNA diploid tumors</td>
<td>[20]</td>
</tr>
<tr>
<td>1994</td>
<td>192</td>
<td>Frozen</td>
<td>Direct sequencing</td>
<td>No</td>
<td>No</td>
<td>Associates with ER status. 22% mutations</td>
<td>[21]</td>
</tr>
<tr>
<td>1994</td>
<td>230</td>
<td>Paraffin</td>
<td>IHC</td>
<td>Yes</td>
<td>Yes</td>
<td>Correlates with ER, proliferation (MIB-1), grade, DO-1 antibody</td>
<td>[22]</td>
</tr>
<tr>
<td>1994</td>
<td>462</td>
<td>Paraffin</td>
<td>IHC</td>
<td>Yes</td>
<td>No</td>
<td>30% cutoff for protein overexpression. Associates with ER negative, high proliferation rate</td>
<td>[23]</td>
</tr>
<tr>
<td>1995</td>
<td>85</td>
<td>Paraffin</td>
<td>IHC</td>
<td>No</td>
<td>No</td>
<td>PAb1801 antibody, Ras/fos status did not correlate. Small sample</td>
<td>[24]</td>
</tr>
<tr>
<td>1995</td>
<td>353</td>
<td>Plasma</td>
<td>ELISA</td>
<td>Yes</td>
<td>Yes</td>
<td>Associates with ER negative, significant survival differences at 5 years. 12% positive rate</td>
<td>[25]</td>
</tr>
<tr>
<td>1996</td>
<td>125</td>
<td>Paraffin</td>
<td>IHC</td>
<td>No</td>
<td>No</td>
<td>p53 status correlated with ER/PR but did not predict prognosis</td>
<td>[26]</td>
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<tr>
<td>1997</td>
<td>375</td>
<td>Fresh</td>
<td>SSCP</td>
<td>Yes</td>
<td>Yes</td>
<td>p53 mutation predicted poor survival independent of lymph node status</td>
<td>[27]</td>
</tr>
<tr>
<td>1998</td>
<td>441</td>
<td>Paraffin</td>
<td>IHC</td>
<td>No</td>
<td>No</td>
<td>p53 status did not predict prognosis. ER, Ki-67 status and patient age were significant predictors. p53 status did predict response to adjuvant therapy</td>
<td>[28]</td>
</tr>
<tr>
<td>1998</td>
<td>329</td>
<td>Paraffin</td>
<td>IHC</td>
<td>No</td>
<td>No</td>
<td>Neither p53 nor HER-2/neu predicted response to chemotherapy</td>
<td>[29]</td>
</tr>
<tr>
<td>1998</td>
<td>634</td>
<td>Fresh</td>
<td>ELISA</td>
<td>Yes</td>
<td>No</td>
<td>Only tumor grade was independent, p53 and uPA status were not</td>
<td>[30]</td>
</tr>
<tr>
<td>1998</td>
<td>998</td>
<td>Fresh</td>
<td>ELISA</td>
<td>Yes</td>
<td>Yes</td>
<td>p53 levels independently predicted relapse and disease-related death</td>
<td>[31]</td>
</tr>
<tr>
<td>1998</td>
<td>345</td>
<td>Paraffin</td>
<td>IHC</td>
<td>Yes</td>
<td>Yes</td>
<td>p53 prognostic significance dependent on the bcl-2 expression status</td>
<td>[32]</td>
</tr>
<tr>
<td>1999</td>
<td>125</td>
<td>Paraffin</td>
<td>IHC</td>
<td>No</td>
<td>No</td>
<td>PAI-1 levels were independent predictors in node-negative patients</td>
<td>[33]</td>
</tr>
<tr>
<td>1999</td>
<td>1245</td>
<td>Fresh</td>
<td>Immunoluminometric assay</td>
<td>Yes</td>
<td>No</td>
<td>p53 status not independent predictor when uPA status was known</td>
<td>[34]</td>
</tr>
<tr>
<td>2000</td>
<td>613</td>
<td>Paraffin</td>
<td>IHC</td>
<td>No</td>
<td>No</td>
<td>Only tumor size and grade were significant prognostic factors</td>
<td>[35]</td>
</tr>
</tbody>
</table>
the use of these highly predictive biomarkers in patients with smaller primary tumors where the entire specimen is processed and no material is available for fresh protein testing. Finally, uPA and uPAR have been detected in nipple aspirate fluid samples: uPA and uPAR were found to be independent predictors of cancer presence and uPAR was also an independent predictor of advanced disease stage [136]. In summary, although highly regarded as prognostic factors for newly diagnosed breast cancer, the plasminogen protease family members have not been widely used to select and predict the response to a specific breast cancer therapy.

