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Breast cancer biomarkers and molecular medicine

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

The first part of this two-part review of established and emerging breast cancer biomarkers and molecular diagnostics considers breast cancer predisposition, screening tests for diagnosis, diagnosis using small specimens and metastatic lesions, micrometastatic disease and breast cancer prognosis assessment. Prognostic factors covered in this review include: cytogenetic markers, DNA ploidy and S phase determination, cell proliferation markers, cell cycle regulators and growth factor measurements including epithelial growth factor receptor, *HER-2/neu* and a variety of other relevant molecules controlling proliferation, differentiation and angiogenesis. The first section of part two will continue the consideration of 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 of part two will consider the prediction of therapy response using the techniques of pharmacogenetics and pharmacogenomics.

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†*Author for correspondence*
Department of Pathology and Laboratory Medicine,
MC 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

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It is estimated that, based upon current incidence rates, an American woman has a one in nine chance of developing breast cancer at some time during her life [1]. This two-part review will consider established and emerging biomarkers and molecular diagnostics in breast cancer predisposition, screening tests for diagnosis, diagnosis using small specimens and metastatic lesions, micrometastatic disease, breast cancer prognosis assessment and the response of breast cancer to treatment with targeted therapies.

Breast cancer predisposition

Familial breast and ovarian cancers account for 5–10% of all breast cancers and represent approximately 1250 of newly diagnosed breast cancers per year in the UK and 9000 cases in the USA [2–5]. Familial breast cancer also accounts for approximately 25% of all cases of the disease occurring in women less than 30 years of age. Genetic abnormalities in either *BRCA1* or *BRCA2* appear to account for approximately 90–95% of familial breast cancer cases with the remainder caused by other, predominantly tumor suppressor, genes (TABLE 1). Substantial interest has recently considered the potential role of the *BRCA1* gene in the development of sporadic breast cancer. Initial studies indicated a loss of heterozygosity in the 17q21 region of the *BRCA1* gene in greater than 50% of sporadic breast and ovarian cancers [2–5].

Breast cancer screening

Although serum tumor marker levels, such as carcinoembryonic antigen (CA) 15.3, 27.29 and others, may reflect disease progression and recurrence, they have not proven to be sensitive for early disease detection [6]. Recently, mammaglobin and maspin have demonstrated promise as potential markers of early breast cancer [7,8]. A panel of three serum biomarkers from early-stage breast cancer patients were identified by protein chip (surface-enhanced laser desorption ionization [SELDI]) arrays that could distinguish women with disease (n = 103) from healthy women with 93% sensitivity [9]. The early detection of circulating breast cancer

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cells by morphologic methods is currently being challenged by ultrasensitive proteomic [10] and PCR-based methods often enhanced by immunomagnetic bead-based cell capture [11,12].

Breast cancer diagnosis

Recent molecular studies of fine needle aspiration (FNA) biopsy specimens by transcriptional profiling have demonstrated that gene expression is similar in FNA specimens and corresponding resected tumors [13] and can be used to study resistance to systemic chemotherapy [14,15]. Cytologic examination of nipple duct fluid after canulation and periductal needle aspiration has been used to diagnose breast cancer [16]. An intriguing pilot study using protein chip (SELDI) analysis of 1 µl nipple aspirate fluid revealed several candidate biomarkers detectable in the majority (75–84%) of women with documented breast cancer. Most notably, the 15,940-Da protein was detected with 80% sensitivity and 100% specificity ($p < 0.001$) in women with breast cancer [17]. Estrogen (ER) and progesterone receptor (PR) expression will not, in all cases, separate breast cancer from other malignancies. A panel of immunohistochemical stains for breast cancer-associated glycoproteins, including B72.3, α-lactalbumin and milk fat globule, have been proposed as being capable of specifically identifying breast origin in a biopsy specimen of metastasis in approximately 75% of cases [18,19]. Recently, new markers, such as mammaglobin [8] and maspin [9,20], have demonstrated promise to serve as additional markers of primary breast cancer and are also being used to detect occult metastasis [21]. Until fully validated by confirmatory trials, these markers are no substitute for histopathology in the definitive diagnosis of breast cancer.

Micrometastasis detection

A wide variety of studies using both immunocytochemical and RT-PCR techniques have been published in an attempt to link the presence of occult micrometastases of breast cancer with disease outcome (FIGURE 1) [22–26]. At this time, consensus has not been reached as to whether the detection of tumor cells or relatively tumor-specific mRNA in sentinel lymph node and bone marrow biopsies independently predicts prognosis and should be used to enhance staging accuracy and plan systemic therapy.

Prognostic & predictive factors in breast cancer

In 1991, McGuire and collaborators described a series of rigorous requirements for adopting a new prognostic marker into clinical practice [27]. Although a wide variety of biomarkers have achieved promise on the basis of preliminary results, only *HER-2/neu* testing has been formally incorporated into standard practice over the past three decades.

Cytogenetics

Complex karyotypes have been associated with unfavorable outcome in breast cancer [28,29], and modern techniques including cDNA microarrays and comparative genomic hybridization (CGH) have further identified complex genetic defects associated with adverse prognosis [30,31].

