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Biomarkers in Cancer

Molecular Profiling for Breast Cancer: A Comprehensive Review

Muaiad Kittaneh[1], Alberto J. Montero[2] and Stefan Glück[3]

[1]Center for Translational Therapeutics, Karmanos Cancer Institute, Detroit, MI, USA. [2]Cleveland Clinic, Cleveland Ohio. [3]Division of Hematology/Oncology, Sylvester Comprehensive Cancer Center, University of Miami, Leonard M. Miller School of Medicine, Miami, FL, USA.

ABSTRACT: In recent years advances in molecular biology have launched disruptive innovations in breast cancer diagnostics and therapeutics. The advent of genomics has revolutionized our understanding of breast cancer as several different biologically and molecularly distinct diseases. This research has led to commercially available polymerase chain reaction (PCR) and microarray tests that have begun to fundamentally change the way medical oncologists quantify recurrence risk in early stage breast cancer patients. The Genomics era has altered the clinicopathologic paradigm of selecting patients for adjuvant cytotoxic chemotherapy. Sufficiently powered prospective studies are underway that may establish these molecular assays as elements of standard clinical practice in breast cancer treatment. In this article, we review the strengths and limitations of currently available breast cancer-specific molecular tests.

KEYWORDS: early breast cancer, molecular profiling, prognostic and predictive tests, Oncotype DX, MammaPrint, PAM50

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CORRESPONDENCE: s.gluck@med.miami.edu

Introduction

We have arrived at an important juncture in the treatment of breast cancer. We stand between the clinical-pathological paradigm, which has been dominant for several decades, and the emerging genomic paradigm.

The clinical-pathological paradigm estimates the probability of breast cancer recurrence using physical characteristics such as tumor size, histological grade, and number of metastatic axillary lymph nodes. Under the clinical-pathological paradigm, estrogen and progesterone receptor (ER/PR) expression levels are determined by immunohistochemistry (IHC). Human epidermal growth factor receptor 2 (HER2) is determined by IHC or in situ hybridization (ISH). The levels of each are used as predictive markers to identify subgroups of patients who are likely to benefit from anti–estrogen- or anti–HER2-directed therapies. They are also used to more precisely quantify risk of recurrence.

By contrast, the genomic paradigm uses only an array of biomarkers. These biomarkers may be identified by scientists and clinicians using, for example, the Oncotype DX (Genomic Health Inc., San Francisco) 21-gene set, by unsupervised analysis of gene clusters via PAM50 (Nanostring Technologies Inc., Seattle, Washington) intrinsic subtyping, or by defining favorable versus unfavorable outcome using cDNA microarrays to identify genes (ie, MammaPrint70-gene analysis Agendica, Inc., Irvine, CA).

One of the most important uses of these data is prognosis, that is, to more accurately estimate the risk of breast cancer recurrence in women with early stage breast cancer and to select patients who would benefit most from cytotoxic chemotherapy, at the same time sparing those who would derive little or no benefit from treatment. Under the current clinical-pathologic paradigm, the typical approach is to use clinical features that are surrogates for metastatic potential such as

tumor size, tumor grade, lymph node involvement, and hormone receptor status to determine the average 10-year risk of recurrence.

The evidence-based software Adjuvant! Online quantifies recurrence risks for patients using standard clinical-pathologic data from large national databases such as the Surveillance, Epidemiology and End Results (SEER) program, from large scale studies, and from the published literature.[1] This prognostic information, for example, a particular patient's 10-year recurrence risk, is then used to estimate the overall magnitude of risk reduction provided by adjuvant cytotoxic chemotherapy and/or endocrine therapy. Because of the potential for severe adverse toxicities with chemotherapy, in particular anthracycline-based regimens, this information is important for both physicians and patients to make informed decisions as to whether chemotherapy should be prescribed.

The limitations of the current clinical-pathologic paradigm cause many women with breast cancer, particularly women with hormone-receptor-positive, HER2-negative tumors, to be overtreated with chemotherapy, a point that was well illustrated by the Austrian Breast and Colorectal Cancer Study Group trial, ABCSG-12. In a cohort of approximately 1,800 premenopausal women with hormone-receptor-positive breast cancer (30% with node-positive disease) who had adjuvant endocrine therapy alone, the reported 7-year overall survival (OS) rate was 95%. Many of these women, particularly in the United States, would have been treated with chemotherapy based on standard clinico-pathologic features because nodal status is a main deciding factor for using cytotoxic chemotherapy—a criterion that leads to overtreatment of many breast cancer patients.[2,3]

Genomic Paradigm—A New Approach to Prognosis

The rapidly evolving genomic paradigm offers a new approach for predicting an individual patient's prognosis by interpreting the expression pattern of a panel of specific tumor-related genes. Transcription of a specific set of genes is used as a surrogate marker for metastatic potential. The gene expression pattern and specific gene expression threshold levels can identify the tumors with more aggressive biology, thereby quantifying risk of recurrence more accurately than the traditional method.

Avoiding unnecessary or ineffective treatments, including cytotoxic chemotherapy, should be a primary goal of modern adjuvant therapy for early stage breast cancer. In this article, we review the published literature documenting the use of genomic assays in the clinical management of patients with early stage breast cancer.