MMPs
MMPs are a group of at least 19 zinc metalloenzymes secreted as proenzymes with substantial sequence similarities that are inhibited by metallochelators and specific tissue inhibitors of MMPs (TIMPs) [137]. The MMPs include the interstitial collagenases, gelatinases, stromelysins and membrane-type MMPs and are involved in breast cancer initiation, invasion and metastasis [137]. High levels of at least three MMPs (-2, -9 and -11) have been found to correlate with poor disease outcome in breast cancer [137–140].

Table 1. Summary of selected studies on the correlation of the p53 tumor suppressor gene with prognosis in breast cancer (cont.).

<table>
<thead>
<tr>
<th>Year of study</th>
<th>Number of cases</th>
<th>Specimen type</th>
<th>Method of analysis</th>
<th>Univariate significance</th>
<th>Multivariate significance</th>
<th>Comment</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000</td>
<td>243</td>
<td>Fresh</td>
<td>Direct sequencing</td>
<td>Yes</td>
<td>Yes</td>
<td>p53 mutation independently predicted outcome in tamoxifen-treated patients</td>
<td>[37]</td>
</tr>
<tr>
<td>2000</td>
<td>297</td>
<td>Fresh</td>
<td>Immunoluminometric assay</td>
<td>Yes</td>
<td>No</td>
<td>Only tumor size and ER status were independent predictors in node-negative patients</td>
<td>[38]</td>
</tr>
<tr>
<td>2000</td>
<td>143</td>
<td>Paraffin</td>
<td>IHC</td>
<td>Yes</td>
<td>Yes</td>
<td>p53 status predicted response to chemotherapy independent of HER-2/neu, ER, PR and Ki-67 expression</td>
<td>[39]</td>
</tr>
<tr>
<td>2001</td>
<td>90</td>
<td>Paraffin</td>
<td>IHC</td>
<td>Yes</td>
<td>No</td>
<td>p53 status predicted prognosis in patients treated with anthracycline-based chemotherapy</td>
<td>[40]</td>
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<tr>
<td>2001</td>
<td>458</td>
<td>Fresh</td>
<td>Immunoblotting</td>
<td>Yes</td>
<td>Yes</td>
<td>p53 status predicted outcome in operable cases</td>
<td>[41]</td>
</tr>
<tr>
<td>2001</td>
<td>514</td>
<td>Frozen</td>
<td>IHC</td>
<td>Yes</td>
<td>Yes</td>
<td>p53 IHC predicted disease-free survival</td>
<td>[42]</td>
</tr>
<tr>
<td>2002</td>
<td>105</td>
<td>Paraffin</td>
<td>IHC</td>
<td>Yes</td>
<td>Yes</td>
<td>p53 but not p21 status predicted prognosis in node-positive patients</td>
<td>[43]</td>
</tr>
<tr>
<td>2001</td>
<td>46</td>
<td>Blood</td>
<td>PCR sequencing</td>
<td>Yes</td>
<td>Yes</td>
<td>p53 mutations in blood DNA in breast cancer patients independently predicted prognosis</td>
<td>[44]</td>
</tr>
<tr>
<td>2002</td>
<td>420</td>
<td>Paraffin</td>
<td>IHC</td>
<td>Yes</td>
<td>Yes</td>
<td>p53 status predicted relapse-free and overall survival</td>
<td>[45]</td>
</tr>
<tr>
<td>2002</td>
<td>94</td>
<td>Fresh</td>
<td>ELISA</td>
<td>Yes</td>
<td>No</td>
<td>HER-2/neu was independent predictor</td>
<td>[46]</td>
</tr>
<tr>
<td>2002</td>
<td>75</td>
<td>Paraffin</td>
<td>IHC</td>
<td>Yes</td>
<td>Yes</td>
<td>p53 and HER-2/neu were independent predictors; bcl-2 was not</td>
<td>[47]</td>
</tr>
</tbody>
</table>

ELISA: Enzyme-linked immunosorbent assay; ER: Estrogen receptor; IHC: Immunohistochemistry; PAI: Plasminogen activator inhibitor; PR: Progesterone receptor; SSCP: Single-strand conformation polymorphism; uPA: Urokinase plasminogen activator.
MMP-9

Studies of MMP-9 in breast cancer have been somewhat conflicting, with some showing an adverse outcome in cases with high MMP-9 expression [145] and others indicating that overexpression of MMP-9 indicated a favorable prognosis [146].

