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Review

REVIEW

Gene Expression Assays for Early-Stage Hormone Receptor–Positive Breast Cancer: Understanding the Differences

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Abstract

Biomarkers are frequently used to guide decisions for treatment of early-stage estrogen (ER) and progesterone (PR) receptor–positive (ER/PR+) invasive breast cancers and have been incorporated into guidelines. The American Society of Clinical Oncology (ASCO) 2016 guideline and a 2017 update were recently published to help clinicians use the tests available. ASCO currently recommends five tests that show evidence of clinical utility based on the parameters defined in the guideline. These include the 21-gene assay (Oncotype DX), Prediction of Analysis of Microarray-50 (PAM50), 12-gene risk score (Endopredict), Breast Cancer Index (BCI), and, most recently, the 70-gene assay (Mammaprint). However, discordance is often seen when the results of these gene assays are compared in a particular patient, for a number of reasons: the assays were initially developed to answer different questions, and the molecular makeup of each signature reflects this; the patient populations that were studied also differed and may not reflect the patient being tested; furthermore, the study design and statistical analysis varied between each test, leading to different scoring scales that may not be comparable. In this review, the background on the development and validation of these assays is discussed, and studies comparing them are reviewed. To provide guidance on which test to choose, the studies that support the level of evidence for clinical utility are presented. However, the choice of a particular test will also be influenced by socioeconomic factors, clinical factors, and patient preferences. We hope that a better understanding of the scientific and clinical rationale for each test will allow patients and providers to make optimal decisions for treatment of early-stage ER/PR+ breast cancer.

Invasive breast cancers are the most common malignancies among women, with 12% of all women diagnosed in their lifetime and a total of 3 million women living with breast cancers in 2013 (1). Sixty-one percent of those cases are women with early-stage breast cancers that are limited to the breast and lymph nodes. Recurrence can occur within five to 10 years, with 15% to 26% of patients developing distant metastases (2–4). While some women with early breast cancer may do well with localized treatment, it is thought that additional systemic therapy may be needed in some subtypes to prevent breast cancer recurrence. Systemic treatments include endocrine therapy, chemotherapy, and, increasingly, targeted therapy, based on molecular and clinical characteristics of the disease. The decision about which patient should receive chemotherapy is challenging as there are clinically significant toxicities, and improved clinical outcome is not realized in all patients (5). Clinicopathologic factors such as the patient's age, race, comorbidity, tumor size, grade, and nodal status factor into the decision-making process (5). Algorithms such as Adjuvant! Online, a web-based tool to determine risk of recurrence, can assist in this regard (https://www.adjuvantonline.com/) (6,7). Of note, the Adjuvant! Online website is currently under construction, and the future ability of providers and patients to assess clinical risk using Adjuvant! Online is unclear. A potential

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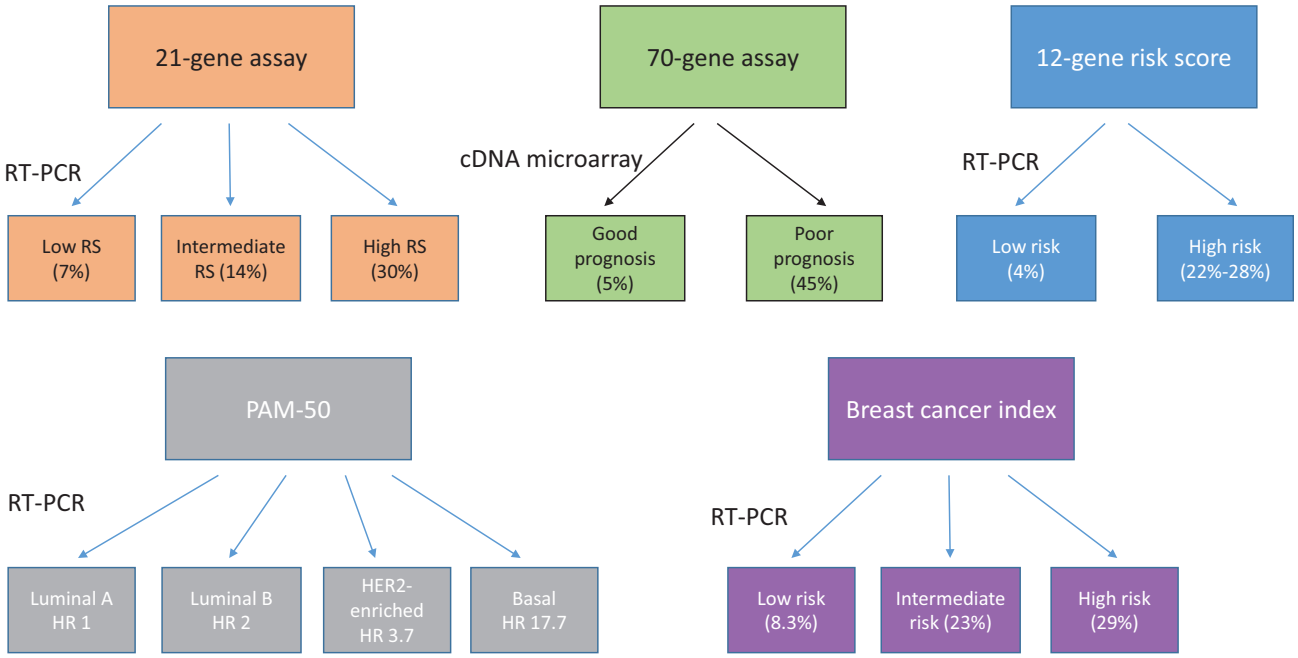

Figure 1. Methods and scoring system of gene expression assays for early-stage breast cancers. Percentages indicate risk of recurrence. HER2 = human epidermal growth factor receptor; HR = hazard ratio; RT-PCR = reverse transcriptase polymerase chain reaction; RS = Recurrence Score.

alternative is the tool "Predict" (http://www.predict.nhs.uk/predict.html), which has similar prognostic accuracy as Adjuvant! (8).