Intrinsic Breast Cancer Subtypes: Advent of the Genomic Paradigm in Breast Cancer

Breast cancer is a heterogeneous group of pathologic entities. Three subtypes of breast tumors with different biologic behaviors were discovered using the traditional IHC techniques: hormone-receptor-positive, triple negative, and Human Epidermal Receptor (HER) 2/*neu*-positive breast cancers. All of these subtypes have distinct natural histories, which require different management approaches.[4–7]

Genome-wide expression profiling and hierarchical clustering have now enabled us to identify additional subtypes. We now know that breast cancer comprises at least 7 different biologic subtypes.[4] They include luminal A, luminal B, luminal C, HER2-enriched, basal-like, claudin-low, and normal breast-like.[8] The distinct features and natural histories of these breast cancer entities have been described in the literature.[8]

Luminal-like Breast Cancer Types

Luminal-like breast cancer derives its name from its similarity to the expression profile of normal luminal breast epithelium. Breast tumors classified as luminal A are known to have overexpression of ER-regulated genes, underexpression of an HER2 gene cluster, and underexpression of proliferation-related genes. These tumors are sensitive to endocrine manipulation. They are less sensitive to cytotoxic agents in both the neoadjuvant and metastatic settings. Approximately 40% of all breast cancers are classified as luminal A. They are associated with a rather favorable prognosis.[9–11]

Luminal B breast tumors have much lower expression of ER-related genes, a variable expression of an HER2 cluster of genes, and a relatively higher expression of proliferation-related genes. They represent about 20% of breast cancers. Luminal B tumors have also been shown to have genomic instability, and to harbor mutations in *TP53*. Luminal B tumors are associated with a relatively higher risk of relapse. Luminal A and B tumors are both known to be much less sensitive to cytotoxic chemotherapy, as evidenced by low pathological complete response rates after neoadjuvant chemotherapy.[12–14] The luminal B subtype is less common than the luminal A subtype, and it carries a poorer prognosis.[4]

The luminal C intrinsic subtype is distinguished from luminal A and B subtypes by its high expression of a different set of genes of presently unknown function. This cluster of genes is also found to be overexpressed in basal-like and HER2-enriched subtypes. Some of the genes that were identified in luminal-C include transferrin receptor (CD71), MYB, *nuclear protein* p40, SQLE, and GGH.[4]

HER2 enriched breast cancer subtype. HER2 enriched breast cancer represents 20% to 30% of all breast tumors. It is characterized by high expression of HER2*heu* proliferation genes and low expression of luminal clusters.[15] Luminal clusters include luminal cytokeratins (CKs) CK7, CK8, CK18, and CK19, and other luminal-associated markers such as human endogenous retrovirus envelope PL1, X-box-binding protein 1, hepatocyte nuclear factor 3, GATA-binding protein 3, Annexin XXXI, and estrogen receptor 1, among others.[15–17] HER2 enriched tumors are usually, but not always, HER2-positive and ER/PR-negative. Clinically, they are associated with a poorer prognosis compared with luminal A tumors.[5]

Basal-like breast cancer subtype. The basal-like intrinsic breast cancer subtype represents about 15% of invasive ductal breast cancers. Its name is derived from shared gene expression patterns with normal basal epithelial cells. The gene expression cluster characteristic of basal epithelial cells includes: keratin 5,6, and 17, integrin-β4, laminin, and fatty-acid binding protein 7.[4,15] These tumors are frequently ER-negative, PR-negative, HER2-negative, CK5/6-positive, and/or EGFR (HER1)-positive by IHC.[18] They are considered ER/PR and HER2/*neu* negative ("triple negative") due to low expression of the luminal and HER2 gene clusters. However, triple negative (TN) and basal breast cancer are not synonymous. TN breast cancers represent a more heterogeneous group of diseases than do basal-like breast cancers. Approximately as many as 30% of TN tumors are not basal-like.[19] This subtype is also characterized by relatively high frequency of *BRCA1* (breast cancer type 1 susceptibility gene) mutations, increased genomic instability, high expression of the proliferation cluster of genes, and a high histologic grade.[20]

Claudin-low breast cancer subtype. The recently recognized claudin-low breast cancer subtype is characterized by overexpression of genes associated with epithelial-to-mesenchymal (EMT) transition. These genes include: (1) cell communication genes, eg, chemokine (C-X-C motif) ligand 12; (2) extracellular matrix formation genes, eg vimentin and fibroblast growth factor 7 genes, which are involved in extracellular matrix formation; (3) cell differentiation genes, eg Krüppel-like factor 2, (4) cell migration genes, eg integrin a5 and moesin; (5) angiogenesis genes, eg vascular endothelial growth factor C, matrix metallopeptidase 9 (MMP-9); (6) immunerelated genes, eg CD79b, CD14, and vav1; and (7) stem-cell like genes, eg CD44+/CD24- and high ALDH1A1.[21]

The majority of claudin-low breast cancers have no expression of luminal differentiation markers, are HER2 and hormone-receptor-negative by IHC, frequently exhibit metaplastic and medullary differentiation, and are often part of the basal intrinsic subgroup.[21]

Gene Expression Profiling in Breast Cancer

Gene expression profiling is a relatively new technology that identifies genes whose activity can be used as a molecular signature in predicting prognosis and guiding therapy. DNA represents the genetic material that gets transcribed into mRNA molecules, which in turn are translated into proteins that define unique cellular functions and properties.[4,22] Oligonucleotide arrays, cDNA, and multiplex polymerase chain reaction (PCR) as well as mRNA level technologies have been used to generate molecular signatures.