MMP-11

The ability of the epithelial tumor cells to elicit the stromal cell elaboration of ECM-digesting enzymes may be a major pathway by which invasion of cancer is facilitated. MMP-11 (stromelysin-3) expression has been associated with poor prognosis in breast cancer [147,148]. Continued interest in MMP and TIMP expression in breast cancer is being fueled, in part, by studies of the potential use of anti-MMP therapies [149]. However, to date, the status of MMP or TIMP expression in breast cancer has not been linked to the response to MMP inhibitors or other specific breast cancer treatment.

Estrogen & progesterone receptor proteins

The role of ER and PR testing as a marker of prognosis and predictor of response to antiestrogen therapy is established as a standard of care for patients with breast cancer [150,151]. Positive ER and PR assays are associated with well-differentiated histology, negative lymph node status, diploid DNA content, low cell proliferation rate and tendency for a relatively indolent clinical course [150-152]. ER/PR-negative tumors are often associated with aggressive disease, including amplification of the HER-2/neu, c-myc and int-2 oncogenes, mutation of the p53 gene, and upregulation of invasion- and metastasis-associated growth factors, growth factor receptors and proteases [150,151]. The microarray approach has also shown that a series of genes are expressed in breast cancer according to the tumor ER status [FIGURE 1A & B] [152]. For example, the GATA transcription factor gene was linked to the expression of the ER pathway by microarray profiling of breast cancer tissue [152]. Expression profiling using microarrays has also shown excellent concordance between protein expression of ER measured by IHC and HER-2/neu gene amplification detected by fluorescence in situ hybridization (FISH) and mRNA levels for both genes detected on the arrays (FIGURE 2). The determination of ER/PR status in newly diagnosed breast cancer is required for selection of patients to receive hormonal...
therapy, and the ER/PR has also been widely used to predict risk for progressive disease [153]. Originally determined on fresh tumor protein extracts and cytosol using a quantitative biochemical competitive binding assay with dextran-coated charcoal, the small size of newly diagnosed primary tumors has required a shift to on-slide IHC methods [154]. Image analysis approaches have further standardized the IHC procedures in some laboratories [155]. In general, patients with positive ER assays by either biochemical or IHC methods will respond to hormonal therapy in proportion to the receptor protein content [156–158]. Conversely, the total absence of ER/PR expression is strongly associated with lack of benefit from hormonal therapy. On occasion, however, patients may fail to show hormonal response despite high levels of receptor protein, which may be functionally defective. As a result, a tumor might produce a positive immunostain due to an abnormal or truncated protein produced by a mutated receptor gene that fails to bind estrogen [156,157]. Thus, despite its limitations, IHC is currently the standard method to determine ER and PR status in breast cancer. In addition, IHC remains a cornerstone of therapy planning for breast cancer and is likely to be utilized clinically in this fashion in the foreseeable future.

Prediction of response to antiestrogen therapy
Although ER/PR testing is the standard approach for predicting tamoxifen response, additional biomarkers including H ER-2/neu and cathepsin D have been proposed to further refine therapy selection [158]. The introductions of specific estrogen response modulators and aromatase inhibitors, such as anastrozole (Arimidex®, AstraZeneca, London, UK), letrozole (Femara®, Novartis, Basel, Switzerland) and exemestane (Aromasin®, Pharmacia, NJ, USA) [159–161], have added new strategies for evaluating tumors for hormonal therapy. For example, it has been recently described that ER/HER-2/neu-positive tumors may be resistant to tamoxifen but may respond to an aromatase inhibitor [162,163]. In summary, the determination of ER and PR status in breast cancer is currently
based on the ability of these markers to predict the response of breast cancer to hormonal-based therapies and is significantly less useful as a general prognostic factor for the disease independent of therapy selection.