DNA ploidy & S phase analysis

Studies on the prognostic significance of ploidy and S phase status have varied greatly with some investigators finding significant prediction of disease-free and overall survival on both univariate and multivariate analysis and others finding no impact on disease outcome (FIGURE 2) [32]. The S phase calculation by flow cytometry has generally outperformed ploidy status as a prognostic factor in breast cancer and is advocated by some investigators as a useful clinical parameter. Despite their continuing clinical use in many institutions, neither the American Society of Clinical Oncologists (ASCO) [33] nor the College of American Pathologists (CAP) [34] include ploidy and S phase measurements in their list of recommended prognostic factors. The lack of a standardized approach to performing this test and interpreting its result is the major reason S phase fraction is not accepted as a standard prognostic marker.

Table 1. Genes associated with breast cancer susceptibility.

Gene	Location	Familial breast cancer association	Sporadic breast cancer association	Other cancers
BRCA1	17q	High (40%)	High	Ovary (colon, prostate)
BRCA2	13q	High (40%)	High	No ovary, male breast (prostate)
p53	17p	Low	High	Carcinomas, sarcomas, leukemias
RAS (HRAS)	11p	Low in young, higher in old	High in old	Carcinomas, sarcomas, leukemias
Ataxia telangectasia	11q	Low	Low	Lymphomas, leukemias
hMSH2 *hMLH1* (lynch II)	2p 3p	Low	Low	Colon, skin, stomach
Neurofibromatosis 1	17q	Very low	Low	Nerve, brain
Androgen receptor	Xq	Only males, low	Only males	Male breast

