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REVIEW

Gene expression profiling: Decoding breast cancer

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Abstract
Gene expression assays that are used in daily clinical practice for treating early breast cancer patients have been introduced in the clinic only recently. This review discusses the development of these arrays, summarizes the validation of those that are commercially available and indicates how the information provided by these assays can help in the care of patients. The review also provides an extensive overview of commercially available assays focusing on MammaPrint, the first and only assay for breast cancer management that has been cleared by the FDA.

Contents

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Introduction

Measuring the expression of thousands of genes at the same time using microarrays has answered many questions that have been impossible to answer previously. A recent Pubmed search for ''microarrays'' generated over 28,000 items, indicating its widespread use. It was anticipated that this technique would quickly find its way into clinical diagnostics, however, only a few are currently in clinical use. As gene expression profiling represents a major change in how we make clinical decisions, it is understandable that clinical adaptation has been slow.

Tumor metastasis is a complex biological process that involves many steps starting at the tumor site and ending at the secondary tumor site. These processes involve biological pathways that are important in tumor formation and metastasis as depicted in Figure 1. There are many intersections on this roadmap along with many side roads and also many one-way streets from which there is no point of return. Thus, the ability of a tumor to survive and metastasize is determined by the molecular roadmap that it is committed to follow.

In breast cancer, the metastasis risk can be predicted by the overall gene expression of the primary tumor. This finding challenged the idea that the metastatic potential is acquired relatively late during the multi step process of tumor formation [1]. However, molecular signatures are preserved throughout the life of the tumor, even after the

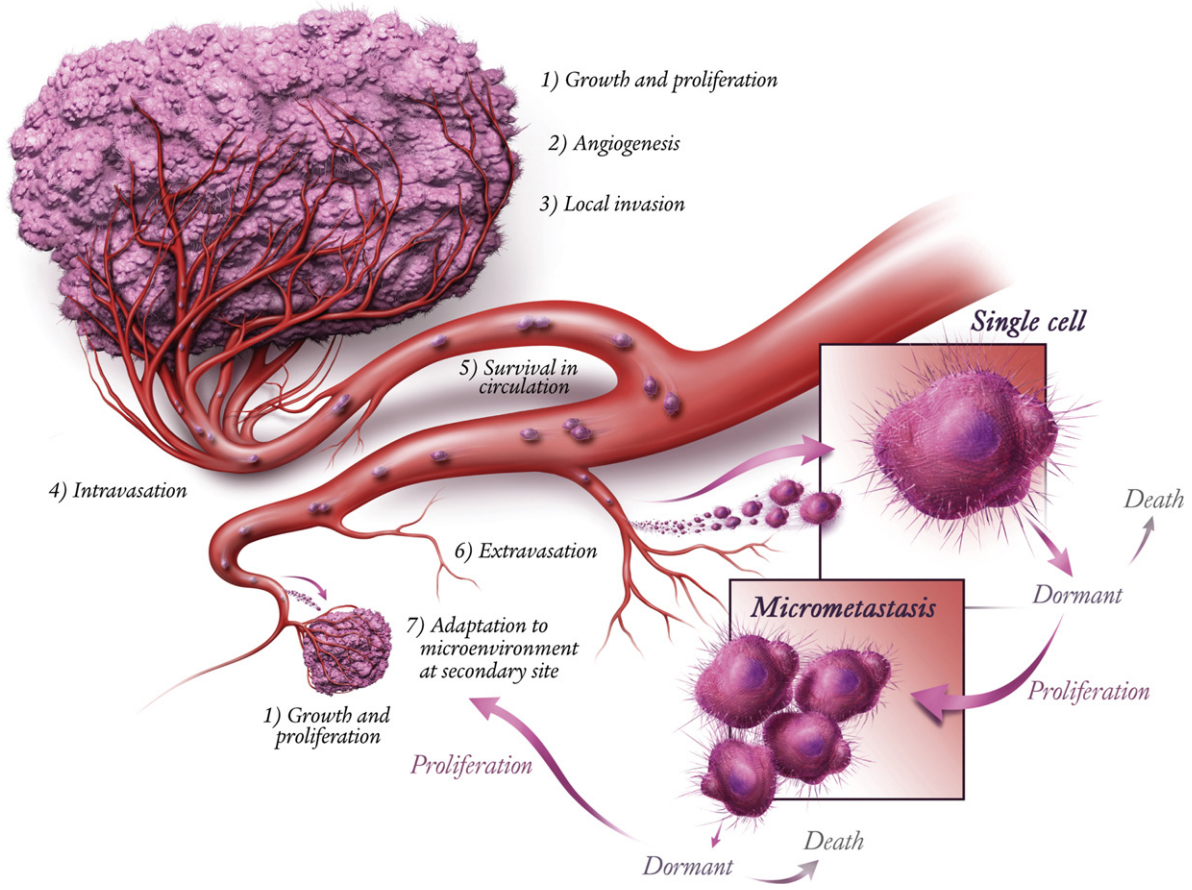

Figure 1 This figure depicts all of the critical genomic pathways associated with breast cancer recurrence; from tumor progression through the metastatic cascade.

tumor has metastasized indicating that the original signature is the tumor's blueprint [2,3].