In addition, family support, geography, and other personal stressors affect the decision and should always be considered. While these factors inform the decision-making process, they are imperfect and imprecise. Refinement of risk prediction is therefore necessary, particularly in lower-risk groups where the likelihood of toxicity may be greater than treatment benefit.

Sequencing the human genome led to an improved understanding of the role of genomic composition on cancer biology and clinical outcome. These molecular characteristics include aberrant activation of cell signaling pathways, epigenetic modifications, and changes in the tumor microenvironment that can promote a more aggressive disease phenotype (9), which, in turn, plays a role in predicting outcome. Several molecular tests, based on gene expression, were developed to improve risk prediction for breast cancer patients. These molecular "signatures" attempt to identify patients at increased risk of recurrence who may benefit from systemic chemotherapy in addition to antiestrogen treatment. In contrast, patients predicted to be at lower risk by these assays may not require additional treatment beyond endocrine therapy (10–12).

This review evaluates five different gene assays and how they relate to treatment decisions: the 21-gene assay (Oncotype DX), Prediction of Analysis of Microarray-50 (PAM50), 12-gene risk score (Endopredict), Breast Cancer Index (BCI), and, most recently, the 70-gene assay (Mammaprint) (Figure 1). While some other gene assays are listed, they have not shown the level of evidence needed to validate their claims. Clinical evidence supporting each assay is compared to facilitate informed decision-making when choosing a particular test. We review initial studies behind each assay and the molecular mechanisms that form the background and predictive basis.

Discordance exists when these tests are compared against each other. A patient can be at high risk of recurrence using one test and low risk using another. These differences can be due to the difference in their origin and associated molecular mechanisms behind the gene signatures. For example, the 21-gene assay was developed in an estrogen and progesterone receptor–positive (ER/PR+) population, evaluating risk of recurrence in ER/PR+. In contrast, the 70-gene assay was initially focused on the evaluation of risk of metastases in node-negative breast cancer. The PAM50 assay distinguished between different types of breast cancer and secondarily found that it was predictive of risk. Each of these assays categorizes women into low- or high-risk groups, and some also include an intermediate-risk category. Risk is also analyzed by both clinical and molecular risk factors.

Based on guidelines for biomarker studies established by Simon, Paik, and Hayes (13), clinical utility is best proven by a prospective randomized trial such as the Microarray in Node-negative and 1 to 3 Positive Lymph Node Disease may Avoid Chemotherapy (MINDACT) study for the 70-gene assay or TAILORx for the 21-gene assay. Because it is often difficult to recruit, evaluate, and randomize based on a biomarker, the prospective-retrospective study is also used to establish clinical utility. This type of study includes patients from a prospective clinical trial and evaluates the marker of interest with archived tissue. To achieve level 1 evidence, two prospective-retrospective studies need to be performed (13). Gene expression assays included in this review have all achieved that goal, as outlined in Table 1.

Gene Expression Signatures Used for Treatment Recommendations for Earl-Stage Breast Cancers

21-Gene Assay (Oncotype DX)

The 21-gene expression assay was developed for use in women with node-negative, ER/PR+ breast cancer, derived from a

Table 1. Clinical studies of gene expression assays demonstrating clinical utility

Gene assay	Study	Type of trial used for LOE determination*	Clinical trial	No.	LN-negative LOE for clinical utility*	LN-positive LOE for clinical utility*
21-gene assay					1A	1B
	Paik et al. 2004 (14)†	Prospective-retrospective	NSABP B14	668	B	N/A
	Paik et al. 2006 (15)†	Prospective-retrospective	NSABP B20	651	B	N/A
	Sparano et al. 2015 (16) †	Randomized prospective	TAILORx	10 253	A	N/A
	Albain et al. 2010 (17)†	Prospective-retrospective	SWOG trial CAF-T S8814	367	NA	B
	Goldstein et al. 2008 (18)	Prospective-retrospective€	ECOG trial E2197	465	B	B
	Dowsett et al. 2010 (19)	Prospective-retrospective€	TransATAC	1231	B	B
	Denduluri et al. 2011 (20)	Retrospective		50	D	D
	Petkov et al. 2016 (21)	Retrospective	SEER	38 568	D	D
	Gluz et al. 2016 (22)	Prospective	Plan B	3198	C	C
	Bartlett et al. 2016 (23)	Randomized prospective	OPTIMA	313	B	B
	Shivers et al. 2013 (24)	Retrospective		148	D	D
	Clough et al. 2012 (25)	Retrospective		67	D	D
70-gene assay					1A	1A
	van't Veer et al. 2002 (26)		None	117		
	van de Vijver et al. 2002 (27)	Retrospective	None, consecutive patients	295	D	N/A
	Bueno-de-Mesquita et al. 2007 (28)	Prospective	RASTER	427	A	N/A
	Mook et al. 2010 (29)	Retrospective	None	148	D	N/A
	Cardoso et al. 2016 (30)†	Randomized prospective	MINDACT	6693	A	A
PAM50					1B	1B
	Parker et al. 2009 (31)		5 different hospitals	189/279	Development/ validation of PAM50	
	Nielsen et al. 2010 (32)	Retrospective	BCCA Series	786	D	D
	Gnant et al. 2013 (33)†	Prospective-retrospective	ABCSG-8	1478	B	B
	Gnant et al. 2015 (34)†	Prospective-retrospective	ABCSG-8, ATAC	543	B	B
	Dowsett et al. 2013 (35)	Prospective-retrospective	ATAC	1017	B	B
	Liu et al. 2016 (36)†	Prospective-retrospective	CALGB 9741	1471	N/A	B
12-gene risk score					1B	1B
	Filipits et al. 2011 (37)†		ABCSG-6 and -8	378 1324	Development/ validation	N/A
	Dubsky et al. 2012 (38)†	Prospective-retrospective	ABCSG-6 and -8	1702	B	B
	Dubsky et al. 2013 (39)†	Prospective-retrospective	ABCSG-6 and -8	1702	B	B
	Fitzal et al. 2015 (40)	Prospective-retrospective	ABCSG-8	1324	B	B
	Buus et al. 2016 (41)	Prospective-retrospective	ATAC	928	B	B
BCI					1B	2B
	Goetz et al. 2006 (42)†	Prospective-retrospective	NCCTG 89-30-52	211	B	B
	Jerevall et al. 2011 (43)	Prospective-retrospective	Stockholm	588	B	N/A
	Zhang et al. 2013 (44)	Prospective-retrospective	Stockholm + multinstitutional	317 + 358	B	N/A
	Sgroi et al. 2013 (45)†	Prospective	TransATAC	665	B	N/A