**Markers of drug resistance**

The multiple drug resistance gene MDR1 encodes an integral transmembrane protein, the P-glycoprotein (Pgp), which functions as an energy-dependent efflux pump, decreases intracellular drug accumulation, is associated with chemoresistance in breast cancer and has been detected by a variety of techniques, including PCR amplification, Southern blotting, in situ hybridization and immunocytochemistry [164]. Despite the occasional negative study [165], a large meta-analysis of published information concluded that MDR1 expression correlated significantly with chemotherapy resistance and adverse prognosis [166]. It should be noted that not all anticancer drugs are substrates for Pgp. For example, anthracyclines, other topoisomerase II-active agents, vinca alkaloids and taxanes are effected by Pgp expression, but many other commonly used cytotoxic drugs are not. The glutathione S-transferase (GST)-π gene localized within the 11q13 amplicon correlates with Pgp expression, enhances intracellular drug detoxification and is associated with multidrug resistance in breast cancer [167,168]. GST-π expression has been associated with resistance to alkylating agents and several investigators have proposed the use of GST-π expression to select chemotherapy regimens for patients with breast cancer. pS2 is an estrogen-inducible small trefoil protein associated with...
with positive Pgp and may be a marker of functioning ER, irrespective of ER status [169,170]. Use of pS2 measurement continues to show promise as a predictor of indolent disease and clinical hormonal responsiveness in breast cancer. The heat shock or stress response proteins HSP 27, HSP 70 and HSP 90 are a family of highly conserved proteins associated with tissue responses to heat, toxins, heavy metals, abnormal pH., certain hormones, drugs and anoxia [171]. HSP 27 and HSP 70 expression have been found to be predictors of disease recurrence and outcome in a number of studies of breast cancer [171-175].

Apoptosis & apoptosis regulators
Apoptosis has been measured in breast cancer specimens using a variety of techniques, including routine morphology, electron microscopy, IHC with the TUNEL assay and PCR-based methods [176]. The majority of studies have linked increased apoptotic index levels with adverse outcome for the disease [177-180]. Proapoptotic members of the Bcl-2 family include Bax, Bak, Bad, Bid and Bcl-xL, and antiapoptotic proteins include Bcl-2 and Bcl-xL [176]. Bcl-2 expression in primary invasive ductal breast carcinoma correlates with ER/PR-positive status and has been associated with improved patient survival [181,182]. However, primary tumor Bcl-2 expression levels are not predictive for response to systemic chemotherapy given after relapse [183]. Bax protein expression has not been clearly linked to outcome [183]. In addition, activated caspases can act as both initiators and effectors of the apoptotic pathway and there is evidence that caspases 3, 6 and 8 are associated with higher levels of apoptosis, histological grade and tumor aggressiveness in breast cancer [184]. Caspase expression in breast cancer has been linked to improved overall survival [185] and chemoresistance [186].

Transcription factors
Nuclear factor (NF)-κB binds to multiple DNA sequences initiating the transcription of a wide variety of genes, including the cytokines interleukin (IL)-1,-6 and -8, tumor necrosis factor (TNF)-α, angiogenesis factors (vascular endothelial growth factor), cell adhesion molecules (intercellular adhesion molecule 1 and vascular cell adhesion molecule 1), enzymes (cylooxygenase [COX]-2 and nitric oxide synthase) and antiapoptotic factors (Bcl-2 and survivin) [187-189]. Proteasome-based degradation releases active NF-κB, which then translocates into the nucleus where it binds to specific DNA sequences on its target genes. Recent studies of the NF-κB pathway in breast and other cancers have led to the concept of NF-κB as a target of anticancer therapy [190,191]. Recently, proteasome inhibitors and IκBα kinase (IKK) inhibitors have targeted the NF-κB pathway in both preclinical models and early clinical trials for patients with breast cancer and other solid tumors [188-191]. Ets-1 regulates the expression of a group of angiogenic and ECM remodeling factors of importance in breast cancer [192]. When measured in a RT-PCR format, Ets-1 expression has shown significant prognostic value for relapse-free survival as an independent predictor of poor prognosis [192]. The Wnt transcription factors have been discussed in the cell signaling section as the signaling pathway of the cadherin/catenin complex. In a recent study, expression of the Wnt-5a protein increased the risk of early relapse and death in breast cancer, supporting its role as a tumor suppressor via its effects on cell adhesion and motility [193].

Telomerase
Telomerase is a cellular RT that maintains the ends of chromosomes. It is activated in over 90% of breast cancers but not in normal adjacent tissues [194]. Early studies using the PCR-based telomere repeat amplification protocol (TRAP) assay to determine enzyme activity revealed conflicting results as to the prognostic significance of telomerase expression in breast cancer [195-197]. More recent reports measuring the catalytic subunit, human telomerase RT (hTERT), and the internal RNA component (hTR) have found independent significance for the prediction of disease outcome [198,199].

DNA repair & microsatellite instability
Microsatellite instability (MSI) is characterized by a mutational process of insertions or deletions in microsatellite repeats and is considered to be a sensitive indicator for genomic instability, increasing the risk for the development of cancer [200]. Compared with other diseases, such as colorectal cancer, MSI appears to be a relatively infrequent finding in breast cancer development [201]. Preliminary reports have linked MSI with prognosis [202], but large controlled studies need to be performed before MSI can be considered as a reliable marker for aggressive disease.