Background: Predictive Versus Prognostic

At this writing, 3-genomic assays are commercially available for use in early stage breast cancer: Oncotype DX, (Genomic Health Inc., San Francisco) MammaPrint, and PAM50 (PAM50 is not yet commercially available in the USA). All 3 tests can provide an overall risk assessment of breast cancer recurrence; however, there are important differences among them. In one sense, all 3 of these genomic assays are prognostic biomarkers as they provide an estimated recurrence risk and appear to provide prognostic information independent of that provided by standard clinical and pathologic factors.

The terms prognostic and predictive are frequently used interchangeably; however, there are some important distinctions. Generally speaking, a predictive biomarker identifies patients who would benefit from a specific intervention. The BRAF V600E mutation, which predicts benefit from tyrosine kinase inhibitor therapy with vemurafinib in metastatic melanoma, is an example of a predictive biomarker.[23] A prognostic biomarker provides information on the likely outcome of the disease irrespective of treatment. An example of a prognostic biomarker is the KRAS mutation, which is associated with poor survival in non–small-cell lung cancer.[24]

Some biomarkers are both predictive and prognostic, such as protein overexpression or gene amplification of HER2, or KRAS mutations in colorectal cancer.

The 3 currently available genomic biomarker assays for breast cancer are not "predictive" of chemotherapy benefit in the same sense as BRAF for melanoma because none of these assays were specifically designed to predict which subset of patients would benefit from chemotherapy.

The first genomic biomarker assay that became available for breast cancer treatment decisions was the Oncotype DX. This assay was initially tested and validated in women with hormone-receptor-positive early breast cancer who were receiving endocrine therapy. This assay, therefore, gives a recurrence score for patients on endocrine therapy. The gene signatures comprising both PAM50 and MammaPrint, by contrast, were derived from patients with all subtypes of breast cancer. Moreover, patients in the initial and validation data sets underwent surgery only and did not receive systemic adjuvant therapy.[41,28,53]

A truly predictive chemotherapy genomic signature for breast cancer would likely be best developed in the neoadjuvant setting correlating signature with pathologic complete response, which is a validated surrogate marker for overall survival. Such predictive biomarkers have been evaluated; however, further elaboration is beyond the scope of this review.[25,26] Datasets showing a significant benefit of adjuvant chemotherapy in breast cancer patients with a high recurrence risk score by both Oncotype DX and MammaPrint are predictive in the sense that they quantify the recurrence risk.[27,32,48] The relative benefit of chemotherapy can then be extrapolated for each risk group.[26]

Oncotype DX

Oncotype DX is a multiplex, 21-gene, real time, PCR-based assay that was developed to quantify the likelihood of disease recurrence in women with stages I and II hormone-receptor-positive, lymph-node-negative, invasive breast cancer, and who had received tamoxifen for 5 years.[28] This genomic assay was developed through selection of a panel of genes with

known function that were thought to be the most relevant to the biology of hormone-receptor breast cancer. This assay was also optimized for quantification of RNA extracted from fixed paraffin-embedded tumor tissue.[28]

Oncotype DX was developed after identifying 250 candidate genes that were analyzed in a total of 447 patients from 3 separate studies, which eventually led to the 21-gene profile and an algorithm for calculating a recurrence score (RS). The 21 genes are divided into 2 groups: 16 are cancer related, and 5 are reference genes that serve as internal controls (Table 1).[28]

A mathematical algorithm was used to generate a RS, which classifies patients as low-, intermediate-, or high-risk. The algorithm calculates the expression for each gene by normalizing the expression of the 16 cancer-related genes to the expression of the 5 reference genes. Genes are grouped on the basis of function, correlated expression, or both. The scores of cancer-related genes including *GRB7*, *ER*, proliferation, and invasion groups are then calculated from individual gene-expression measurements. An increased expression of a certain cancer-related gene is associated with an increased risk of recurrence. A RS of <18 is defined as low risk, while a score of ≥ 31 is defined as high risk, with 18 to 30 being intermediate risk.

In summary, a low level of ER expression and a high level of proliferation/invasion gene expression and/or HER2 expression predict a higher risk of recurrence. Higher expression of estrogen-associated genes and GSTM1 and BAG1 genes were associated with longer, relapse-free survival. A score of <18 was considered low risk on the bases of National Surgical Adjuvant Breast and Bowel Project clinical trial B-20 (NSABP-B20) results, where patients with this score had estimated rates of distant recurrence of $<10\%$ (6.8%) at 10 years. These patients were found to have derived minimal benefit from the addition of chemotherapy.[27]

Validation of Oncotype DX in clinical studies. The Providence St. Joseph's Hospital study was a single institution study that analyzed tissue from 136-breast cancer patients, irrespective of nodal status, whether they received chemotherapy or not, and who had ER positive or negative tumors.[29] Using the expression pattern of 250 identified candidate genes and linking them to outcome data led to the identification of 16 additional specific cancer-related genes and 5 reference genes constituting the 21 genes used to establish the Oncotype DX Recurrence Score (ODRS) algorithm.