*Level of evidence that demonstrates clinical utility is based on Simon-Paik-Hayes criteria, either through prospective (1A) or two prospective-retrospective trials (1B). Refer to Simon RM, Paik S, Hayes DF. Use of archived specimens in evaluation of prognostic and predictive biomarkers. *J Natl Cancer Inst.* 2009;101(21):1446–1452, 2009.
LN = lymph node; LOE = level of evidence.
†A study that established clinical utility for that particular gene assay.

combined cohort of patients from three independent clinical trials, using RNA extracted from formalin-fixed, paraffin-embedded (FFPE) tissue (14). From 250 candidate genes, the authors developed a real-time reverse transcriptase polymerase chain reaction (RT-PCR)–based signature of 16 cancer-related genes (*Ki67*, *STK15*, *Survivin*, *CCNB1*, *MYBL2*, *MMP11*, *CTSL2*, *ER*, *PR*, *BCL2*, *SCUBE2*, *HER2*, *GRB7*, *GSTM1*, *CD68*, *BAG1*) and five reference genes (*GAPDH*, *ACTB*, *RPLO*, *GUS*, and *TFRC*). The Recurrence Score (RS) is a numeric score that represents risk of recurrence for patients who receive endocrine therapy. Patients with an RS of 0 to 18 are predicted to have a low risk of recurrence (4% to 9.6%), an RS of 19 to 30 is labeled as intermediate risk, and an RS of greater than 31 suggests a higher risk (23.6% to 37.4%).

To validate these findings, Paik et al. used tumor tissue from 675 node-negative ER+ patients enrolled in the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14 trial who were randomly assigned to receive tamoxifen or not. They found that the Recurrence Score consistently and independently predicted recurrence-free survival in these patients and could be used as a continuous function to predict outcome in patients treated with tamoxifen (14). These results were

confirmed in the subsequent NSABP-B20 study (15) and the transATAC study (19). Flaws in this study included patients whose tissue samples were used to design the initial 21-gene assay. Additionally, this study did not address treatment for patients in the intermediate-risk group.

For patients who fall in the intermediate-risk (8.3% to 20.3%) cohort, it remains unclear whether or not they derive benefit from chemotherapy. In an effort to resolve this issue, the Trial Assigning Individualized Options for Treatment ($TAILOR_X$) was designed to evaluate whether women with node-negative ER/PR+ breast cancer and an intermediate RS between 11 and 25 benefit from the addition of chemotherapy. The high-risk group (RS $\geq$ 31) was also allocated to receive chemotherapy and endocrine therapy, whereas the low-risk group (RS between 0 and 10) was treated with endocrine therapy alone. The study enrolled 10 253 patients, and in the first report of $TAILOR_X$, Sparano et al. looked at 1626 patients (15.9%) in the low-risk arm (16). The authors found freedom from distant recurrence to be 99.3% at a five-year median follow-up, suggesting that low-risk patients have an excellent prognosis with endocrine therapy alone and that they may be spared chemotherapy. The results of the study in patients in the intermediate-risk group are still pending and eagerly awaited (46,47).

The role of the 21-gene assay for lymph node–positive patients remains controversial. Emerging evidence, however, supports the use of the 21-gene assay in the node-positive population (17,18,48). Petkov et al. used the SEER database to prospectively evaluate data from 38 568 patients with early-stage invasive breast cancers. Of those patients, 4691 had lymph node–positive disease. In the low-risk group, the breast cancer–specific mortality approached that of lymph node–negative patients, suggesting that low-risk node-positive patients might forgo the use of chemotherapy (21). In the prospective Phase III Plan B Trial, Gluz et al. evaluated 3198 patients with node-positive or node-negative disease and found that those with a low recurrence score might be spared chemotherapy (22). We await the results of RxPONDER, a randomized phase III trial for women with ER/PR+, one to three node–positive disease, and RS under 25, to definitively answer this question (49,50).

70-gene assay (Mammaprint)

In 2002, Van't Veer et al. generated oligonucleotide microarray data from 117 patients with lymph node–negative breast cancer in an effort to develop a gene expression profile that could predict recurrence in this group (27). The authors compared patients who remained metastasis free for at least five years with those who developed metastases within a five-year period. Using "leave-one-out" cross-validation, they were able to generate a 70-gene assay with a highly significant odds ratio of 15 for freedom from distant recurrence in the low-risk group. The signature surpassed clinical variables in this analysis and contained genes involved in cell cycle, invasion, metastasis, angiogenesis, and signal transduction, providing a biologic rationale for these findings (26).

In a retrospective study of 295 consecutive patients with early-stage ER/PR+ tumors, the authors compared their signature with the St. Gallen and National Institutes of Health clinical criteria. They found that the 70-gene assay more accurately identified prognosis with a 10-year overall survival (OS) of 54.6% in the poor-risk group vs 94.5% in good-risk patients (27). Several study flaws existed, including 61 of the lymph node–negative patients in this analysis who were from the original cohort, leading to sample contamination. Hence, a prospective trial, listed below, was performed to determine if this signature could perform in an independent data set.

The MINDACT trial was designed to definitively test the 70-gene assay prospectively and to determine its clinical utility (30). This randomized phase III trial included 6693 women with ER/PR+ early breast cancer and used a modified version of Adjuvant! Online (7) to determine low or high clinical risk.

Once clinical risk was established in the MINDACT trial, the authors then used the 70-gene assay to stratify patients into low– and high–genomic risk groups to determine choice of therapy. Those patients who had both high clinical risk and high genomic risk received chemotherapy, whereas those with both low risk categories received no chemotherapy. Patients with discordant results were randomly assigned to chemotherapy or not. Initially, the study allowed only node-negative patients to enroll; however, two years into the trial, the protocol was amended and women with up to three positive axillary lymph nodes were included.