The concept of individual molecular signatures can be illustrated by the fact that the approximately 400 different cell types in the body each have different gene expression profiles. These profiles reflect their distinct cellular functions even though they all belong to one individual. Of importance, profiles have been shown to retain part of their gene expression patterns in the metastatic setting and these profiles can be used to determine the primary tissue of origin. Thus, the genomic signatures of metastatic cancers of unknown primary can be used to help characterize their respective primary sites of origin. In addition, it has been shown that poorly differentiated and undifferentiated tumors of a given cancer type retain expression patterns observed in their particular well-differentiated tumors [4,5].

In the past decade, efforts have been directed at determining gene expression profiles for diagnosis, prognosis and prediction. Whole genome microarrays have become readily available and have enabled characterization of profiles for use in the clinical oncology setting.

The natural history of breast cancer is changing as the benefits of screening mammography and adjuvant chemotherapy are becoming evident with earlier diagnosis of smaller tumors without lymph node involvement. Thus, the need for better stratification of patients is becoming increasingly important in order to identify those patients who will not need to be treated with adjuvant chemotherapy after optimal locoregional treatment, as well as identifying those high-risk patients who will benefit from certain chemotherapy regimens (Figure 2).

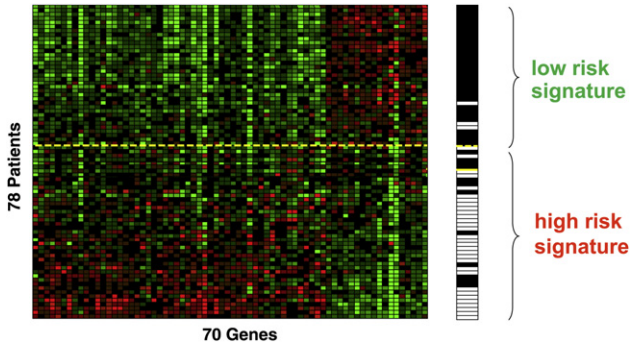

Figure 2 Heatmap depicting expression data of the 70 MammaPrint genes from tumors of 78 breast cancer patients. Each row represents a tumor and each column a gene. The dashed line indicates the optimized sensitivity. Above this line patients have a good prognosis signature (low risk of recurrence), below the line the prognosis signature is poor (high risk of recurrence). The white boxes in the panel on the right indicate patients who developed distant metastases within 5 years after primary diagnosis; black boxes depict patients who had remained disease free for at least 5 years after diagnosis. First published by Van 't Veer et al, 2002 [6].

Genetic profiles

MammaPrint is a genetic profile for breast cancer prognosis and prediction, developed in 2001 at the Netherlands Cancer Institute (NKI) in Amsterdam to help clinicians decide how to treat a growing population of patients with early stage breast cancer [6]. Researchers set out to develop a genetic signature that could correctly distinguish patients with a high risk of developing metastases from those who could be safely be spared adjuvant chemotherapy treatment as their long term distant metastasis risk was sufficiently low that chemotherapy would provide little benefit. The Amsterdam signature was the world's first gene expression profile designed to predict the clinical outcome of breast cancer patients and fill this clinical need.

The initial development of this profile used patients from a comprehensive tissue bank at the NKI and divided them in two groups: 34 patients who developed a distant metastasis within 5 years following the diagnosis of invasive breast cancer and 44 patients who remained free of metastasis. A genome wide analysis was performed in which the expression of all genes in the human genome (25,000) was measured by microarray technology. This expression was then correlated with disease outcome and a supervised unbiased approach used to select those genes that could accurately distinguish between the two patient groups. 231 genes were identified as most significant in breast cancer recurrence and the top ranked 70 genes were selected for the profile that has become the diagnostic MammaPrint test. The profile was designed with the aid of clinicians defining ''low'' and ''high'' risk as determined by which untreated patients would benefit from the addition of adjuvant chemotherapy given a 25–30% benefit in distant disease free survival and overall survival and which patients could be managed with endocrine treatment alone [7].