DNA methylation
One of the most common molecular alterations in human neoplasia is altered methylation of DNA, with the CpG island hypermethylation of gene promoter regions believed to be one of the most frequent mechanisms of loss of gene function [203]. The most frequently methylated genes in breast cancer are the G2 checkpoint regulatory gene 14-3-σ at 91%, followed by ERα and E-cadherin at 50%, PR at 40%, GST-σ at 30%, RARβ2 and TIMP-3 at 25% and BRCA1 and p16 at 15% [204]. DNA methylation of ERα has been associated with hormone-refractory disease, which has been reversed in preclinical studies using demethylation drugs [205]. GST-σ methylation leading to genomic instability and BRCA1 and p16 methylation leading to cell proliferation may contribute to breast carcinogenesis [204]. Methylation of E-cadherin and TIMP-3 may facilitate invasion and metastasis [204].

Miscellaneous biomarkers
Overexpression of the S1004A calcium binding protein has been reported as an independent prognostic factor in breast cancer [206]. The polymorphic epithelial mucins Muc-1 and Muc-2 have shown some promise as potential predictive biomarkers in both tissue-based assays and serum diagnostics [207].
### Table 2. Review of major ancillary/molecular prognostic factors in breast cancer.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Assay</th>
<th>Target of therapy</th>
<th>Therapy</th>
<th>Current status</th>
<th>Future prospects</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER/PR</td>
<td>IHC Binding</td>
<td>Yes</td>
<td>Tamoxifen</td>
<td>Standard of care</td>
<td>Improved IHC with antibodies that are negative when ERα is truncated to reduce false positives</td>
</tr>
<tr>
<td></td>
<td>assay</td>
<td></td>
<td>SERMs</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Aromatase inhibitors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HER-2/neu</td>
<td>IHC FISH</td>
<td>Yes</td>
<td>Trastuzumab</td>
<td>Standard of care</td>
<td>CISH assay may replace both IHC and FISH</td>
</tr>
<tr>
<td>DNA ploidy</td>
<td>Cytometry</td>
<td>No</td>
<td></td>
<td>Common use</td>
<td>Decreased use</td>
</tr>
<tr>
<td>S-phase</td>
<td>Cytometry</td>
<td>No</td>
<td></td>
<td>Common use</td>
<td>Maintained use</td>
</tr>
<tr>
<td>Cell proliferation index</td>
<td>IHC</td>
<td>No</td>
<td></td>
<td>Common use</td>
<td>Increased use of Ki-67 IHC</td>
</tr>
<tr>
<td>Cyclin D</td>
<td>IHC</td>
<td>Possible</td>
<td>Flavopyridol Translocation targets</td>
<td>Clinical trials</td>
<td>May select new drug use, such as proteasome inhibitors</td>
</tr>
<tr>
<td>Cyclin E</td>
<td>IHC Western</td>
<td>No</td>
<td></td>
<td>Ruo</td>
<td>Prognostic significance must be validated</td>
</tr>
<tr>
<td>EGFR</td>
<td>IHC FISH</td>
<td>Yes</td>
<td>Gefitinib</td>
<td>Increasing use</td>
<td>Targeting the anti-EGFR drugs likely combined with pharmacogenomics</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Erlotinib</td>
<td>Clinical trials</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cetuximab</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>IHC FISH</td>
<td>Yes</td>
<td>Bevacizumab</td>
<td>Increasing use</td>
<td>Increasing use for prognosis. Initial targeted therapy disappointing</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Small molecules</td>
<td>Clinical trials</td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>IHC SSCP</td>
<td>Yes</td>
<td>Gene therapy</td>
<td>Increasing use</td>
<td>Targeted therapies disappointing to date</td>
</tr>
<tr>
<td></td>
<td>sequencing</td>
<td></td>
<td></td>
<td>Clinical trials</td>
<td></td>
</tr>
<tr>
<td>E-cadherin</td>
<td>IHC Methylation-PCR</td>
<td>Yes</td>
<td>5-azacytidine Demethylation</td>
<td>Increasing use</td>
<td>Diagnosis of pleomorphic lobular carcinoma</td>
</tr>
<tr>
<td>CD44 v6</td>
<td>IHC</td>
<td>No</td>
<td></td>
<td>Ruo</td>
<td>Predictive significance of v6 splice variant requires validation</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>Immunoassay</td>
<td>No</td>
<td></td>
<td>Common use in Europe</td>
<td>IHC studies disappointing; will continue to fade from view</td>
</tr>
<tr>
<td>uPA/PAI-1</td>
<td>Immunoassay</td>
<td>Yes</td>
<td>Small molecules</td>
<td>Common use in Europe</td>
<td>Targeted therapies in early stages. IHC assays not validated to date restricting use in the USA</td>
</tr>
<tr>
<td>MMPs 2, 9, 11</td>
<td>IHC</td>
<td>Yes</td>
<td>Marimistat</td>
<td>Clinical trials Ruo</td>
<td>Early results of targeted therapy disappointing</td>
</tr>
<tr>
<td>MDR</td>
<td>IHC</td>
<td>Yes</td>
<td>Small molecules</td>
<td>Clinical trials Ruo</td>
<td>Continued use</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>IHC</td>
<td>Yes</td>
<td>Genasense Proteasome inhibitors</td>
<td>Increasing use</td>
<td>Initial results of targeted therapies disappointing</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Clinical trials</td>
<td></td>
</tr>
<tr>
<td>Telomerase</td>
<td>TRAP IHC ISH</td>
<td>Yes</td>
<td>Small molecules</td>
<td>Ruo</td>
<td>Increased use if slide-based assays are successful prognostic factors</td>
</tr>
<tr>
<td>NF-κB</td>
<td>IHC Western</td>
<td>Yes</td>
<td>Proteasome inhibitors</td>
<td>Ruo</td>
<td>Will be used if targeted therapies are successful alone or in combination with cytotoxic drugs</td>
</tr>
<tr>
<td>Transcriptional profiling</td>
<td>cDNA array Oligonucleotide array</td>
<td>No</td>
<td></td>
<td>Ruo</td>
<td>Continued major expansion of use. Predictive marker sets will require multiple cross validation. Could become standard if initial results are confirmed</td>
</tr>
</tbody>
</table>