In a large retrospective validation set, the ODRS was obtained on tumor blocks from patients enrolled in the NSABP-14 (a clinical trial to assess tamoxifen in patients with primary breast cancer and negative axillary nodes, whose tumors were positive for estrogen receptors). Women with early stage, lymph-node-negative, and hormone-receptor-positive breast cancers were randomized to receive either tamoxifen or placebo. Clinically, the ODRS were translated into a risk percentage for the development of distant metastatic disease at 10 years, with a score of 18 representing a 10% risk, and a score of 31 representing a 20% risk. Fifty-one percent of the patients on the NSABP-14 tamoxifen-treated trial arm (postmenopausal, ER-positive, node negative breast cancer) were categorized low-risk, 22% intermediate-risk, and 27% high-risk. Women with low ODRS tumors were found to have a 10-year distant relapse-free survival (DRFS) rate of 93.2% compared with women with high-scoring tumors, whose DRFS rate was 69.5%.[28] In a subgroup analysis of patients classified as low-risk by National Comprehensive Cancer Network (NCCN) guidelines, the ODRS reclassified 28% of these patients as having a higher recurrence risk (intermediate/high-risk). Likewise, analysis of the NCCN high-risk subgroup using ODRS reclassified 49% of these patients as low risk.[39]

Oncotype DX was subsequently retrospectively evaluated in another randomized control trial, the NSABP-B20, a study that explored the benefit of adding adjuvant chemotherapy to tamoxifen over tamoxifen alone in managing patients with primary invasive breast cancer, negative axillary nodes, and estrogen-receptor-positive tumors. One arm of this 2-arm study was already used in the training set of the profile. In this trial, patients with nonmetastatic, hormone-receptor-positive breast cancer were randomized to receive either nonanthracycline-based chemotherapy (CMF or MF) plus concurrent tamoxifen or tamoxifen alone. Tamoxifen was administered daily, 20 mg orally, for 5 years. Fifty-four percent of these patients had an ODRS putting them in the low risk group ($RS < 18$); 21% in the intermediate-risk group ($18 \leq RS \leq 30$); and 25% in the high-risk group ($RS \geq 31$). High risk patients received the maximum benefit from adjuvant chemotherapy with a 27.6% risk reduction of distant metastasis at 10 years, whereas low risk patients received minimal benefit (3.78% risk reduction of distant recurrence at 10 years) from chemotherapy. Patients in the intermediate-risk group who received chemotherapy did not appear to have significantly different distance recurrence rates over patients receiving tamoxifen alone, but the possibility of clinical benefit could not be eliminated due to uncertainty in the estimate.[27]

The purpose of the TAILORx (Trial Assigning IndividuaLized Options for Treatment) prospective study[32] was

Table 1. Oncotype DX 21-gene profile.

CANCER RELATED GENES (16)	REFERENCE GENES (5)
Proliferation genes: *Ki67; STK15; Survivin; CCNB1 (Cyclin B1); MYBL2*	*ACTB (b-actin)*
Invasion genes: *MMP11 (Stromolysin 3); CTSL2 (Cathepsin L2)*	*GAPDH*
HER2 genes: *GRB2; HER2*	*RPLPO*
Estrogen genes: *ER; PGR; BCL2; SCUBE2*	*GUS*
Other cancer related genes: *GSTM1; CD68; BAG1*	*TFRC*

to determine whether adjuvant cytotoxic chemotherapy is significantly beneficial in improving clinical outcomes in the intermediate-risk group. Patients with a score of 11 to 25 formed the primary study group. These patients were randomly assigned to 1 of 2 groups, 1 receiving adjuvant hormonal therapy with chemotherapy and 1 receiving no chemotherapy. Patients with an RS > 25 were assigned to chemotherapy plus hormonal therapy. Patients with an RS < 11 were assigned to hormonal therapy alone. The basis for this trial was to investigate the inconsistent benefit of chemotherapy in hormone-receptor-positive breast cancer patients with intermediate RS and to answer specifically whether these patients would benefit from chemotherapy in addition to adjuvant endocrine therapy.

TAILORx redefined the intermediate-risk group as having an ODRS of 11 to 25 (~45% of all trial subjects) rather than the 18 to 31 parameter of the initial validation. The upper limit of the low-risk score was reduced from 18 to <11 in this trial because a RS of <11 is correlated with a recurrence risk of 5% to 10% on endocrine therapy alone. A recurrence risk of 5% to 10% is considered the minimum threshold at which cytotoxic chemotherapy would be considered clinically justified. Conversely, the lower end of the high-risk ODRS was reduced from 31 to >25 because an ODRS of 30 is correlated with a 10-year recurrence risk on endocrine therapy of approximately 20%.