The study met its primary objective of event-free survival at five years in patients with high clinical risk and low genomic risk. These patients who were randomly assigned to no chemotherapy had a 94.4% (95% confidence interval [CI] = 92.3% to 95.9%) median survival from distant metastases, while for those who received chemotherapy, 95.9% (95% CI = 94.0% to 97.2%) survived over five years. Patients with low clinical risk and high genomic risk had no significant difference in outcome, whether or not they received chemotherapy. This suggested that patients with clinically high-risk tumors who scored low on the 70-gene assay can avoid chemotherapy, with these caveats: the 1.5% nonsignificant (but underpowered) difference in metastasis- free survival (51) may matter to some patients, and the trial results may not apply to all patient subgroups. Indeed, the majority of patients had ER/PR+ tumors with underrepresentation of human epidermal growth factor receptor 2–positive (HER2+) or triple-negative subsets, and the overall results may not apply to these small subgroups. Consequently, the 70-gene assay is not recommended in HER2-positive and triple-negative groups.

Based on these results, the ASCO guidelines were recently updated to include the 70-gene assay in the recommendations. This assay may be used to determine the utility of chemotherapy in patients with ER/PR+ node-negative disease and also in patients with one to three positive lymph nodes (52).

Prediction Analysis of Microarray-50 (PAM50, PAM50 ROR Score, or Prosigna)

In 2000, Perou et al. published a seminal paper classifying breast tumors based on gene expression patterns. Using tumor tissue from 42 patients, they analyzed cDNA microarrays and used a hierarchical clustering method to group the samples, based on their similarity in gene expression. They identified tumors in classes termed "intrinsic breast cancer subtypes" that included luminal A, luminal B, HER2-enriched, and basal-like subtypes (53,54). In the ER/PR+ groups, luminal A is associated with a better prognosis because these tumors are generally low grade with a lower proliferative fraction. In contrast, luminal B tumors are associated with less favorable prognosis and are more proliferative, likely due to the activation of alternative signaling pathways such as HER2 (31,32,55,56).

To apply the intrinsic breast tumor subtypes to patient prognosis, Parker et al. developed a gene expression signature termed PAM50. Tumor tissue from 189 patients with both

node-negative and node-positive disease was used to develop the 50-gene signature using both gene expression microarray and quantitative RT-PCR methodologies. The intrinsic groups were then stratified by outcome, generating a risk of recurrence (ROR) score. These findings were then validated in a second cohort of FFPE tissue from 761 patients and stratified based on ER/PR and HER2 status, pathologic stage, and intrinsic subtype (31).

In a follow-up analysis comparing the PAM50 with clinicopathologic features, 786 ER/PR+ invasive node-positive or node-negative breast cancer patients were assigned a PAM50 ROR score and weighted for tumor size and proliferation. In node-negative patients, PAM50 ROR score was found to be more accurate than Adjuvant! Online (32).

Gnant et al. used FFPE tumor tissue from 1478 women with ER/PR+ early-stage node-positive and node-negative breast cancers. In a multivariable analysis, they found that the PAM50 ROR score added additional prognostic information to clinical variables in both the one node (<0.0001)– and two to three node–positive groups ($P = .0002$). The luminal A cohort had a significantly lower ROR score compared with luminal B at 10 years ($P < .0001$) in both nodal groups (33). In a subsequent prospective-retrospective study of the CALGB 9741 study, Liu et al. verified that the intrinsic subtypes were independently prognostic for recurrence-free survival (RFS) and OS ($P < .0001$) (36). This was also true of PAM50 ROR scores, with the greatest prognostic difference seen between the low (five-year RFS 85%) vs intermediate/high-risk groups (five-year RFS = 74% and 70%, respectively).

To further determine if the PAM50 ROR score was prognostic in patients with node-positive disease, Gnant et al. evaluated 543 patients with node-positive disease on the ABCSG-8 and ATAC trials. Using the ROR score, they were able to stratify patients reliably into either high-risk or low-risk groups. The 10-year absolute risk for distant recurrence was 25.5% (95% CI = 17.5% to 36.1%) for patients with one lymph node in the high-risk group compared with 6.6% (95% CI = 3.3% to 12.8%) for patients with one node in the low-risk group (34).

In summary, PAM50 has shown evidence of clinical utility in node-negative patients. However, results are inconsistent in those with node positive, ER/PR-positive, HER2 negative (HER2-) breast cancer. Therefore the PAM50 ROR score is not currently recommended in those patients (11,13).

12-Gene Risk Score (EP, EP Score, EPclin, or Endopredict)

The 12-gene risk score was developed from two Austrian Breast and Colorectal Cancer Study Group trials, ABCSG-6 and ABCSG-8, using the tamoxifen-only arm from each study. The training set used 964 ER/PR+, HER2-negative (HER2-) patients from ABCSG-6 with a prespecified threshold to divide samples into low or high risk of distant recurrence, based on 10-year distant disease-free survival (DFS). This 12-gene risk score includes eight cancer-related genes, *BIRC5, UBE2C, DHCR7, RBBP8, IL6ST, AZGP1, MGP*, and *STC2*, and three reference genes, *CALM2, OAZ1*, and *RPL37A*. Two validation studies were performed using tumor tissue from 378 additional patients on ABCSG-6 and 1324 on ABCSG-8. In multivariable analyses, the 12-gene risk score was an independent predictor of distant recurrence in both ABCSG-6 and ABCSG-8. In subgroup analyses, there was no evidence of heterogeneity by clinical variables or trial cohort (37).

In an effort to harmonize these results with those of clinical guidelines, the group assessed whether there was a benefit to integrate the gene assay with clinical parameters to predict risk of recurrence. They developed an algorithm, termed EPclin, that incorporated nodal status and tumor size with the 12-gene assay and found that 58% to 61% of patients classified as high-/intermediate risk by clinical variables were reclassified as low risk according to EPclin, with a 5% risk of distant metastasis at 10 years (38).