Several other groups have also developed profiles for breast cancer prognosis [8–11] as well as for breast cancer subgroup profiling [12]. Some experts have raised concerns

Unbiased profile development

Profiles for predicting tumor recurrence can be developed by comparing whole genome expression profiles of tumors that either metastasize or that do not recur. Those genes that are significantly different between the two tumor groups are probably the ones that can discriminate good and poor prognosis patients. The genes have been extracted in an unbiased way; there have been no human assumptions as to why certain genes end up in the profile. The next phase of a gene expression profile development is to validate whether the developed profile can be used in patient populations other that the patients the profile was developed in. It is the independent validation studies that determine the strength of a diagnostic profile.

as to why these profiles share so few genes in spite of being used for more or less identical indications [13]. This seeming contradiction has to do with the complexity of the human genome, where many genes can essentially be an indicator of the same message. Molecular profiling enables the development of tests that can more accurately assess the tumor's biology and clinical behavior, even though tests with identical outcomes may contain completely different gene sets. Thus, many genetic profiles can examine the same molecular roadmaps, given that so many genes that are responsible for controlling the many biochemical pathways are 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 tamoxifen response, can be examined in several ways, including ELISA, IHC and gene expression. On an expression level, we know that ER status can be determined by measuring the single gene expression level of ER itself or it can also be measured by a gene profile not even containing ER [14].

The other profiles that have been developed include the 76-gene Rotterdam signature [10], the wound-response profile [15], invasiveness signature [16] and p53 [17]. However, only a few of these are available commercially (Table 1). Part of the limited availability of genetic profiling test has to do with the many important steps that are required before a multigene expression test can be implemented as a routine diagnostic tool. These include developing a customized array along with designing control systems to closely monitor the reproducibility, robustness, accuracy and stability over time [40]. Other important steps limiting development include the cost of development, the availability of tumor material and patient datasets with sufficient follow-up.

OncotypeDX

A genomic profile that has been on the market for several years is the Oncotype DX recurrence score. The genes that make up this test were selected from a predefined set of candidate genes. This set of genes had been found to be important in breast cancer development and recurrence based on published studies and included several that were reported in the original MammaPrint study [6]. A total of 21 genes were selected for this profile of which 5 are used for normalization.

Oncotype DX has been validated in 3 independent study populations using different study designs. The first study population was a subset of patients from a randomized clinical trial, NSABPB-14, that originally included almost 3000 patients randomized to assess tamoxifen benefit in lymph node negative, ER-positive breast cancer patients [8]. Thus, all study patients received 5 years of tamoxifen therapy. It demonstrated that patients classified as having a low Recurrence Score (51% of patients) have a significantly different 10-year rate of distant recurrence (6.8%; 95CI 4.0—9.6%) than patients (27%) classified as having a high Recurrence Score (30.5%; 95CI 23.6—37.4). However, the low Recurrence Score group of patients had overlapping confidence intervals with patients (22%) having an intermediate RS (14.3%; 95CI 8.3—20.3%).

In the second validation study conducted at MD Anderson [28], the three patient groups defined by the Recurrence Score did not show a significant correlation with the 10-year rate of distant recurrence as the confidence intervals of all three groups overlapped. The low risk patients had a rate of distant recurrence of 18% (95CI 7—30%), the intermediate risk patients of 38% (95CI 15—61%) and the high-risk patients of 28% (95CI 13—32%). These patients were all untreated.

The third validation study was conducted by Kaiser Permanente [29] and was designed as a case control study, where 220 patients with breast cancer who had died of the disease were matched with three controls per case (i.e. the total number control patients was 570) being alive at the time their matched index patient had died. The statistically approximated 10-year recurrence rate was 2.8% (95% CI 1.7—3.9) for patients classified as ''low risk'' and is statistically different from the ''intermediate risk'' patients who had a 10-year recurrence rate of 10.7% (95% CI 6.3—14.9). However, patients classified as ''high risk'' did not significantly differ from ''intermediate risk'' patients in 10-year recurrence rate (15.5%; 95% CI 7.6—22.8).

A recent presentation by Dowsett et al. reviewed Oncotype DX validation in the ATAC trial, which was designed to determine patient outcome in patients treated with Tamoxifen alone or those initially treated with an aromatase inhibitor (AI) [30]. Even though Oncotype DX could not distinguish between AI-treated patients classified as high, intermediate or low risk, the recurrence rate was significantly different in the three Oncotype DX risk groups when patients from both treatment arms were included. This suggests that Oncotype DX may have value in patients treated with an AI initially.

Mapquant

Another commercially available gene expression profile for breast cancer prognosis is the Genomic Grade test (Mapquant DX). This profile was developed by defining 97 genes that are associated with tumor differentiation and tumor grade ascertained by comparing expression profiles from histologic grade 3 and histologic grade 1 tumors in a training set of 64 estrogen receptor positive tumor samples. The profile was validated in previously published and publicly available datasets and was found to be more strongly associated with relapse free survival than was histological grade [34]. In addition, the Genomic Grade index appeared to reclassify patients with histologic grade 2 tumors into two groups with high versus low risks of recurrence (hazard ratio 3.61, 95% confidence interval = 2.25—5.78; $P < 0.001$, log-rank test). A second study validated the Genomic Grade test in 650 ER-positive patients who were untreated or were only treated with tamoxifen. The majority of these patients were also derived from previously published and publicly available datasets [35]. The Genomic Grade outcome was tested in 229 patients with Her2-negative breast cancer in the neoadjuvant setting and a high Genomic Grade appeared to be associated with a higher response to neoadjuvant chemotherapy [36].