Oncotype DX in conjunction with aromatase inhibitor adjuvant therapy. Oncotype DX has also been evaluated in postmenopausal breast cancer patients treated adjuvantly with aromatase inhibitors. Analysis of the ODRS of 1231 ER-positive and/or PR-positive patients from the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after 9 years of follow-up disclosed DR rates of 4% in patients with low ODRS (<18), 12% for intermediate ODRS (18–30), and 25% for high ODRS (≥31) in node-negative patients. In women with lymph-node-positive breast cancer, the distributions of ODR scores were higher at 17%, 28%, and 49% for low, intermediate, and high ODRS, respectively.[33] The prognostic value of ODRS appeared to be similar for women treated with either anastrozole or tamoxifen. ODRS was also found to be an independent predictor of recurrent disease in patients with hormone-receptor-positive disease irrespective of their nodal status.[33]

Oncotype DX limitations. Like any biomarker, Oncotype DX has limitations. It has only been validated in hormone-receptor-positive breast cancer. There are no data on the utility of Oncotype DX for other breast cancer subtypes. There is a relatively high false negative rate for HER2, which could lead to underestimation of risk since HER2 is heavily weighted in the RS.[34] Emerging data suggest that Oncotype DX does not provide independent prognostic information over that provided by IHC for ki-67, ER, PR, and HER2.[35] In the NSABP B-20 study, tamoxifen was concomitantly given with adjuvant chemotherapy, which in subsequent trials was found to be associated with decreased efficacy of adjuvant cytotoxic chemotherapy. In the current definition of intermediate-risk score, ODRS is uninformative in about a third of patients. This currently being investigated to further classify these patients.

A recent study evaluated the discordance rate between IHC/FISH and Oncotype DX RT-PCR HER2 assays. The Oncotype DX RT-PCR HER2 assay is usually reported separately from ODRS. In this retrospective study, 4% of women tested positive for HER2 by IHC/FISH, of which 39% were falsely negative by RT-PCR. Additionally, 3% of women who were HER2 equivocal by IHC/FISH were all reported as HER2 negative by Oncotype DX RT-PCR. These results corresponded with a >50% HER2 false-negative rate for Oncotype DX.[34] These data contradict a previously reported concordance rate of 97% between HER2 expression by FISH and HER2 expression by RT-PCR using Oncotype DX.

HER2 testing by IHC and FISH, the current standard method for determining outcomes and response to trastuzumab has been validated in multiple clinical trials. However, Paik et al reported that among 104 patients who were entered in NSABP Protocol B-31, up to 18% of IHC test results may be inaccurate as a central testing facility was unable to confirm these community-based assays by HercepTest (Dako North America, Inc. 6392 Via Real Carpinteria, CA) IHC or fluorescence in situ hybridization (FISH).[37]

Conflicting data make the cause of this discrepancy between HER2 testing results by RT-PCR and IHC/FISH difficult to determine. One unanswered question remains: since HER2 is an important and heavily weighted component of the 21-gene score, does underestimation of HER2 transcription levels by RT-PCR lead to underestimation of breast cancer recurrence through the assignment of lower ODRS? It is important to note that at this time, HER2 testing by IHC- and FISH-validated assays remains the standard practice for making decisions about anti-HER2 therapy. The use of genomic assays for determination of HER2 expression and potential use of adjuvant trastuzumab is not currently recommended or suggested by any consensus guidelines.[66]

The robustness of ODRS as an independent prognostic test in early breast cancer was further challenged by a recent study that compared ODRS results to the prognostic value of 4 widely measured IHC markers (IHC4).[35] Cuzick et al created a prognostic score based on 4 widely measured IHC markers (IHC4): ER, PR, HER2 (including fluorescent in situ hybridization in the 2+ group), and Ki-67. Those IHC markers were evaluated by using tumor blocks collected from patients enrolled in the ATAC trial, and the score was used to determine the extent to which the 4 markers provide additional prognostic information not captured by the classical clinical and pathologic variables like patient's age, nodal status, tumor grade, size, and hormonal treatment. The added information in this score was compared with that added by the predefined RS in predicting the 10-year risk of distant recurrence. Prognostic information provided by the IHC4 score was similar to that provided by ODRS, and little if

any additional independent prognostic value was seen in the combined use of scores. Thus, it was concluded that the IHC4 score may constitute a simpler and less expensive alternative prognostic biomarker that provides similar prognostic data to the recurrence score = RS (Oncotype).[35]

MammaPrint

MammaPrint is a 70-gene expression profile that was initially developed from whole-genome-expression (25,000 genes) arrays of consecutively collected breast cancer specimens from a cohort of women who had undergone definitive surgery only, with no systemic therapy and with known long term clinical outcomes.[38] The overall approach for developing this prognostic profile was distinct from that used in the development of the previously discussed Oncotype DX assay, which was derived from a set of 250 preselected candidate genes believed to have prognostic importance in hormone-receptor-positive breast cancer.

MammaPrint has been shown to be a prognostic marker, independent of conventional clinical and pathologic factors such as tumor size, hormone receptor status, and HER2 status. MammaPrint was cleared for use by the FDA in 2007, and at publication, was the only FDA-approved breast cancer genomic assay.