CISH: Chromogenic in situ hybridization; EGFR: Epidermal growth factor receptor; ER: Estrogen receptor; FISH: Fluorescence in situ hybridization; IHC: Immunohistochemistry; ISH: In situ hybridization; MDR: Multidrug resistance; MMP: Matrix metalloprotease; NF: Nuclear factor; PAI: Plasminogen activator inhibitor; PR: Progesterone receptor; RUO: Research use only; SSCP: Single-strand conformation polymorphism; SERM: Specific estrogen receptor modulator; TRAP: Telomere repeat amplification protocol; uPA: Urokinase plasminogen activator; VEGF: Vascular endothelial growth factor receptor.
Overexpression of COX-2 protein has achieved prognostic significance in one study [208]. The nuclear matrix protein (nmp)-66 serum assay has recently been introduced as a proteomics-based serum marker for the early detection of breast cancer, but has not been evaluated as a prognostic factor [209]. The cancer testis antigens melanoma antigen E (MAGE) and GAGE family gene products encompass tumor-associated antigens recognized by human leukocyte antigen (HLA)-restricted specific T-cells [210,211]. Assays for the MAGE genes in breast cancer have been correlated with prognosis [212] and used in sensitive molecular assays designed for early detection of primary and relapsed disease [213].

Transcriptional profiling
Whole-genome transcriptional profiling has been introduced as a technique for determining prognosis in breast cancer [214–217]. The transcriptome is a complete set of transcribed genes expressed as messenger RNAs that may code for proteins that define an individual or a specific breast cancer [218–222]. Of human genes, 95% are normally repressed in a given cell, with this control occurring at either the transcriptional or the translational level. Gene expression profiles can define cellular functions, biochemical pathways, proliferative activity and regulatory mechanisms. Transcriptional profiles of diseased tissues compared with their normal counterparts may promote the understanding of disease biology, predict disease outcome and identify new therapeutic targets [221].