Theros

The H/I and molecular grade index, also known as Theros, was specifically developed for ER positive breast cancer patients treated with tamoxifen. In the initial development

Table 1 Commercially available multigene assays for breast cancer prognosis and/or prediction.

	MammaPrint	OncoTypeDX	Mapquant DX	Theros	Bioclassifier	MammoStrat
Also known as	70-gene signature	21 gene recurrence score	Genomic grade test	H/I and Molecular grade index	PAM50	Five antibody test
Manufacturer	Agendia	Genomic Health	Ipsogen	Biotheranostics	ARUP	Applied Genomics
Assay method	Gene expression array	RT-PCR	Gene expression array	RT-PCR	RT-PCR	IHC
Number of genes	70	16	97	2+6	50	5
Development	Van 't Veer, 2002 [6]	No published manuscripts	Sotiriou, 2006 [34]	Ma, 2004 (H/I) [9] Ma, 2008 (MGI) [37]	Parker, 2009 [11]	Ring, 2006 [38]
Validation on prognosis	Van de Vijver, 2002[18] Buyse, 2006 [19] Bueno-de-Mesquita, 2008 [20] Wittner, 2008 [21] Mook, 2008 [22] Mook, 2009 [23]	Paik, 2004 [8] Esteva, 2005 [28] Habel, 2006 [29] Dowsett, 2008 [30]	Sotiriou, 2006 [34] Loi, 2007 [35]	Ma, 2006 (H/I) [4]	Parker, 2009 [11]	Ross, 2008 [39]
Validation on prediction	Knauer, 2009 [24] Bender, 2009 [25] Straver, 2009 [26]	Paik, 2006 [31] Albain, 2007 [32]	Symmans, 2008 [36]	No published manuscripts	Parker, 2009 [11]	No published manuscripts
Feasibility in clinical practice	Bueno-de-Mesquita, 2007 [27]	Oratz, 2007 [33]	No published manuscripts	No published manuscripts	No published manuscripts	No published manuscripts
Prospective randomized trial	MINDACT Ongoing	TAILORx Ongoing	no	no	no	no
FDA status	FDA cleared, safe and effective	Not cleared	Not cleared	Not cleared	Not cleared	Not cleared

study of 60 patients, the ratio of HOXB13 to IL17R expression was found to predict the risk of distant metastasis in tamoxifen-treated patients [9]. In a validation study with samples from 852 tamoxifen-treated and untreated breast cancer patients, the two gene ratio validated in both patient groups, providing evidence that the ratio could correctly stratify patients into high and low risk [4].

The tumor grade signature, or molecular grade index (MGI), has been developed to compliment H/I by starting with 39 previously identified genes and narrowing this gene list by looking at functional annotation and association with clinical outcome. Similar to the Mapquant genomic grade test, the MGI stratifies grade 2 tumors into a high and low risk groups. In this same study, the two genomic grading profiles were compared using publicly available datasets and were found to perform equally well. It should be noted that 4 of the 5 genes that make up the MGI profile are also part of the 97 genes that make up the Genomic Grade test also referred to as Mapquant. This same study also considers the prognostic power of combining the gene ratio test and MGI in 84 ER positive breast cancer patients treated with tamoxifen [37].

PAM50

The PAM50 signature or Breast Bioclassifier has recently been published and is based on the subgroups formed by performing gene expression profiling with unsupervised clustering [11]. The subgroups specified are the basal-like subtype, which is predominantly estrogen receptor (ER)-negative, progesterone receptor (PR)-negative and ERBB2-negative (often referred to as triple negative), the ERBB2-like subtype, characterized by the increased expression of *ERBB2* (HER2) and two luminal-like subtypes, called the luminal A and B subtypes both of which are ER positive [12]. These molecularly defined subgroups have distinct clinical outcomes and responses to therapy [41]. The PAM50 signature is a risk model that incorporates a predictor for the Risk of Relapse (ROR) based on tumor size with the molecular subtypes providing prognosis and prediction of chemotherapy benefit. Even though the PAM50 signature appears to provide information additive to currently used clinico-pathological parameters, all patient results on which this signature was validated were derived from publicly available in silico data.