The biological functions of the 70 genes in the MammaPrint signature are associated with the essential steps necessary for tumor progression and metastasis. These genes are the hallmarks of cancer-related biology, regulating cell cycle, invasion, metastasis, proliferation, local invasion, survival in circulation, extravasation, and adaptation to the microenvironment as well as angiogenesis. They reflect the acquired malignant characteristics of a cancer cell along with tumor progression and metastasis-related biological activities.[39]

The MammaPrint signature was designed based on overall expression levels to divide patients into low and high risk groups that correspond with 10-year distant metastasis-free survival rates of >90% or <90%, respectively, in the original datasets involving breast cancer patients who underwent surgery alone without any systemic therapy.

MammaPrint was first validated in a series of 295 consecutive invasive breast tumors from patients with early stage breast cancer who were all part of the tumor bank at the Netherlands Cancer Institute (NKI). The 70-gene profile was found to be a strong independent predictor of clinical outcome, and added to the predictive power of standard clinical-pathologic parameters.[40] In a multivariate analysis, MammaPrint was the strongest predictor of 10-year distant metastasis-free survival with a hazard ratio of 4.6 (95% CI, 2.3–9.2).[40]

The second independent validation study for MammaPrint was performed by the TRANSBIG Consortium.[41] The 5 participating European hospitals evaluated 302 patients who had received loco-regional therapy but no systemic adjuvant therapy. The median follow-up in this dataset was 13.6 years. The median distant metastasis-free survival at 10 years was 90% and 69% for low- and high-risk groups, respectively.[42] On multivariate analysis, MammaPrint was found to provide independent prognostic information beyond what could be determined from patient age, tumor grade, size, or hormone receptor status in a population of node-negative breast cancer patients, none of whom had received any adjuvant endocrine or chemotherapy. The MammaPrint profile was found to be a better prognostic biomarker than Adjuvant! Online and provided an independent risk assessment with a 28% to 35% discordance between MammaPrint and Adjuvant! Online in low- and high-risk groups. These results suggest that the discordant patients had clinical outcomes that were more accurately predicted by MammaPrint; therefore, 34% of Adjuvant! Online high-risk patients could have avoided chemotherapy because they had low-risk MammaPrint results. Similarly, 14% of patients who were categorized as low risk by Adjuvant! Online had high-risk profiles as determined by MammaPrint and might have benefited from additional treatment.[41]

Bueno-de-Masquita et al[43] reported the results of 123 breast cancer patients with pT1-T2N0 disease, <55 years of age, who were followed up for a median of 5.8 years. In this dataset, 48% of patients had high-risk MammaPrint scores, which corresponded to a median 5-year OS of 82%, (95% CI ± 5%), with low-risk score patients having a corresponding median OS of 97% (±2%).[43]

MammaPrint was shown to have a high negative predictive value for distant recurrence after adjuvant treatment (both endocrine and chemotherapy) in 100 postmenopausal breast cancer patients treated at the Massachusetts General Hospital.[44] In this patient population, the 70-gene signature correctly identified 100% of women at low-risk for distant metastases at 5 years.[44] Additional work revealed that MammaPrint has a strong prognostic value in patients with up to 3 positive lymph nodes.[45] The 10-year distant metastasis-free survival was 91%, for the good prognosis-signature group (99 patients), and 76% for the poor prognosis-signature group (142 patients). Further work by Mook et al[45] demonstrated that MammaPrint can accurately select postmenopausal women at low-risk of breast cancer-related death within 5 years of diagnosis and can be used clinically to identify postmenopausal women who would benefit most from adjuvant chemotherapy.[46]

MammaPrint appears to be effective in classifying patients into either a low-risk (<10%) or high-risk of developing distant metastases. Corresponding hazard ratios for time to distant metastasis adjusted for clinical risk in patients with high-risk MammaPrint tumors in the first 5 years following curative treatment vary from 4.5 to 4.7.[41] It is important to note that it is in these same years that chemotherapy exerts its maximal beneficial effect.[47] Patients who received adjuvant treatment clearly show a lower risk of recurrence compared to untreated patients in this same 5-year period, whereas beyond this interval the difference in risk of recurrence stabilizes.

The predictive value of MammaPrint for chemotherapy benefit in addition to endocrine therapy has been analyzed from pooled study series. In a study involving 541 patients who received either endocrine treatment (n = 315) or chemotherapy followed by endocrine treatment (n = 226), distant disease-free survival at 5 years was determined for MammaPrint high- and low-risk groups. In MammaPrint low-risk patients, distant disease-free survival (DDFS) was 93% for patients who received endocrine therapy alone compared with 99% for those patients who received chemotherapy plus endocrine therapy, with a hazard ratio of 0.26 (95% CI, 0.03–2.02; $P = 0.20$). In MammaPrint high-risk patients, DDFS for patients who received endocrine therapy versus endocrine therapy plus chemotherapy was 76% versus 88% with a hazard ratio of 0.35 (95% CI, 0.17–0.71; $P < 0.01$). Results were similar in multivariate analysis.[48]