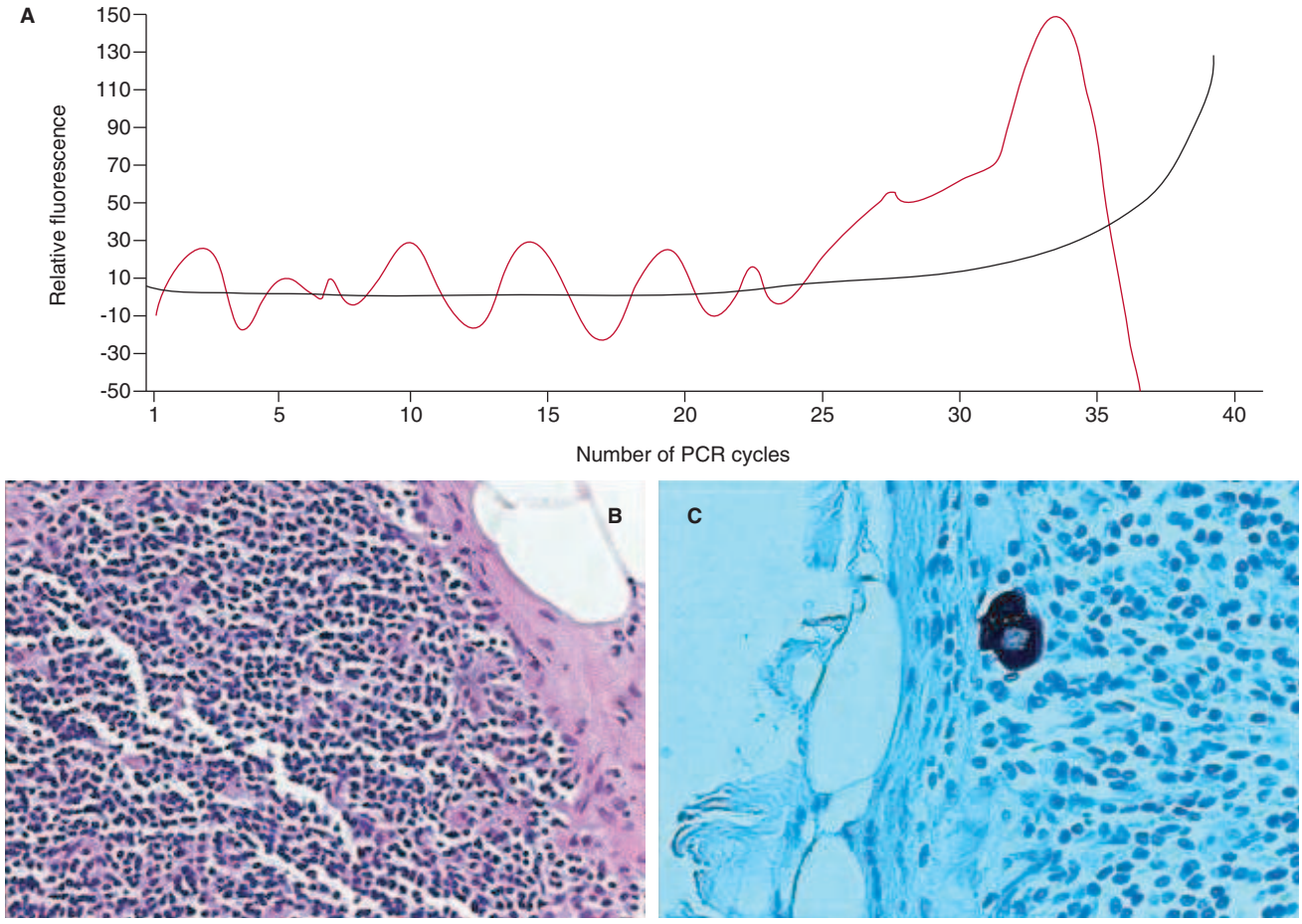

Figure 1. Micrometastasis detection of breast cancer in a sentinel auxiliary lymph node biopsy. (A) Real-time PCR (TaqMan™) detection of cytokeratin 19 mRNA after 25 cycles of amplification (red) normalized to internal control dye (black). **(B)** Hematoxylin and eosin stained section of one-half of the same lymph node showing no evidence of tumor cells. **(C)** Immunohistochemical stain for cytokeratin 19 demonstrates a single aggregate of two malignant cells beneath the surface in the subcapsular sinusoid.

Cell cycle associated markers

Cell proliferation labeling measured by Ki-67 immunostaining correlates with the S phase levels calculated by flow cytometry but is generally higher, reflecting the fact that the Ki-67 antigen is also expressed in late G1 as well early G2/M phases of the cell cycle (FIGURE 3) [35]. Ki-67 staining has achieved a more consistent significant correlation with breast cancer outcome both on univariate and multivariate analysis than DNA ploidy alone. Amplification or overexpression of cyclin D1 (*PRAD1* or bcl-1), localized to chromosome 11q13, has also been identified in 20% of clinical breast cancers [36], and has been linked to the expression of the ER [37] and the transition from *in situ* to invasive ductal breast cancer [38]. In a recent study, high levels of the low-molecular-weight isoforms of cyclin E, measured by western blotting, correlated strongly with decreased disease-specific survival [39]; moreover, levels of total cyclin E were also highly correlative with poor outcome, which is consistent with prior studies performed by immunohistochemistry (IHC) [40]. The p21 protein (p21/WAF1/Cip1) is an inhibitor of cyclin-dependent kinases (CDKs) and serves as a critical downstream effector in the p53-specific pathway of cell growth control [41]. Some studies have linked altered expression of p21 with adverse outcome in breast cancer [42,43], whereas others have not [44]. p27 (kip1) is a cell cycle regulator that acts by binding and inactivating CDKs [44]. Low p27 expression has been correlated with poor prognosis in many (but not all) studies of patients, especially those with small primary tumors [45–48]. The S phase kinase-associated protein Skp2 is required for the ubiquitin-mediated degradation of various proteins including the CDK inhibitor p27 [49]. Skp2 expression is inversely proportional to the expression of p27. A recent report suggests an important role for skp2 overexpression in the pathogenesis of ER-negative/HER2-negative breast carcinoma [49], which is consistent with the proposal that *skp2* can serve as a proto-oncogene.

Growth factors & receptors

The epidermal growth factor receptor (EGFR), also known as c-*erb*-B-1 and HER-1, is a member of a family of transmembrane receptors that also includes HER-2, -3 and -4. HER-1 shares significant homology with the HER-2/neu protein, featuring an intrinsic tyrosine kinase active intracellular domain that is activated by the ligand(s) binding to the EGFR. EGFR is

overexpressed in 14–90% of breast cancers, depending on the material tested and the method used to detect or quantitate the receptor. EGFR overexpression has been linked to adverse prognosis in a variety of tumors including breast cancer [50,51]. Studies of EGFR in breast cancer have conflicted with some groups with some finding correlation with prognosis [52] and others finding no correlation [53]. The frequent finding of either gene amplification, gene mutation and/or protein overexpression of EGFR in breast cancer which has ranged from 67 to as high as 90% [54] has prompted numerous clinical trials employing small molecule inhibitors [55] and antibodies [56] targeting the EGFR pathway. To date, the clinical trials have yielded some evidence of efficacy but have failed to establish this strategy as a new type of successful therapy in the marketplace. There is no standardized test for EGFR and despite the enthusiasm for this molecule as a therapeutic target, it is not considered a prognostic factor for routine use.