MammoStrat

To address the need for specialized laboratories to ensure the quality assurance required for gene expression-based assays, Ring et al designed a multiple marker test using fewer genes which employed a readily available technology, namely IHC [38]. They investigated the possibility of developing an IHC test using the insights from many published gene expression studies and tested 700 gene targets chosen on the basis of interesting gene expression patterns in published datasets from three patient cohorts with 466, 299 and 344 patients. Twenty antibodies were found to have a significant association with patient outcome in the 195 ER-positive, node-negative patients from the first training cohort. Several models were found to have prognostic power and were subsequently tested in the two independent cohorts. This initial study found a minimum set of 5 antibodies that could be combined using a Cox proportional hazards ratio (i.e. the Cox model prognostic index) and used to predict outcome in ER-positive breast cancer patients. Their first study was underpowered in the node-negative subsets of patients and prompted a further validation study of the five-antibody IHC test using patient samples from the NSABP trials B-14 and B-20 [39]. From the B-14 study (initiated to establish clinical benefit of adjuvant tamoxifen), 287 placebo and 550 tamoxifen-treated patients were included, a subset from a total of 1414 and 2615 patients respectively. From the B-20 trial, initiated to establish the clinical benefit from adjuvant chemotherapy added to tamoxifen, 161 tamoxifen-treated patients and 296 tamoxifen plus chemotherapy treated patients were included. These patients were a subset of a total number of 771 and 1535, respectively. The test classifies patients into low, moderate and high-risk patients, and shows considerable differences in outcome predictions for various age groups. Younger patients classified as low risk still had a 20% risk of disease progression, whereas this was only 6% for patients 60 years and older. An absolute 21% decrease in recurrence rate was seen for the high-risk patients treated with chemotherapy. This age stratification still needs confirmation in additional studies as the study was not a prespecified analysis. Further, as the test was developed in a predominantly postmenopausal cohort, it could be that this IHC test was population specific.

Comparing genetic profiles

A direct comparison among a number of existing profiles was carried out by Fan et al. [45]. This study includes 295 patients from the Netherlands Cancer Institute and compared MammaPrint, the wound—response model, the Oncotype recurrence score, the intrinsic subtype model, and the two-gene-ratio model. For this study, the expression data for sufficient number of genes was assessed to permit the simultaneous analysis of the five profiles that have almost no gene overlap. The analysis revealed these several models gave similar predictions in this patient cohort, suggesting the absence of a unique prognostic gene set.

A more recent study attempts to elucidate how the different genes are related to one another and examines the contribution of well-known biological processes of breast cancer tumorigenesis to their prognostic performance.

The investigators studied publicly available gene expression and clinical data from 2833 breast tumors. They show that the 9 studied prognostic signatures exhibit similar prognostic performance and suggest that proliferation plays an important role in breast cancer prognosis [46].

How genetic information can enable a personalized approach

After optimal locoregional treatment, which may include a variety of therapeutic modalities such as breast conserving surgery, mastectomy with or without immediate reconstruction and sentinel node sampling with nodal dissection if metastases are encountered, the patient is a candidate for adjuvant therapy. Ideally one would like to forego adjuvant chemotherapy in those patients who are likely to be cured by the locoregional treatment alone.

NSABP trails

The National Surgical Adjuvant Breast and Bowel Project (NSABP) is a clinical trials cooperative group supported by the National Cancer Institute (NCI) that started some 50 years ago. NSAPB B-14 is a clinical trial to assess tamoxifen in patients with ER positive, node-negative breast cancer and was originally designed to treat patients for a total of 2 years but was subsequently modified to maintain treatment through 5 years [42]. The trial included 2892 patients randomized for tamoxifen and an additional 1235 patients all treated. A total of 2617 patients were treated with tamoxifen. The genes in the Oncotype DX test were first selected in several patient cohorts, including the tamoxifen-treated patients from the NSABP B-20 trial [43]. The test was hence developed on a subset of 668 patients (668/2617 patients) from the NSABP B-14 trial [8]. Patients of this subset had similar age and tumor size distribution as the total patient population of the NSABPB-14 trial. As for other patient characteristics and such as grade and HER2 status no information is available.
The NSABP B-20 trial is a trial to determine the value of chemotherapy and tamoxifen over tamoxifen alone in ER positive, node-negative breast cancer patients. The tamoxifen-treated patients of this trial have been used both to develop the Oncotype DX test as well as to determine whether the test would be predictive of chemotherapy response [31,43]. The use of samples for validation of the test that have also been used in developing that same test can influence the outcome of the study [44].

Gene expression profiling enables the clinician to identify patients with a low risk for recurrence and treat them with hormones alone. Conversely, for high-risk patients one would like to know which patients respond to a particular therapy and which patients will show responsiveness to targeted molecular therapies. Finding gene profiles that would provide such targeted response prediction is the promise of personalized medicine.