In a recent DNA microarray analysis on primary breast tumors of 117 node-negative young patients using a supervised classification to identify a poor prognosis gene expression signature, aberrant expression of genes regulating cell cycle, invasion, metastasis and angiogenesis strongly predicted a short interval to distant metastases [217]. In a follow-up study, the poor prognosis gene expression profile outperformed all currently used clinical parameters in predicting disease outcome, including lymph node status, with an estimated hazard ratio for distant metastases of 5.1 (95% confidence interval 2.9–9.0; p < 0.001) [217]. Although these results require further validation with larger groups of patients treated at multiple institutions before they will achieve wide

Figure 3A. Gene expression profiling of fine needle aspirations of breast cancer identifies genes associated with complete pathological response to neoadjuvant paclitaxel/5-fluorouracil, doxorubicin and cyclophosphamide (FAC) chemotherapy. Supervised clustering of the top 500 signal-to-noise ratio markers associated with pathological response from the 24 training samples. All the pathological complete responders (pCR) cluster together and are separated from the samples that had incomplete pathological response (>pCR). In this study, an 81% accuracy of predicting the presence or absence of pathologic complete response after preoperative chemotherapy with sequential weekly paclitaxel and FAC in breast cancer was achieved. More importantly, 75% of the patients who were predicted to have complete pathologic response based on their gene expression profile indeed experienced complete response. This compares very favorably with the 25–30% chance of complete response that unselected patients typically expect with this treatment regimen (data derived from [234]).
The powerful prediction of breast cancer disease outcome demonstrated by these transcriptional profiling studies strongly suggests that this technique may discover a marker set that in the future will be used to help customize the therapy for each individual patient.

**Proteomics**

Matrix-assisted laser desorption ionization and surface-enhanced laser desorption ionization mass spectrometry and other proteomics strategies have shown preliminary success for the early detection of ovarian cancer [223], and have recently been applied to breast cancer for the discovery of new and better biomarkers both in serum and nipple aspirate specimens [223,224].

**Breast cancer prognosis summary**

A summary of the major prognostic factors for breast cancer is included in Table 2. The ancillary biomarkers currently approved by the CAP and ASCO guidelines include ER/PR and HER-2/neu testing.

**Pharmacogenetics**

Greater than one million genetic markers known as single nucleotide polymorphisms (SNPs) have recently become available for genotyping and phenotyping studies [225]. SNP genotyping and gene sequencing have uncovered a variety of familiar cancer predisposition syndromes based on single and multiple gene variants [226] and the discovery of variations in drug metabolism associated with genomic variations in drug metabolizing enzymes such as the cytochrome system [227]. Pharmacogenetic strategies have been used to reduce the incidence of toxicity from such anticancer drugs as amonafide, 5-fluorouracil (5-FU), 6-mercaptopurine, irinotecan (Campto®, Aventis, NJ, USA), epirubicin (Pharmorubicin®, Pharmacia) and flavopiridol [228]. The application of genotyping strategies to predict anticancer drug efficacy has recently emerged in a variety of clinical settings [229,230]. Relevant to breast cancer, recent publications have suggested that overexpression of thymidylate synthase was associated with resistance to 5-FU and related compounds [231].

**Pharmacogenomics**

Two important challenges in the diagnosis and management of breast cancer are: how to identify patients who are at sufficiently low risk for recurrence and can therefore be spared from systemic adjuvant therapy; and how to select the optimal systemic therapy for an individual who is at high risk for recurrence. Pharmacogenomics is an application of whole-genome and protein expression data designed to predict the sensitivity or resistance of an individual’s disease to a single agent or combination therapy. Recently, powerful new technologies emerged that can measure simultaneously the expression of several thousands of mRNA species in a biological specimen. With high-density
DNA microarrays, technically it may be possible to monitor almost all human genes present in a biological sample. The hierarchial clustering technique of data analysis from transcriptional profiling of clinical samples known to have responded or been resistant to a single agent or combination of anticancer drugs has recently been employed as a guide to anticancer drug therapy in cancers of the breast and other organs. Using transcriptional profiling on nylon membranes, the microarray technique has been able to generate 81% accuracy for predicting the presence or absence of pathologic complete response after preoperative chemotherapy with sequential weekly paclitaxel (Taxol®, Bristol-Myers Squibb, NY, USA) and 5-FU, doxorubicin and cyclophosphamide (FAC) in breast cancer. More importantly, 75% of the patients who were predicted to have complete pathologic response based on their gene expression profile indeed experienced complete response. This compares very favorably with the 25–30% chance of complete response that unselected patients may expect with this treatment regimen. In another study using commercial oligonucleotide microarrays with the mRNA extracted from core needle biopsies, different patterns of gene expression significantly correlated with docetaxel (Taxotere®, Aventis) response in invasive breast cancer.

The use of pharmacogenomics to identify subgroups of breast cancers and individualize their treatment has enormous appeal to physicians and patients. However, the challenges concerning the standardization of both the microarray hybridization and associated data interpretation procedures are significant and must be solved before this complex assay can be widely applied at the bedside. Nonetheless, it is likely that the DNA microarray technology will be applied in the clinic in the near future to predict important clinical outcomes that cannot currently be assessed by existing standard methods.