The 12-gene risk score classifies breast tumors into low risk and high risk of distant recurrence. This score has been validated using two prospective-retrospective studies and should be added to the growing list of gene expression assays that show evidence of clinical utility for predicting the risk of recurrence in ER/PR+, node-negative breast cancer (10).

Breast Cancer Index

The Breast Cancer Index includes two independent gene expression markers, the two-gene expression ratio of homeobox gene HOXB13 and interleukin 17B receptor (IL17BR), known as the H/I ratio, and the five-gene tumor grade signature called the molecular grade index (MGI). The BCI plays a role in patients with early-stage breast cancers in predicting likelihood of recurrence and has been assessed for its ability to predict benefit for an additional five years of endocrine therapy. The H/I ratio was initially identified from a 22 000-gene oligonucleotide microarray from tissue samples of 60 patients, with node-negative early-stage ER/PR+ breast cancer. In this cohort, 28 patients developed distant metastases within four years and 32 remained disease free at 10 years. HOXB13 was expressed in the tumors of patients who had recurrent disease, while IL17BR was overexpressed in those without evidence of recurrence (57).

The MGI was developed from 79 tissue samples of patients with recurrent disease and 160 matched controls, all from patients with stage I, II, or III invasive ER/PR+ breast cancer. From the previous cohort that was used to develop the H/I ratio, 39 genes were overexpressed in high-grade tumors. From this group, 5 genes (BUB1B, CENPA, NEK2, RACGAP1, and RRM2) were selected based on their involvement in the cell cycle and proliferation. This was validated with a retrospective study of 239 patients and found that the MGI was prognostic for metastasis-free survival (MFS) (58).

To further validate these findings, investigators measured the H/I ratio in archived samples from 206 women with early-stage breast cancers who received adjuvant tamoxifen on a controlled clinical trial. In 130 patients with node-negative disease, a high H/I ratio was associated with poor prognosis and worse RFS (hazard ratio [HR] = 1.98, $P = .031$) and OS (HR = 2.4, $P = .014$). These results were confirmed in a retrospective study of 1252 breast tumor tissue samples. In this cohort, as previously shown, a high H/I ratio was significantly associated with worse DFS and progression-free survival (45,59). However, this was not observed in patients with node-positive disease (42).

Jerevall et al. conducted a retrospective analysis of BCI from tissue samples from 588 women with ER/PR+ invasive node-negative early-stage breast cancer. The authors found that BCI classified patients as low, intermediate, and high risk, which was independently associated with the rate of distant recurrence at 10 years (43).

In summary, the BCI assay has shown clinical utility to predict recurrence in patients with node-negative, ER-positive patients; however, benefit from extended adjuvant therapy should be validated in a second prospective-retrospective or

prospective trial to meet the criteria of Simon-Paik-Hayes for those end points (13).

Comparison and Contrast of Genomic Signatures for Early Invasive Breast Cancers

In this era of predictive medicine, we have an embarrassment of riches—five commercially available gene expression assays that patients and providers can consider when making treatment decisions. The development of each assay was distinct, and here we compare and contrast where each may be most applicable.

Risk Assessment Comparisons

The following studies compare different gene expression assays to determine which assay would better predict the risk of distant recurrence in women with invasive early-stage breast cancers. In each case, the 21-gene assay was compared with the other tests as it was the first commercially developed biomarker test.

In a prospective-retrospective study using archived tissue from 665 women with node-negative ER/PR+ breast cancer from the TransATAC tissue bank, the 21-gene assay and the BCI were compared with another test called Immunohistochemical 4 (IHC4). The IHC4 assay is an additional assay that uses an algorithm with immunohistochemistry (IHC) staining of ER, PR, HER2, and Ki67 to calculate a risk of recurrence score (23). Of these three assays, the BCI prognostic test was more likely to predict distant recurrence compared with the 21-gene assay (BCI: HR = 2.77, 95% CI = 1.51 to 2.56; 21-gene assay: HR = 1.80, 95% CI = 1.42 to 2.29) or IHC4 (45).

In another comparison, the 12-gene risk score was compared with the 21-gene assay in 928 women with node-positive (n = 248) or node-negative (n = 680) ER/PR+ HER2- breast cancer. Both the 12-gene risk score and EPclin (incorporates clinical risk into the gene assay score) low- and high-risk scores were highly prognostic, and they were similar to the 21-gene assay from zero to five years. In the 10-year follow-up, EPclin was more prognostic than the 21-gene assay, as it calculates in the risk associated with tumor size and nodal status, in addition to the 12-gene assay (likelihood ratio of EP = 49.3, EPclin = 139.3, RS = 29.1) (41).

A number of studies have also compared the assays and showed significant discordance between the risk assignments of the assay (20,24,25,35,41). Denduluri et al. compared the 70-gene assay score with the 21-gene assay in a cohort of 50 patients. They found the concordance to be 0.64 (95% CI = 0.29 to 0.98, P = .0013), with five cases classified as low risk on the 70-gene assay score and intermediate/high on the 21-gene RS (20). In a study of 148 patients with ER/PR+ early-stage breast cancer, comparison of the 70-gene with the 21-gene assays showed discordance in 30% of cases, classifying the same sample as low risk for one assay and high risk for another (24). Clough et al. examined a cohort of 67 patients with low and intermediate risk and found that 45% of the patients with a high risk score using the 70-gene assay had a low recurrence score using the 21-gene assay (25). In another comparison of PAM50 and the 21-gene assay, Dowsett et al. found that in node-negative patients, PAM50 provided more prognostic information than the 21-gene assay and more cases scored as high risk instead of intermediate risk with 21-gene assay (HR = 7.20 vs 6.60) (35). Using the TransATAC cohort, Sestak et al. compared the 21-gene assay, 50-gene assay, EPclin, BCI, IHC4, and another test called the Clinical Treatment Score (CTS) to assess risk of distant recurrence up to 10 years following treatment cessation. They found that assays that included clinical risk such as CTS and EPclin were the most likely to predict recurrence (60).