HER-2/neu

Amplification and overexpression of the *HER-2/neu* gene and protein have been identified in 10–34% of invasive breast cancers [57]. The ligand for the HER-2/neu protein receptor has not been identified and its activation may occur through homo- and heterodimerization with other family members (EGFR, HER-3 and -4). Both morphology-based and molecular-based techniques have been used to measure HER-2/neu status in breast cancer clinical samples (TABLE 2) [57]. The vast majority of these studies have linked either gene amplification or protein overexpression of HER-2/neu with adverse prognosis in either node-negative or node-positive disease [57]. In general, when specimens have been carefully fixed, processed and embedded, there has been excellent correlation between gene copy status and protein expression levels [57–60]. IHC staining, which has been the predominant method used, can be significantly impacted by technical issues, especially in archival fixed paraffin-embedded tissues (FIGURE 4A). Advantages of IHC testing include its wide availability, relatively low cost, easy preservation of stained slides and use of a familiar routine microscope. Disadvantages of IHC include the impact of preanalytic issues, including storage, duration and type of fixation, intensity of antigen retrieval, type of antibody (polyclonal vs. monoclonal), nature of system control samples and, most importantly, the difficulties in applying a subjective slide scoring system. Two commercially available HER-2/neu IHC kits, the Dako Herceptest™ (DakoCytomation, Glostrup, Denmark) and the Ventana Pathway™ (Ventana Medical Systems Inc., AZ, USA), are approved for sale by the US Food and Drug Administration (FDA) for determining eligibility for patients to receive the antiHER-2/neu therapeutic antibody trastuzumab (Herceptin®, Genentech, CA, USA). Problems with standardization in slide scoring have been recently highlighted in reference to the best method for using HER-2/neu status to predict response to trastuzumab [61]. Slide scoring can be improved by avoiding specimen edges, retraction artifacts, under- or overfixation, cases with significant staining of benign elements and tumor cells lacking a complete membranous staining pattern (the so-called chicken wire appearance). The use of a computerized image analysis system can reduce slide-scoring variability among pathologists and improve the reproducibility of the IHC technique [62]. Finally, in a recent study, the use of an antibody designed to detect phosphorylated HER-2/neu receptor demonstrated significant promise as a more powerful prognostic factor [63].

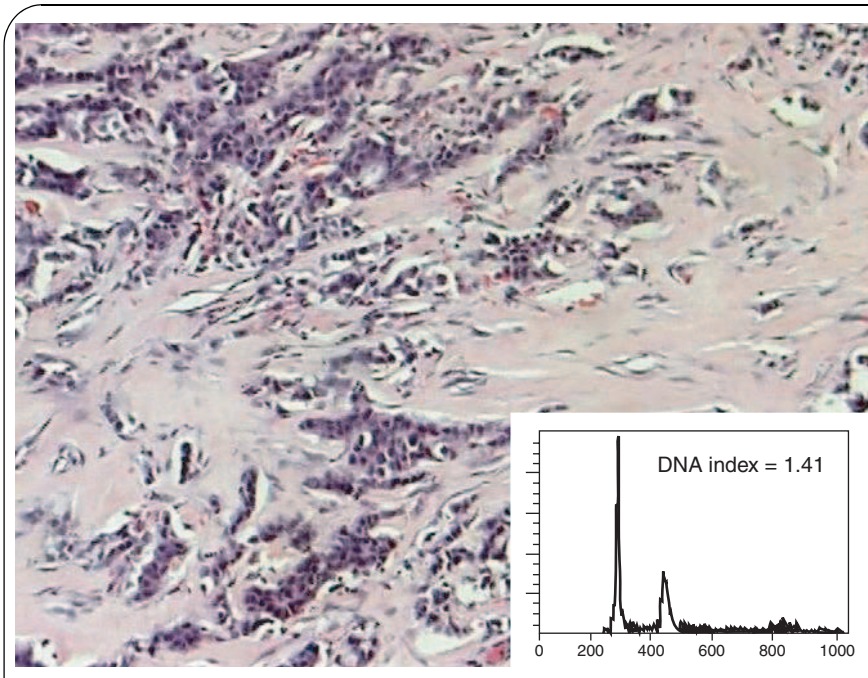

Figure 2. DNA ploidy in breast cancer. Infiltrating ductal breast cancer with high nuclear grade. Inset shows aneuploid DNA content determined by flow cytometry with DNA index of 1.41.

Southern and slot blotting are significantly impacted when tumor cell DNA extracted from the primary carcinoma sample is diluted by DNA from benign breast tissue and inflammatory cells.

The fluorescent *in situ* hybridization (FISH) technique, which is morphology-driven and like IHC can be automated, has the advantages of an objective scoring system and the presence of a built-in internal control consisting of the two *HER-2/neu* gene signals present in all cells in the specimen (FIGURE 4B). Disadvantages of FISH testing include the higher cost of each test, longer time required for slide scoring, requirement of a fluorescence microscope and the inability to preserve the slides for storage and review. Two versions of the FISH assay are FDA-approved: the Ventana Inform™ test (Ventana Medical Systems Inc.), that measures only *HER-2/neu* gene copies and

the Abbott–Vysis Pathvysion™ test (Abbott Laboratories, IL, USA; Vysis Inc., IL, USA) that includes a chromosome 17 probe in a dual-color format. Published studies indicate that the two assays are highly correlative [64]. However, the Inform system cannot distinguish true *HER-2/neu* gene amplification from chromosome 17 polysomy and the Pathvysion test will classify significant chromosome 17 polysomies (e.g., 6 or more copies) as negative in cases which may ultimately prove to be trastuzumab responsive. The chromogenic *in situ* hybridization (CISH) method features the advantages of both IHC (routine microscope, lower cost, familiarity) and FISH (built-in internal control, objective scoring, the more robust DNA target) but is not, to date, FDA-approved for selecting patient eligibility for trastuzumab treatment (FIGURE 4C) [65–67]. A recent CISH-based study found that *HER-2/neu* gene amplification detected by this method was an independent predictor of adverse disease outcome [68].