The predictive value of MammaPrint in the neoadjuvant setting has also been explored. In one study involving 167 breast cancer patients who received neoadjuvant chemotherapy, 144 (86%) had tumors characterized as high-risk and 23 (14%) as low-risk. No patients with low-risk tumors achieved a pathological complete response (pCR) (0/23) versus 29/144 patients (20%) with high-risk tumors who did achieve pCR ($P = 0.015$).[49] These results suggest that tumors with good prognostic signatures (low-risk group) are unlikely to respond to chemotherapy, while tumors with poor prognostic signature (high-risk group) are more sensitive to chemotherapy.[49]

The MicroarRAy PrognoSTics in Breast CancER (RASTER) trial evaluated the impact of MammaPrint in assisting with adjuvant treatment decisions. In this prospective study, 427 women with primary breast carcinoma were enrolled between 2004–2006. In this study, physicians were encouraged to use chemotherapy based on MammaPrint scores. Patients were not randomized. The prognostic information provided by MammaPrint was found to have had a meaningful impact, leading to a change in adjuvant treatment decisions for 20% of patients.[50] Overall, 51% of patients had low-risk tumors. The 5-year DMFS rate was 96.1% for low-risk patients and 89.8% for high-risk patients. The vast majority of the high-risk patients (85%) received chemotherapy.[51] The RASTER study was the first to present prospective data in early stage breast cancer patients whose treatment decision was made in the context of available MammaPrint scores.

MINDACT MammaPrint trial with Adjuvant! Online. MINDACT (Microarray In Node-negative and 1–3 node-positive Disease may Avoid Chemo Therapy) is an international prospective, randomized, phase III trial comparing MammaPrint with a common clinical-pathological prognostic tool (Adjuvant! Online) in selecting patients with negative or 1 to 3 positive nodes for adjuvant chemotherapy in breast cancer. This trial has enrolled 6600 patients and completed recruitment in July 2011.[52] Women with breast cancer categorized as high risk by both MammaPrint and clinical-pathologic guidelines are advised to receive adjuvant chemotherapy. Women categorized as low-risk are recommended to undergo endocrine therapy alone. However, patients with discordant MammaPrint and clinical-pathologic assessments (high-risk by MammaPrint and low-risk by Adjuvant! Online or vice versa) are randomized to receive either chemotherapy plus endocrine therapy or endocrine therapy alone. The primary objective of this trial is to confirm that breast cancer patients with a low-risk molecular prognosis by MammaPrint and high-risk clinical prognosis can be safely spared chemotherapy without affecting DMFS.

MammaPrint and Oncotype DX: dichotomous, trichotomous or continuous scales. MammaPrint provides a dichotomous (binary) test result, which means the patient has either a low or high risk for developing distant metastases. The Oncotype DX provides a trichotomous test result. In addition to the low-risk (<18) and high-risk (>31) categories, approximately 33% of patients are classified as intermediate-risk. The ODRS is also a continuous predictor of the risk of distant recurrence. Treatment decisions made in early stage breast cancer are almost never "black and white." Clinicians and patients need a test that provides information on a continuous scale. The continuous RS, as provided by Oncotype DX, on top of the trichotomous system, might give additional information to individual patients, but only if each single scoring point were supported by their clinical data.

PAM50

As previously discussed, invasive breast cancers can be classified by whole gene arrays into at least 4 major biological "intrinsic" subtypes—referred to as luminal A, luminal B, HER2-enriched, and basal-like—and 3 subtypes that are less used clinically: luminal C, normal like, and claudin-low. These subtypes have been reproducibly identified in the research setting by microarray and RT-PCR. In 2009, Parker et al proposed a 50-gene set, a Prediction Analysis of Microarrays (PAM50), for standardizing subtype classification.[53] The PAM50 Breast Cancer Intrinsic Classifier is the clinical manifestation of this gene set that uses a quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assay that has been validated on formalin-fixed paraffin-embedded tissues (FFPE). The test measures the expression of 50 classifier genes and 5 control genes to identify the intrinsic subtypes of breast cancer known as luminal A, luminal B, HER2-enriched, and basal-like. Multivariate analyses have shown that the PAM50 is an independent predictor of survival in breast cancer, that is, independent of clinicopathologic variables like nodal status, ER, tumor grade, etc.[53–55]

The PAM50 test provides additional information about the biology of the tumor and quantitative data on biomarkers already used for treatment decisions. Along with a categorical classification of breast cancer subtype, the clinical PAM50 test provides quantitative values for proliferation, luminal gene expression, ESR1, PGR, and ERBB2.

One study reported the following distribution of breast cancer subtypes among the population: 73.4%, luminal A; 11.6%, luminal B; 3.7%, HER2 overexpressing; and 11.3%, triple negative.[56]

Luminal A tumors usually have intermediate-to-high expression of ESR1 (Estrogen Receptor 1) and ER-regulated genes, and rarely have high ERBB2 expression. Luminal B tumors usually have intermediate-to-high expression of ESR1 and estrogen-regulated genes, and often have a higher proliferation rate than Luminal A tumors. HER2-enriched tumors usually have intermediate-to-high expression of the ERBB2 gene, and intermediate-to-low expression of ESR1 and estrogen-regulated genes. Approximately one-third of tumors subtyped as HER2-enriched are not HER2+ by IHC (2+ or 3+ HER2 score) or fluorescence in situ hybridization (DNA amplified for ERBB2). Basal-like tumors usually have low expression of ESR1, PGR (progesterone receptor gene), ERBB2, and estrogen-regulated genes but have a high proliferation rate. In the neoadjuvant setting, PAM50 has been associated with differential outcomes, as shown by the endpoint of pathologic complete remission – pCR (no residual invasive tumors after neoadjuvant therapy in the surgically removed breast tissue = ypT0) and near pCR (residual invasive tumors after neoadjuvant therapy in the surgically removed breast tissue is remaining as 5 mm or less = ypT1a).[14]