The Optimal Personalized Treatment of early breast cancer using Multiparameter Analysis (OPTIMA) trial was a randomized, prospective clinical trial that attempted to clarify which biomarker assay best determines risk of recurrence. In this study, 313 women with early-stage breast cancers were randomly assigned to standard treatment with chemotherapy and endocrine therapy vs test-directed therapy. Those in the test-directed arm had 21-gene assay testing performed. If the RS was more than 25, they received chemotherapy and endocrine therapy. If the RS was 25 or less, patients received endocrine therapy. The standard treatment arm used the 21-gene assay, and both arms additionally performed the 70-gene assay and PAM50, IHC4, Automated Quantitative Immunofluorescence (IHC4-AQUA), and MammaTyper Breast assays. There was only 39.4% (n = 119) tissue specimen agreement of either low/intermediate-risk or high-risk categories. Within a test, they also showed a concerning discordance among 183 (60.6%) of the tumor samples (61).

These comparison studies make it clear that there is significant discordance between the tests. This is likely due to differences in molecular features, patient cohorts and calculations of risk used while developing each assay. In addition, some assays include clinical risk, which adds another layer of complexity. It is important, therefore, to consider all features of the patient population in which the assay has shown clinical utility to make the most appropriate choice (Table 1).

Comparison of Molecular Mechanisms

To understand the differences in molecular features between the assays, it is helpful to put them in the context of the Hallmarks of Cancer (62). These include the fundamental processes that are dysregulated in malignant cells such as inhibition of apoptosis, proliferation, replicative immortality, evasion of growth suppression, metastasis, and angiogenesis (Figure 2, Table 2). As each assay was developed from a different set of patients, and often for a different purpose, the specific genes in the signatures do not usually coincide. But it is notable that most of the assays have genes in common pathways, which may not be surprising as these pathways are necessary for cancer survival (56,62,63). The genes listed below provide examples of these common pathways and are referred to in Table 2—the specific assays that use each pathway are also described.

Mechanisms to Inhibit Apoptosis

Apoptosis is the process of naturally occurring programmed cell death. Impaired apoptosis plays a key role in tumorigenesis and is often critical for cancer cell survival, including breast cancer (64,65).

B-cell lymphoma 2 (BCL-2) acts to inhibit cell death and is a significant contributor to tumorigenesis. The 21-gene assay, 70-gene assay, and PAM50 all use BCL2, BCL2-associated athanogene 1 (BAG1), or BCL-2 binding component (BBC3) in the gene expression assays, reflecting the aggressive nature of tumors with BCL-2 overexpression. In healthy cells, BCL-2 is an integral membrane protein and acts to regulate the intrinsic pathway of apoptosis. Through cytotoxic stimuli, BCL-2 family members

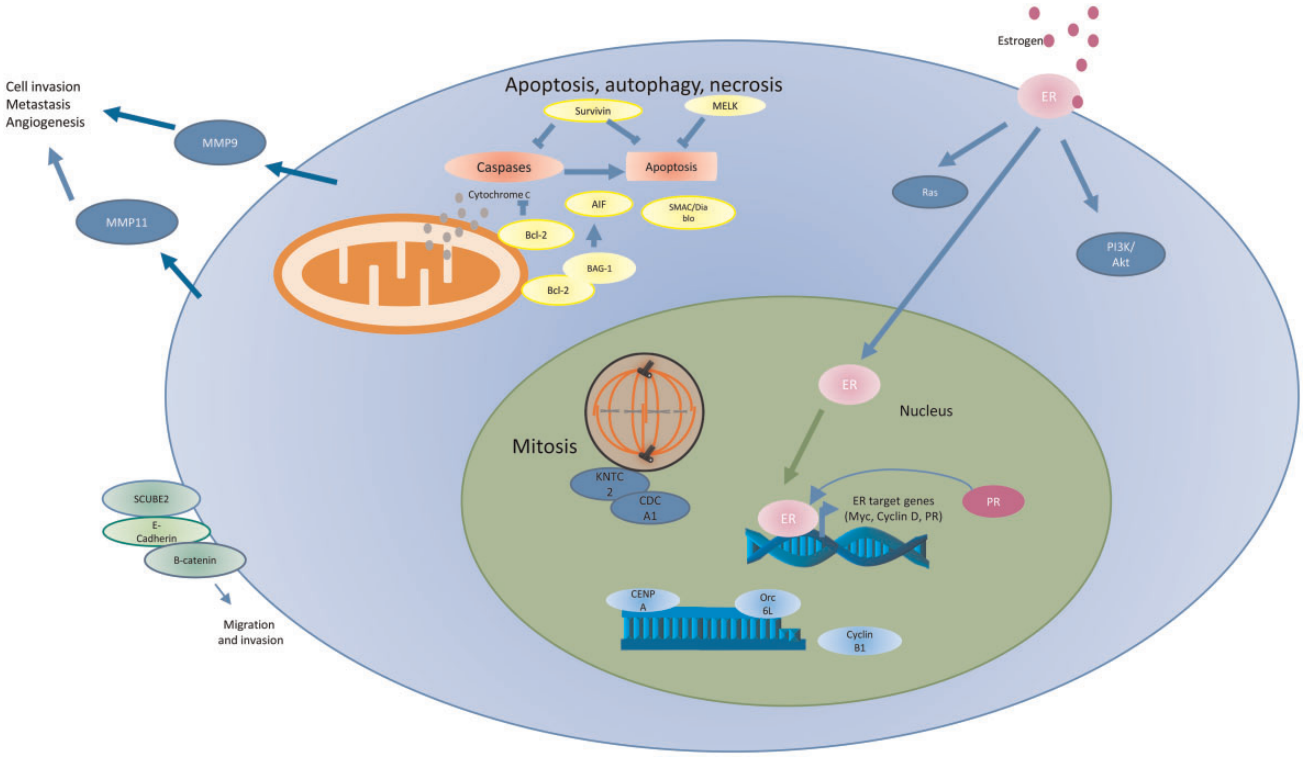

Figure 2. Hallmarks of Cancer pathways aberrantly expressed in gene expression assays used in early-stage breast cancer.

including Bax and Bak become activated, resulting in outer mitochondrial membrane permeabilization. The disruption of this membrane releases apoptogenic proteins such as cytochrome c and Smac/DIABLO. When these proteins are released into the cytoplasm, they promote cell death either through caspase activation or through independent death effectors (66–68). In malignant cells, BCL-2 and its homologs are often constitutively expressed, increasing cell survival and acting with c-myc to promote proliferation (69) and increase chemotherapy resistance (68).