The RT-PCR technique has predominantly been used to detect *HER-2/neu* mRNA in peripheral blood and bone marrow samples [69,70]. It has correlated more with gene amplification status than IHC levels [71] and failed to predict survival, however, did correlate with ER/PR and tumor grade status in one breast cancer outcome study of 365 patients [72].

The enzyme-linked immunosorbent assay (ELISA) technique, when performed on tumor cytosol made from fresh tissue samples, avoids the potential antigen damage associated with fixation, embedding and uncontrolled storage. However, the small size of breast cancers associated with expanded screening programs in the USA generally precludes tumor tissue ELISA methods because insufficient tumor tissue is available to produce a cytosol.

HER-2/neu status & the prediction of response to trastuzumab therapy

Using recombinant technologies, trastuzumab, a monoclonal immunoglobulin G1 class humanized murine antibody, was developed to specifically target patients with advanced relapsed breast cancer that overexpressed the HER-2/neu protein [73]. Since its launch in 1998, trastuzumab has become a major therapeutic option for patients with HER-2/neu-positive breast cancer and is being used not only for its approved indication as second-line treatment for advanced metastatic disease but also in earlier stage disease as well as in neoadjuvant treatment protocols [74–76]. The best method to identify patients for trastuzumab therapy has been a source of controversy. The original IHC technique used as the clinical trial assay was succeeded by the commercial Herceptest. This assay was originally criticized for yielding false-positive results [77], although better performance was ultimately achieved when the test was performed exactly according to the manufacturer's instructions. Concern over IHC accuracy using standard formalin-fixed paraffin embedded tissue sections has encouraged the use of the FISH assay for its ability to predict trastuzumab response rates [78]. Reports that FISH could outperform IHC in predicting trastuzumab response [79] and well-documented lower response rates of 2+ IHC staining versus 3+ staining tumors [80] has resulted in an approach that either uses IHC as a primary screen with FISH testing of all 2+ cases or primary FISH-based testing (FIGURE 5) [81,82]. In a recently published study where trastuzumab was used as a single agent, the response rates in 111 assessable patients with 3+ IHC staining was 35% and the response rate for 2+ cases was 0%. The response rates in patients with and without *HER-2/neu* gene amplification detected by FISH were 34 and 7%, respectively [79]. In a study of trastuzumab plus paclitaxel (Taxol®, Bristol–Myers Squibb, NY, USA) in patients with HER2/neu-overexpressing tumors, overall response rates ranged from 67 to 81%, compared with 41 to 46% in patients with normal expression of HER2/neu [83]. However, there are currently no published studies describing the response to trastuzumab in patients that were classified for HER-2/neu status by FISH testing alone. Moreover, the original comparison study of IHC and FISH included both 2+ and 3+ cases in the IHC analyses and when only 3+ IHC cases are evaluated, the response rates to trastuzumab therapy either

Table 2. HER-2/neu testing techniques.

Method	Target	FDA-approved	Slide-based
IHC	Protein	Yes[§]	Yes
FISH	Gene	Yes[§]	Yes
Southern blot	Gene	No	No
PCR	Gene	No	No
RT-PCR	mRNA	No	No
Tumor ELISA	Protein	No	No
Serum ELISA	Protein	Yes	No

[§]Approved for trastuzumab selection.
ELISA: Enzyme-linked immunosorbent assay; FDA: US Food and Drug Administration; FISH: Fluorescence *in situ* hybridization; IHC: Immunohistochemistry.

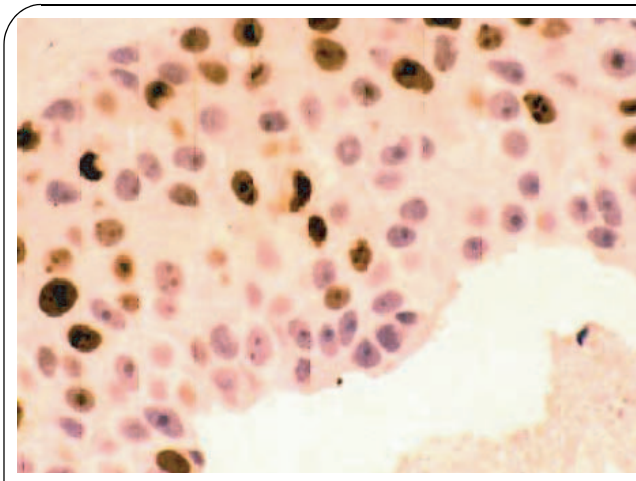

Figure 3. Cell cycle analysis in breast cancer. Intraductal component of a poorly differentiated infiltrating ductal breast cancer with high Ki-67 labeling index (greater than 25%) demonstrated by immunohistochemistry.