Storing RNA

To enable the development of representative response profiles, the genomic information from which these profiles are developed needs to be properly preserved in a non-contaminated, non-degraded fashion. In this regard, proper fresh tissue banking has become important not only in the clinical research setting, but also for routine daily clinical practice. Information contained in the RNA of the tumor cells is degraded when the tissue is processed and embedded in paraffin, the most common procedure for tumor storage. Not only is the integrity of this information of critical importance to the development of contemporary response profiles, but individual patients might profit from this information 5—10 years from now as therapeutic frontiers advanced.

Retrieving fresh specimens for gene expression analysis is feasible in community hospital settings. The RASTER (microarRAy PrognoSTics in Breast CancER) study was performed in Dutch community hospitals on a series of 400 unselected patients with node-negative breast cancer [27]. The median implementation time was only one month and the addition of MammaPrint profile information was perceived as beneficial for patient management by the treating physicians [47].

Current risk stratification of breast cancer patients

Many tumor characteristics affect the outcome of patients and many different classification systems have been devised for classifying patients according to clinico-pathologic criteria. The clinical guidance of these classification systems in the HER2 negative, early stage breast cancer patients according to NCCN, St Gallen and other consensus guidelines differ significantly and the clinical guidance offered for patients differs according to guidelines being used.

Retrieving fresh tissue specimen for gene expression analysis

Both for small and larger tumors it is important to take a fresh specimen with sufficient percentage of tumor cells within approximately one hour after surgery (see Figure 3). To facilitate handling of the specimen we recommend hardening the tissue by storing it in a refrigerator (4 °C) for 20 min or freezer (−20 °C) for 10 min. This hardening process not only makes slicing the tumor easier, but it also makes visualization of the tumor margins as reliable as if the tumor were immediately embedded in paraffin.
We recommend slicing the tumor in sections of approximately 5 mm so that even small tumors are cut into multiple slices. After slicing the tissue, one can use the 3 mm biopsy punch from the kit or use a scalpel to take a sample. The sample is put in an RNA stabilizing solution (such as RNA Retain). The sample should be taken from the core to middle diameter from the tumor, not including the margins of the tumor. When dealing with a larger (>2 cm) tumor, the biopsy should not be taken from the core of the tumor, since this could potentially contain only necrotic tissue. As for core needle biopsies the recommendation is to save at least 2 biopsies in the RNA stabilizing solution, given that there is a higher likelihood of single samples containing no tumor cells.

Figure 3 Pictures of taking a fresh tissue biopsy: The tissue of the specimen is hardened by storing it in a refrigerator (4 °C) for 20 min or freezer (−20 °C) for 10 min prior to slicing. The tumor is sliced in sections of approximately 5 mm. A 3-mm biopsy punch is used to take a sample. The sample is put in an RNA stabilizing solution.

MammaPrint validation studies

MammaPrint was developed and initially validated in a series of 295 consecutive (i.e. to ensure no selection bias) women with breast cancer collected according to an NKI protocol [18]. The patients were all part of the tumor bank at the Netherlands Cancer Institute (NKI) which included all patients seen for any cancer diagnosis and from which all patients with a breast cancer diagnosis who were untreated and stages 1—3 were included. This tumor bank dates from 1986 when the NKI was founded and has preserved tissue from all cancer patients seen there since that time. In the subset of 151 patients with lymph node negative disease (of whom 95% received no adjuvant chemotherapy), the proportion of patients who remained free from distant metastases at ten years was 87% in the ''low risk'' group and 44% in the ''high risk'' group. The gene profile was a statistically independent predictor of outcome and added to the power of standard clinico-pathologic parameters; HR = 4.6 (95% CI 2.3—9.2).

As patients were collected from the same institute as the original discovery patient group [6], there was some overlap between patients in the development and initial validation study potentially biasing the validation. However, when the overlapping patients were excluded, the profile could still distinguish between ''low'' and ''high'' risk patients in a more sensitive way than using accepted clinical guidelines, such as St Gallen or Adjuvant! Online.

The second independent validation study for Mamma Print was performed by a European clinical research group, the TRANSBIG Consortium [19]. The five participating European hospitals accessioned 302 untreated patients with at least 10 years of follow-up. The proportion of patients who remained free from distant metastases at ten years was 88% in the ''low risk'' group and 71% in the ''high risk'' group. MammaPrint was found to provide prognostic information beyond what could be determined from patient age, tumor grade, tumor size, and ER status in a population of node-negative patients none of whom received any adjuvant endocrine or chemotherapy. It performed better than outcome assessments derived from Adjuvant! Online and provided independent risk assessment with 28—35% discordance between MammaPrint and Adjuvant! Online in ''low'' and ''high'' risk groups, indicating their independent predictiveness. The discordant patients had clinical outcomes most accurately predicted by MammaPrint. As such, 34% of Adjuvant Online! ''high risk'' patients could have avoided chemotherapy in that they had ''low risk'' MammaPrint results. Similarly, 14% of Adjuvant Online! ''low risk'' patients had ''high risk'' Mammaprint profiles and merited additional treatment based on outcome data.