Comparison of Multigene Assays for Early Breast Cancer Treatment

Several multigene assays are commercially available. Most assays have only a few genes in common, even though all are used for more or less identical indications. This seeming contradiction has to do with differences in the developmental processes among the assays as well as the complexity of the human genome, where many genes can be indicators of the same message or predictors of similar outcome. Molecular profiling allows us to develop assays that can more accurately assess tumor biology, pathways that determine growth dependence (eg estrogen), and clinical behavior.

Tests may contain completely different gene sets and deliver identical outcomes. Thus, many genetic profiles can be used to examine the same molecular pathway because so many genes are responsible for controlling the multiple biochemical pathways expressed by the tumor. For example, the ER status of a tumor, which we know to be highly prognostic for outcome and an important determinant for response to endocrine therapy, can be examined in several ways, including ELISA, IHC, and gene expression. At the expression level, we know that ER status can be determined by measuring the single gene expression level of ER itself. ER status can also be measured by a gene profile or even by a signature that does not contain ER but rather levels of genes downstream to ER activation.

Only a few of the originally developed multigene assays, such as the 76-gene Rotterdam signature,[57] the wound-response signature profile,[58] invasiveness signature,[59] and Mammostrat[60] for breast cancer prognosis are available commercially. The limited availability of genetic profiling tests is in part due to the many important steps that are required before a multigene expression test can be implemented as a routine diagnostic tool. These steps include developing a customized array along and designing control systems to closely monitor the reproducibility, robustness, accuracy, and stability of the tests over time. Other important development-limiting factors are cost, availability of tumor tissue, and patient datasets with sufficient follow-up to provide level I evidence.

With gene expression analysis, additional profiles can be developed and read from the tissue submitted for the original classification. For instance, the tissue submitted for MammaPrint analysis can also be used to determine additional gene profiles, including BluePrint. BluePrint is a molecular sub typing profile that determines the mRNA levels of 80 genes that best discriminate among the 3 distinct molecular subtypes (basal-type, luminal-type, and HER2-type). Combining MammaPrint and BluePrint allows patients to be stratified into the following subgroups: luminal-type/MammaPrint low-risk (similar to luminal A); luminal-type/MammaPrint high-risk (similar to luminal B); HER2-type and basal-type. Several studies have been performed that measure chemosensitivity by pCR in patients classified according to molecular subgroups by MammaPrint and BluePrint. The results are shown in Table 2.[49,61,62,67]

Even though no standard test is available and no one technology is uniformly accepted, many clinicians have embraced

Table 2. Summary of three independent studies, using molecular profiling (MammaPrint and BluePrint[67]) as a predictive marker for chemosensitivity. In each study, the rate of pathologic complete response is given of the total population of patients in each intrinsic subtype. As a summary of all 3 studies, a percentage is given for each subtype from all patients enrolled in these 3 studies in the far right hand column. The luminal subtypes clearly have less chemo-sensitivity compared with HER2 and basal subtypes as shown by the endpoint pCR.

	STRAVER[49]		SOMLO[62]		HESS[63]		TOTAL		
	n	pCR	n	pCR	n	pCR	n	pCR	%
Luminal-type/MP low risk	21	0	14	1	29	1	64	2	3%
Luminal-type/MP high risk	67	3	16	0	53	6	136	9	7%
HER2-type	41	13	18	10	24	12	83	35	42%
Basal-type	38	13	20	4	27	15	85	44	52%

multigene assays because they are an effective tool for making treatment decisions in early stage breast cancer cases. Multigene assays and technology are constantly evolving.

Several other tests not covered in this review are being developed to objectively stratify patients into the proper risk category and possible therapeutic intervention. Validation, reproducibility, and evaluation of these tests and their limitations should continue as more data and technologies emerge, for example, data from the Cancer Genome Atlas Network.[68]

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Author Contributions

Analyzed the data: MK, SG. Wrote the first draft of the manuscript: MK. Contributed to the writing of the manuscript: MK, AM, SG. Agree with manuscript results and conclusions: MK, AM, SG. Jointly developed the structure and arguments for the paper: MK, AM, SG. Made critical revisions and approved final version: MK, AM, SG. All authors reviewed and approved of the final manuscript.

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As a requirement of publication the authors have provided signed confirmation of their compliance with ethical and legal obligations including but not limited to compliance with ICMJE authorship and competing interests guidelines, that the article is neither under consideration for publication nor published elsewhere, of their compliance with legal and ethical guidelines concerning human and animal research participants (if applicable), and that permission has been obtained for reproduction of any copyrighted material. This article was subject to blind, independent, expert peer review. The reviewers reported no competing interests. Provenance: the authors were invited to submit this paper.

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