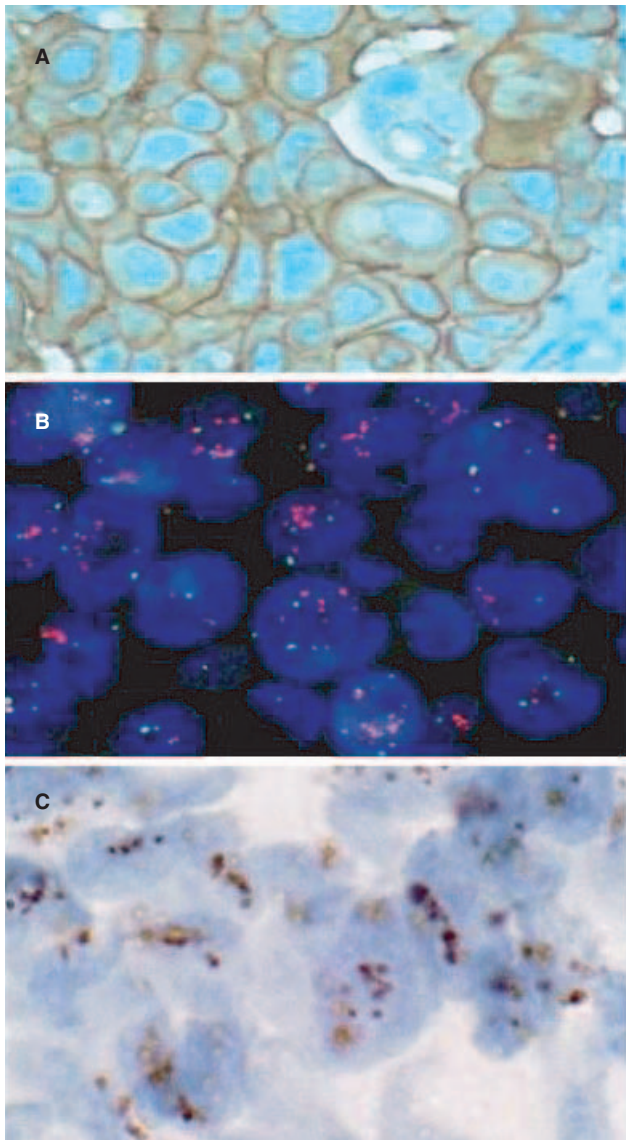

Figure 4. HER-2/neu testing in breast cancer.
(A) Immunohistochemistry using Herceptest™ system with continuous membranous 3+ positive immunostaining for HER-2/neu protein. **(B)** *HER-2/neu* gene amplification detected by fluorescence *in situ* hybridization (Pathvysion™ system). **(C)** *HER-2/neu* gene amplification detected by chromogenic *in situ* hybridization (Zymed System). (Figure prepared in collaboration with Ken Bloom of US Labs, Inc., Irvine, CA, USA).

as a single agent or in combination with cytotoxic drugs in the 3+ IHC group was virtually identical to that observed in the FISH positive group [79]. In summary, while the superiority of one method versus the other remains controversial [84,85], most laboratories are either screening all cases with IHC and triaging selected cases for FISH testing or using FISH as the only method for *HER-2/neu* testing.

Prediction of response of breast cancer to other therapies

The best established correlate between *HER-2/neu* status and nontrastuzumab therapy response is the reported resistance of *HER-2/neu*-positive patients to hormonal therapy alone [86–89]. Tumors that overexpress *HER-2/neu* are more likely to be ER and PR negative than tumors that do not demonstrate overexpression. In fact, when measured as continuous variables, the expression of *HER-2/neu* appears to be inversely related to the expression of ER and PR, even in hormone receptor-positive tumors [90]. In some studies, *HER-2/neu* positive tumors were specifically resistant to tamoxifen therapy [91–93]. However, in other studies, *HER-2/neu* status failed to predict tamoxifen resistance in ER-positive cases [94]. In another study, ER-positive and *HER-2/neu*-positive tumors were not only resistant to tamoxifen but single-agent tamoxifen treatment actually had an adverse impact compared with untreated patients [95]. However, this finding has not, to date, been confirmed by large intergroup studies in the USA [96]. Most recently, data from a relatively small study of ER-positive/*HER-2/neu*-positive tumors suggested that there was a relatively better response to alternative hormonal therapies, such as an aromatase inhibitor, compared with tamoxifen in a neoadjuvant setting [97]. Importantly, while HER-2-overexpression/amplification is correlated with resistance to tamoxifen, resistance is partial and not complete, and it is uncertain whether resistance extends to other hormonal interventions. Studies of the association of HER-2/neu protein overexpression with response of tumors in patients treated with cyclophosphamide, methotrexate, 5-fluorouracil (CMF) adjuvant chemotherapy [94], as well as to taxane-based regimens [98–100], have been controversial with some reports claiming that HER-2/neu status impacted disease outcome while others found no significant differences [101,102]. In the pivotal randomized trial, the response rate to paclitaxel in HER-2-positive tumors was 14 months and the median time-to-progression was 3 months when given as first-line therapy for metastatic breast cancer. This is far below the expected performance of paclitaxel in unselected patients with metastatic breast cancer. However, in another study, HER-2/neu-positive breast cancers were three-times more sensitive to paclitaxel [103]. HER-2/neu overexpression has also been associated with enhanced response rates to anthracycline-containing chemotherapy regimens in some but not all studies [104–108]. Since anthracyclines are topoisomerase inhibitors and topoisomerase IIα is frequently coamplified with *HER-2/neu*, it has been suggested that *HER-2/neu* may be serving as a surrogate marker. Cell lines transfected with *HER-2/neu* and then exposed to doxorubicin (Adriamycin®, Pharmacia, NJ, USA) *in vitro* did not show enhanced sensitivity to the chemotherapy relative to the parent cell lines [109]. At this time, however, it is not clear whether HER-2/neu protein expression, as demonstrated in one study which lacked a control arm [110], or topoisomerase IIα expression is the better predictor of the response of breast cancer to the antitopoisomerase anthracycline epirubicin (Pharmorubicin®, Pharmacia). Other studies have consistently linked coexpression and coamplification of the topoisomerase IIα and *HER-2/neu* genes with adverse prognosis and sensitivity to anthracycline drugs [111–116].