Additional supporting studies have been published in which these results were further validated. One provides an independent validation and studied 123 patients aged <55 years from two Dutch institutes [20]. A second revealed that MammaPrint has a very high negative predictive value for distant recurrence after adjuvant treatment in older American breast cancer patients studied at Massachussetts General Hospital [21]. Additional work revealed that

Table 2 MammaPrint validation studies.

	Number of patients	Details of study	Treatment specifics	Patient age	DMFS by MammaPrint at 5 years		DMFS by MammaPrint at 10 years	
					Poor prognosis	Good prognosis	Poor prognosis	Good prognosis
Van de Vijver et al, (2002) [18]	295 patients, of which 151 patients LN-	Consecutive patient series	5% adj treatment*	<53 years	56%*	93%*	44%*	87%*
Buyse et al, (2006) [19]	302 patients	FDA clearance validation	No adj treatment	<61 years	83%	96%	71%	88%
Wittner et al. (2008) [21]	100 patients	US validation of MammaPrint	45% adj treatment	Median 63 years	90%	100%	90%	100%
Mook et al, (2008) [22]	241 patients	1–3 positive LN patients	91% adj treatment	All ages	80%	98%	76%	91%
Bueno-de-Mesquita et al. (2008) [20]	123 patients	Validation	37% adj treatment	<55 years	78%	98%	n.a.	n.a.
Mook et al. (2009) [23]	148 patients	Validation	18% adj treatment	>55 years	73%	93%	68%	81%
Bueno-de-Mesquita et al, (2007) [27]	427 patients	Prospective clinical trial		<61 years	n.a.	n.a.	n.a.	n.a.

* For LN- patient subgroup ($n = 151$).

MammaPrint has strong prognostic value in patients with 1, 2 or 3 positive lymph nodes [22] and in patients over 55 years [23]. Table 2 depicts all MammaPrint validation studies.

MammaPrint result

The risk thresholds for the MammaPrint profile were determined by discussions with medical oncologists who felt that a ~10% risk of recurrence in untreated patients would translate into a 5–6% recurrence risk if hormonal therapy were used. This was deemed sufficiently low that such patients would not be considered candidates for adjuvant chemotherapy. Conversely, the ''high risk'' threshold was set at ~30% for untreated patients which the same group of physicians deemed sufficiently high that all such patients would be appropriate candidates for adjuvant chemotherapy based on their risk of developing metastases and the accepted ~30% benefit of adjuvant treatment [7].

Hazard ratios and chemotherapy benefit

MammaPrint is effective at distinguishing patients with a ''good'' prognosis from patients who develop early metastases. The hazard ratios for MammaPrint are exceptionally high in the first 5 years following curative treatment ranging from 4.5 to 4.7 for time to distant metastasis adjusted for clinical risk [19]. Of importance, it is in these same years that chemotherapy exerts its maximal salutary effect [7]. Patients who received adjuvant treatment clearly show a lower risk of recurrence compared with untreated patients in this same 5-year period, whereas after this interval the difference in risk of recurrence stabilizes. For treatment with anthracycline-based chemotherapy, this benefit may even be restricted to the first 2 years following treatment [48]. MammaPrint has been developed to distinguish those patients who are likely to develop metastasis in the same time frame that overlaps chemotherapy benefit (Figures. 4 and 5).

Dichotomous, trichotomous or continuous end result

MammaPrint provides a dichotomous (binary) test result: the patient is either at Low Risk or High Risk of developing distant metastases. Over 97% of patients receive a result with more than 90% sensitivity.
The OnctypeDX provides a trichotomous test result: in addition to the Low and High Risk categories, the majority of patients are classified as Intermediate Risk. The continuous Recurrunce Score (RS), as provided by Oncotype on top of the trichotomous system, might add information to individual patients, if only each single scoring point would be supported by their clinical data. The 95% confidence intervals indicate that this is not the case. Even the subdivision into the 3 risk groups cannot significantly be stratified in each validation study; with overlapping 95% confidence intervals for the Low and Intermediate Risk group in the first validation study. The trichotomous classification system is still leading in prognosis of disease outcome and guiding therapy, as RS scores of 2 and 16 imply different risks, but provide the same outcome.