Survivin (BIRC5) is a member of the inhibitor of the apoptosis protein family. In breast cancer, expression of survivin is correlated with BCL-2 expression and associated with a poor prognosis (70). Survivin binds effector cell death proteases caspase-3 and -7, preventing apoptosis and permitting the accumulation of gene mutations (71,72) and chemotherapy resistance (73).

Maternal embryonic leucine zipper kinase (MELK) contributes to many diverse pathways including apoptosis, mitosis, and proliferation and is frequently upregulated in cancer (74–76). This kinase is measured in the 70-gene and PAM50 gene assays. Dysregulation of MELK is associated with clinical progression of breast cancer and more aggressive tumors such as triple-negative breast cancer or basal-like tumors (77,78).

Estrogen Receptor and Progesterone Receptor

Anti-estrogen therapy acts by reducing ER/PR activity and receptor levels. Constitutive activity of ER or PR is associated with endocrine resistance, relapse of disease, and an overall poor prognosis (79–81). PAM50 and the 21-gene assay reflect this by including ESR1 and PGR, the genes that code for these receptors. In these assays, ER overexpression is associated with disease relapse and resistance to endocrine therapy.

In patients with ER+ breast cancer, estrogen and inappropriate activation of ER can promote cell proliferation and inhibit cell death. Estrogen can internalize ER to the nucleus where it binds to co-activators and histone acetyl transferases, facilitating transcription of regulators such as MYC, cyclin D1, cyclin E1, and cyclin E2. This regulation of multiple pathways that upregulate growth, proliferation, and survival is harnessed in breast cancer cells (80–83).

ER directly upregulates expression of PR, causing a feedback loop with PR activation that affects ER transcriptional activity and regulation of proliferation and apoptosis (84). In the presence of ER, progesterone and PR can act to influence ER transcription and activity. Overexpression of PR can result in increased VEGF levels (85), stimulating vascular growth and proliferation (86,87).

Proliferation and Replicative Immortality

Cell growth and proliferation are key regulatory factors that exhibit aberrant behavior in early tumorigenesis. Antineoplastic agents act to prevent cell proliferation; however, resistance and relapse often occur when efforts to halt cell division fail. This hallmark is crucial to determining risk of recurrence in breast cancers; therefore, expression of proliferation genes is measured in the 21-gene, 70-gene, and PAM50 assays. Regulators of cell proliferation seen commonly in these assays include SCUBE2, Cyclin B1, KNTC2, CENPA, and ORC6L.

SCUBE2 is a tumor suppressor gene that complexes with E-cadherin, increasing β-catenin expression and inhibiting transforming growth factor β (TGF β), important for cell migration and invasion (93). SCUBE2 is expressed in breast cancer tissue, increasing proliferation and tumor progression (91,92).

Table 2. Select genes and their functions that are frequently expressed in gene assays used for early-stage, ER-positive breast cancers*

	Gene expression assays	Normal function	Role in breast cancer
Apoptosis			
BCL-2, BCL2- associated athanogene (BAG1), BCL-2 binding component (BBC3)	21-gene assay 70-gene assay PAM50	Membrane protein, regulates intrinsic pathway of apoptosis, caspase activation (66–68)	Act with c-myc to promote proliferation (69) Increase resistance against conventional chemotherapy by preventing mitochondrial membrane permeabilization (68) Helps cell survival during mitotic arrest with taxanes or vinca alkaloids (88)
Survivin (BIRC5)	21-gene assay 12-gene assay PAM50	Member of the inhibitor of apoptosis protein (IAP) family Normally undetectable and only present in fetal tissue	Overexpressed in 70% of breast carcinomas (70) Binds caspase-3 and caspase-7 preventing apoptosis (71) Permits accumulation of gene mutations (72) Correlates with BCL-2 expression, increases chemotherapy resistance (70)
MELK	70-gene assay PAM50	Acts in apoptosis, mitosis and proliferation Regulates proliferation and stem cell self-renewal in neonate (89) In mitosis plays a role in cytokinesis	Associated with more aggressive tumors (78) Overexpressed in cells with nutrient starvation Promotes survival by suppressing and inhibiting pro-apoptotic BCL-G_L (74, 77)
Estrogen and progesterone receptor			
Estrogen receptor	21-gene assay PAM50	Acts in cell growth, proliferation and survival Facilitates transcription of Myc, cyclin D1, cyclin E1, and cyclin E2 (81–83)	Increase endocrine therapy resistance (80,81) Increase proliferation and decrease apoptosis (90)
Progesterone receptor	21-gene assay PAM50	ER acts in a feedback loop with PR, affecting proliferation and apoptosis (84)	Influences ER transcription stimulating growth and proliferation (87,90) Increases VEGF levels (85)
Proliferation and replicative immortality			
Signal peptide complement protein C1r/C1s, Uegf, and Bmp1- epidermal growth factor–like domain-containing protein 2 (SCUBE2)	21-gene assay 70-gene assay	Expressed on ductal epithelial and vascular endothelial breast tissue cells	Expression associated with a favorable prognosis Overexpression suppresses proliferation (91,92) Complexes with E-cadherin suppressing cell migration and invasion (93)
Cyclin B1 (CCNB1)	21-gene assay PAM50	Acts at transition from G2 to M phase p53 inhibits cyclin B1, preventing apoptosis (95)	When p53 inactivated cyclin B1 levels increase (94) Elevated levels seen in aggressive breast cancer
Kinetochore associated 2 (KNTC2)	70-gene assay PAM50	Acts at spindle checkpoint in mitosis to ensure segregation and alignment of chromosomes (96)	Upregulation increases cell proliferation, seen in tumorigenesis (97–99)
Origin replication complex 6L (Orc6L)	70-gene assay PAM50	Initiation of DNA replication Help unwind DNA duplex (100)	DNA replication
Centromere protein A (CENPA)	BCI	Ensures genome stability (101)	Increased risk of metastasis
Metastasis			
Matrix metalloproteinase (MMP), MMP 9	70-gene assay	Gelatinase, degrades extracellular matrix	Promotes angiogenesis through increase in VEGF, tumor cell invasion and metastasis (102,103)
MMP11	21-gene assay PAM50	Stromelysin, found in high levels in surrounding breast tissue	Acts in initial tumor dissemination (104,105)

*ER = estrogen receptor.