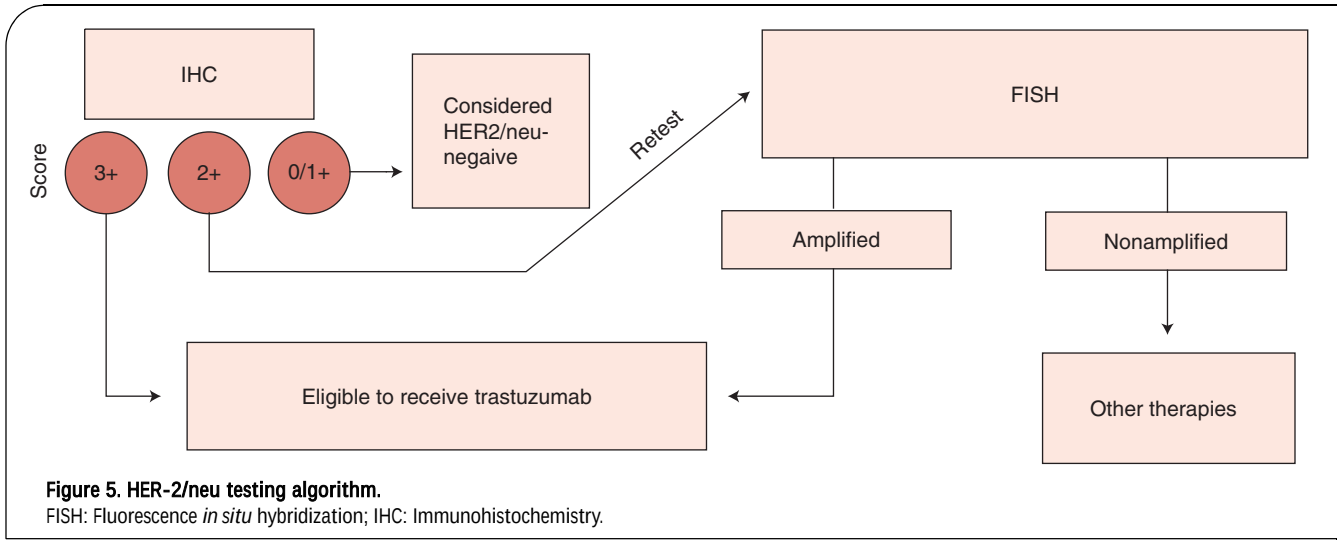

Figure 5. HER-2/neu testing algorithm.
FISH: Fluorescence *in situ* hybridization; IHC: Immunohistochemistry.

HER-2/neu immunostaining has successfully predicted local recurrence in patients receiving surgery and radiation [117]. In summary, although strong trends have been presented in the published studies, including the resistance to tamoxifen and sensitivity to anthracycline regimens for *HER-2/neu*-positive tumors, more studies are needed using appropriate control arms to confirm these important associations. Should this be accomplished it would seem likely that *HER-2/neu* testing, which achieved standard-of-care status in the ASCO breast cancer clinical practice guidelines in 2001, would be of even greater value in the management of breast cancer patients.

Serum HER-2/neu antigen levels as a tumor marker

Circulating levels of the cleaved extracellular domain of the HER-2/neu receptor protein have successfully predicted the presence and progression of HER-2/neu-positive breast cancer. Serum HER-2/neu levels have correlated with decreased survival and absence of clinical response to hormonal therapy in ER-positive tumors in some studies [118,119] but not in others [119].

HER-2/neu expression & breast pathology

HER-2/neu overexpression has been consistently associated with the more aggressive and extensive forms of ductal carcinoma *in situ* [120–122] and both mammary and extramammary Paget's disease [123,124]. The majority of studies that have compared the HER-2/neu status in paired primary and metastatic tumor tissues have found an overwhelming consistency of the HER-2 status in both invasive and noninvasive tumors, regardless of the method of testing (IHC vs. FISH) [125–129]. In the largest published study comparing the paired primary tumor and distant metastatic lesions, 94 and 93% of samples had concordant HER-2/neu status when analyzed by IHC or FISH, respectively [130]. *HER-2/neu* amplification and overexpression has been associated with adverse outcome in some studies of male breast carcinoma [131–134], but not in others [135,136]. Finally, low-level HER-2/neu overexpression has been identified in benign breast disease biopsies and associated with an increased risk of subsequent invasive breast cancer [137].

Other growth factors.