Expert opinion

An earlier and more specific diagnosis and more accurate method to predict response to therapy of breast cancer will continue to challenge the molecular diagnostics industry. Progress in genomics and proteomics will eventually lead to the discovery of new serum-based biomarkers that will compete for disease detection and monitoring applications. The measurement of the ER/PR and HER-2/neu status will remain a cornerstone of this approach along with the determination of HER-2/neu. The ER/PR testing strategy may move towards a more functional rather than static approach (e.g., IHC) with expanded use of high-density genomic microarrays capable of assessing the ER/PR pathway, including downstream events that may aid in the choice of traditional anti-estrogen (tamoxifen-based) versus novel antiestrogenic agents (aromatase inhibitors). Microarrays will also continue to be tested for their ability to predict multigene chemotherapy response in both adjuvant and neoadjuvant settings. Other prognostic factors including p53, plasminogen proteases and MMPs, cell adhesion molecules and signal transduction factors will continue to be evaluated predominantly in research settings, with their likelihood of becoming a standard in clinical practice dependent on their ability to select the best therapy for each individual patient in a new era of personalized medicine.

Key issues

- Can expression profiling and microarray systems designed to assess prognosis and predict specific responses to therapy reach the bedside or will the high cost and current lack of platform or data analysis standardization impede clinical utility?
- Can high-technology proteomics overcome initial problems with sample handling, limited disease biology availability and high cost to overtake gene expression profiling as the method of choice for selecting therapy?
- Will new targeted therapies be developed that will require new pharmacodiagnostic tests for the treatment of breast cancer and further expand the role of molecular diagnostics in the management of patients with this disease?

References

Papers of special note have been highlighted as:

- of interest
- of considerable interest


** O verview of p53 status and correlation with other parameters.


** T horough review of p53 testing in clinical samples: cautions against the use of immunohistochemistry (IHC).
Breast cancer biomarkers and molecular medicine


Ross, Linette, Stec. 672–676 (2001).


Breast cancer biomarkers and molecular medicine


**Initial interest in cathepsin D testing faded when it became clear that the assay was only useful when performed on fresh tissue or protein extracts.**


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Breast cancer biomarkers and molecular medicine


**Initial discovery study of gene expression and breast cancer biology.**


**Transcriptional profiling of breast cancers with long-term follow-up links patterns of gene expression with overall and disease-free survival.**


**Further details and expansion of data of clinical outcome study.**


Can barcode surface-enhanced laser desorption ionization-based proteomics with neural network data analysis detect occult cancer in serum far earlier than currently available cancer detection methods?


Ingelman-Sundberg M. Genetic susceptibility to adverse effects of drugs and environmental toxicants. The role of the CYP family of enzymes. Mutat. Res. 482, 11–19 (2001).


First demonstration of the use of transcriptional profiling to predict the response to multianti breast cancer chemotherapy in the neoadjuvant setting using pathologic complete response as the study end point.


Affiliations

Jeffrey S Ross, MD
Department of Pathology and Laboratory Medicine, M C 80 Albany Medical College, 47 New Scotland Avenue, Albany, NY 12208, USA
Tel.: +1 518 262 5461
Fax: +1 518 262 3663
rossj@mail.amc.edu
Division of Molecular Medicine, Millennium Pharmaceuticals Inc., Cambridge, MA, USA

Gerald P Linette, MD, PhD
Division of Molecular Medicine, Millennium Pharmaceuticals Inc., Cambridge, MA, USA

James Stec, BS
Division of Molecular Medicine, Millennium Pharmaceuticals Inc., Cambridge, MA, USA

Edwin Clark, PhD
Division of Molecular Medicine, Millennium Pharmaceuticals Inc., Cambridge, MA, USA

Mark Ayers, BS
Division of Molecular Medicine, Millennium Pharmaceuticals Inc., Cambridge, MA, USA

Nick Lechly
Division of Molecular Medicine, Millennium Pharmaceuticals Inc., Cambridge, MA, USA

W Fraser Symmans, MD
Depts of Breast Medical Oncology and Pathology, University of Texas MD Anderson Cancer Center, Houston, TX, USA

Lajos Pusztai, MD, PhD
Depts of Breast Medical Oncology and Pathology, University of Texas MD Anderson Cancer Center, Houston, TX, USA

Lejos Pusztai, MD, PhD
Depts of Breast Medical Oncology and Pathology, University of Texas MD Anderson Cancer Center, Houston, TX, USA