Cyclin B1 (CCNB1) acts at the transition from G2 to M phase in the cell cycle and is required to initiate mitosis. The tumor suppressor gene, p53, is able to arrest cell division by inhibiting transcription of cyclin B1, preventing the cell from undergoing mitosis (95). With inactivation of p53, cyclin B expression becomes aberrant (94) and overexpressed in aggressive breast cancers (106).

ORC6L initiates DNA replication by binding directly to the specific nucleic sequencing on the DNA (100). CENPA acts to ensure genome stability (101). At the time of cell division, KNTC2 and CDCA1 form a complex necessary for the spindle checkpoint in mitosis. These pathways are often upregulated in tumorigenesis and metastasis (97–99).

Metastasis

Distant recurrence in breast cancer is a poor prognostic marker and is often associated with treatment resistance and a reduction in OS. The 70-gene assay was developed with the express purpose of determining risk of metastasis at five years (27). This assay includes matrix metalloproteinase (MMP) 9, while both the 21-gene assay and PAM50 include MMP11. These proteinases are secreted by the tumor cells and degrade the extracellular matrix, allowing the cell to disseminate into the circulatory and lymphatic systems. Both MMP9 and MMP11, in addition to other MMPs, have increased expression in breast cancers compared with normal tissue and are associated with a poor prognosis (104,107,108). In 270 tumors from patients with lymph node–negative breast cancer, 59.6% had IHC staining positive for MMP9. In this study, high levels of MMP9 directly correlated to tumor grade and poor relapse-free survival (109). Another study found that increased expression of MMP9 coincided with tumor invasion (102). Activation of MMP9 resulted in upregulation of angiogenesis through VEGF increases in tissue adjacent to tumor cells (103). MMP11 expression is also increased in breast cancer tissue and plays a role in initial tumor invasion and dissemination, acting on the extracellular matrix and associated with a poor prognosis and increased metastasis (105,110,111).

Discussion

The decision to treat an invasive early-stage breast cancer patient with chemotherapy, in addition to endocrine therapy, is often difficult. Clinicians consider clinicopathologic factors, socioeconomic factors, and, more recently, the molecular features of the tumor. A number of gene expression assays are now available for use, and several have been recommended by the ASCO Tumor Marker Guidelines Committee as they have demonstrated clinical utility. However, the choice between assays is not clear, and often discordant results are seen when more than one assay is performed in an individual patient.

To understand why these tests provide differing results, we reviewed the five gene assays that are currently sanctioned by ASCO: the 21-gene assay, PAM50, 12-gene risk score, BCI, and, most recently, the 70-gene assay. Prospective-retrospective studies, using archived tissue from prospective clinical trials, provided a method to evaluate prognostic and predictive value for each assay (13). In all cases, clinical utility was demonstrated for determining prognosis in node-negative ER/PR+ patients treated with adjuvant endocrine therapy. However, when these gene tests are compared in the same patient population, there is often discordance between the level of risk, as best illustrated by the OPTIMA trial, which compared the five different biomarker assays prospectively. They found less than 60% concordance for assignment to low/intermediate and high groups and a similarly concerning discordance between sample studies with a particular test. In addition, none of the assays was found to be superior at predicting risk, and therefore it is difficult to recommend one over the other based on this information (61).

The discordance is due to several different factors. To begin with, the signatures were initially developed to answer different clinical questions, and these questions differed between studies. The 70-gene assay was designed to predict metastasis in node-negative and one to three node–positive patients whereas the 12-gene risk score and 21-gene assay were developed to assess risk of recurrence in ER/PR+ early-stage breast cancer patients with an effort to determine who might need chemotherapy. As would be expected, the patient cohorts were different in these studies, not only by ER/PR status but also by nodal status, and the resulting gene sets reflect hormonal influences and the biology of metastasis (Table 2). Furthermore, the study design and statistical analysis differed between tests, leading to different scoring scales that may not be easily comparable.

By evaluating the molecular features of each test, we begin to understand the differences and similarities between them and why this may lead to discordant results. The heterogeneity of breast cancer, both clinically and at the molecular level, not only influences the results in a specific patient but makes comparison between patients difficult. It may be advisable not to utilize more than one of these assays for a particular patient as this can lead to disparate results and difficulty with decision-making. However, if it is felt by the patient and their provider that having the results of more than one test is useful, that is also a consideration.

In conclusion, there are several gene expression assays that reliably identify a group of ER/PR+ early-stage breast cancer patients who might be spared chemotherapy (Table 1). However, the preferred choice between tests is still unclear in node-negative ER/PR+ breast cancer. Decisions are likely to be made based on cost, availability, and which patient population the assay is approved for until evidence for proof of superiority in outcomes of one test over another can be established by ongoing prospective trials. In the meantime, the physician should carefully evaluate the evidence and choose the test that provides the highest level of evidence of clinical utility in that particular patient, and proceed with caution at ordering more than one test. Of note, the assays listed in this review have all been shown to have clinical utility for node-negative ER/PR+ breast cancer; however, the 70-gene assay shows the highest level of evidence (level 1A) for patients with one to three positive nodes (52). This underscores the need for physicians to be as informed as possible about the pros and cons of each gene expression assay and ultimately to use clinical judgment to choose the test that provides optimal information for patient decision-making.

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