The expression of transforming growth factor (TGF)-α, an activating ligand for EGFR, has been associated with disease recurrence and adverse prognosis in breast cancer [138,139], and may mediate its effects through activation of the ER pathway [140]. TGF-β is a regulatory peptide that, in addition to a role as a cell growth mediator, is a potent stimulator of fibroblasts and extracellular matrix production [141]. TGF-β expression has been linked to stromal proliferation in breast tissues [142], although it has not been implicated as a prognostic factor for epithelial breast malignancy. Insulin and insulin-like growth factors (IGF)-I and -II and their receptors have been associated with cell proliferation and linked to overall survival in breast cancer [143,144]. Platelet-derived growth factor (PDGF) has been linked to the desmoplastic stromal response in breast cancer [145] and has been identified as a prognostic factor for the disease [146]. Fibroblast growth factors (FGF), including the related *int-2* and *HST-1* genes, have been linked to breast cancer prognosis in some studies [147–149] but not in others [150]. Vascular endothelial growth factor (VEGF), the most potent endothelial cell mitogen and a regulator of vascular permeability, and its various receptors have been extensively studied in breast cancer and associated with adverse prognosis in some studies [151–154] but not in others [155–157]. The number of microvessels in the richest vascular area of invasive breast cancer has been an inconsistent predictor of prognosis in breast cancer [158,159]. Tumor VEGF expression may be more reliable than microvessel density measurements as a predictor of angiogenesis and adverse prognosis [160]. To date, antiangiogenesis therapies including small molecules, ribozymes and antibodies have failed to achieve significant efficacy for the treatment of metastatic breast cancer.

Conclusion & expert opinion

An earlier and more specific diagnosis of breast cancer will continue to challenge the molecular diagnostics industry with nipple aspiration techniques, proteomics methods, enhanced RT-PCR protocols and immunomagnetic bead cell capture

procedures assisting and competing with enhancements in breast imaging. Genomic and proteomic discoveries will lead to the discovery of new serum-based biomarkers, such as maspin and mammoglobin, which will compete for disease detection and monitoring applications and seek to become the 'prostate-specific antigen' for breast cancer.

Five-year view

Over the next 5 years, further clarity will be reached concerning the best method for the early detection of breast cancer with the nipple aspiration technique finding a clinical application or becoming discarded. Serum-based breast cancer detection research will continue and the potential of proteomics methods, enhanced RT-PCR techniques and immunomagnetic bead cell capture procedures will compete with enhanced breast imaging for their ability to detect the disease earlier, while reducing the high false-positive rate of current screening procedures. Biomarkers, such as maspin and mammoglobin, will be evaluated in large cohorts of patients as potential new serum assays for disease detection and monitoring. Emphasis will be placed upon therapy-specific tests and not on stand-alone prognostic factors. For this reason, DNA ploidy and cell proliferation assays are likely to continue to lose popularity. Given the established efficacy for trastuzumab for the treatment of metastatic breast cancer, HER-2/neu testing will continue as a standard-of-care with CISH replacing both IHC and FISH as the preferred measurement technique. EGFR testing will likely not achieve widespread use unless future research, unlike currently available data, finds that either gene or protein status can guide the use of antiEGFR targeted therapies.

Key issues

- Will high-throughput genomics, proteomics, nipple duct aspiration, RT-PCR and magnetic cell capture techniques generate new and clinically useful stand-alone biomarkers for breast cancer early diagnosis and monitoring?
- Are micrometastases in lymph nodes and bone marrow clinically significant and will serial section, immunohistochemistry (IHC), RT-PCR and other enhanced detection methods become standard-of-care?
- Will stand-alone prognostic tests that do not impact specific therapy selection continue to be offered in their traditional roles for selection of patients who need to be treated with adjuvant chemotherapy?
- Will the chromogenic *in situ* hybridization method of *HER-2/neu* gene amplification detection, combining the best aspects of fluorescence *in situ* hybridization and IHC, achieve a trastuzumab eligibility equivalence with currently approved tests and become the prevalent clinical assay?
- Will epithelial growth factor receptor (EGFR) testing be used to guide anti-EGFR therapy in breast cancer?

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- •• of considerable interest

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Affiliations

- *Jeffrey S Ross, MD*
Department of Pathology and Laboratory Medicine,
MC 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
Department of Medicine,
Washington University,
St. Louis, MO, USA
- *James Stec, BS*
Division of Molecular Medicine,
Millennium Pharmaceuticals, Inc.,
Cambridge, MA, USA
- *EdwinClark, 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 Leschly*
Division of Molecular Medicine,
Millennium Pharmaceuticals, Inc.,
Cambridge, MA, USA
- *W Fraser Symmans, MD*
Departments of Breast Medical Oncology and Pathology,
The University of Texas MD Anderson Cancer Center,
Houston, TX, USA
- *Gabriel N Hortobagyi, MD*
Departments of Breast Medical Oncology and Pathology,
The University of Texas MD Anderson Cancer Center,
Houston, TX, USA
- *Lajos Pusztai, MD, PhD*
Departments of Breast Medical Oncology and Pathology,
The University of Texas MD Anderson Cancer Center,
Houston, TX, USA