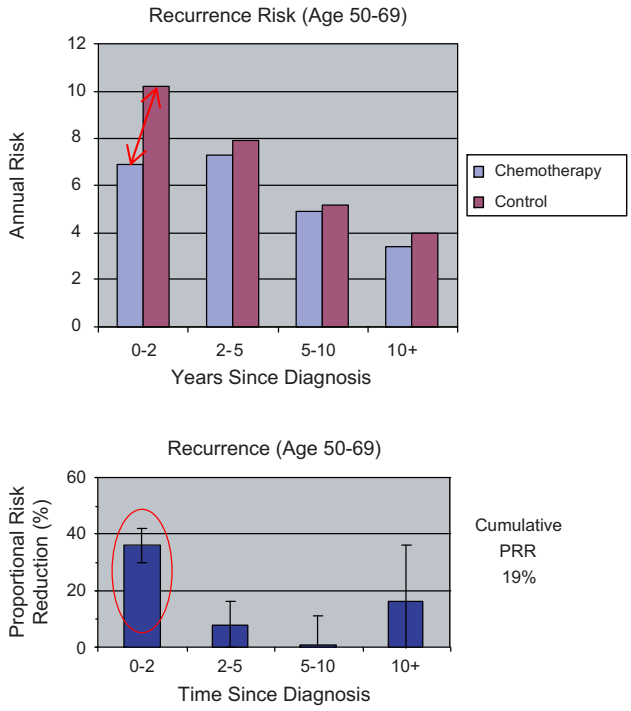

Figure 4 Figures from Ravdin et al. [48] depicting how adjuvant chemotherapy exerts its salutary effects mainly in the first 2—5 years after diagnosis.

MammaPrint predictiveness

We have analyzed the ability of MammaPrint to help physicians predict chemotherapy responsiveness for patients with ''high risk'' and ''low risk'' profiles. The results were reported for both neoadjuvant chemotherapy [26] and for adjuvant chemotherapy as presented by Bender et al [25]. Straver et al. [26] reported that MammaPrint predicted chemotherapy response in 167 patients with Stage II—III disease who received neoadjuvant chemotherapy with regimens which included either AC × 6 cycles, dose dense AC, A-docetaxel × 6 cycles or docetaxel-capecitabine × 6 cycles if HER2 negative. For HER2 positive patients, dose dense AC plus PTC × 6 cycles was the standard of care. The patient group included 144 (86%) with a ''poor'' prognosis signature of whom 29 (20%) had a pathologic complete response (pCR) to treatment, whereas none of the ''good'' prognosis patients experienced a pCR.

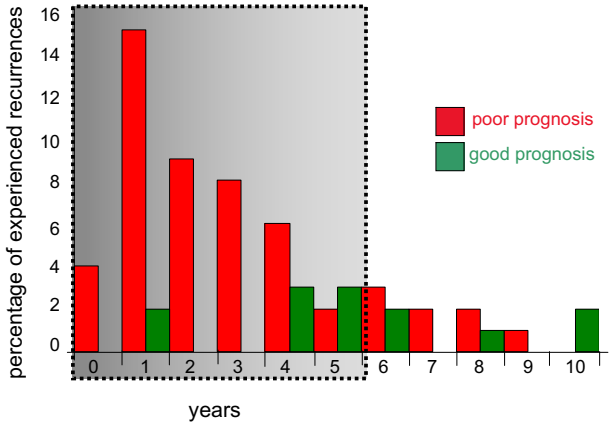

Figure 5 MammaPrint has been developed to recognize those patients that have a high likelihood of developing distant metastases in the first 5 years after diagnosis. This figure depicts the percentage of experienced recurrences of patients from the second MammaPrint validation study [19], stratified for good and poor prognosis by MammaPrint. These patients did not receive any adjuvant treatment.

The predictiveness of MammaPrint for patients receiving adjuvant chemotherapy was presented at ASCO by Bender et al. [25] (also presented earlier at the St. Gallen international symposium by Knauer et al. 2009 [24]). These presentations review a meta-analysis of 1696 patients from 7 previously reported studies with a median follow-up of 7.1 years. Of these, 315 received hormonal therapy alone and 226 received hormonal therapy plus chemotherapy. Adjuvant chemotherapy was CMF or an anthracycline with or without taxane-based chemotherapy. MammaPrint assigned 252 (47%) patients to ''low risk'' and 289 (53%) patients to ''high risk'' of recurrence. In the MammaPrint ''high risk'' group, a significant (HR = 0.35, $p < 0.01$) benefit for the combined treatment of 12% was observed. These results remained robust in a multivariate analysis (HR = 0.38, $p = 0.04$). Conversely, there was no significant benefit for hormonal therapy plus chemotherapy versus hormonal therapy alone in the ''low risk'' patient group.

From prognosis to response

The rationale for the development of gene expression profiles for prognosis and chemotherapy response prediction lies in the hypothesis that the natural history of a tumor is determined by its underlying regulatory gene pathways. By comparing genome wide expression data from patients who have developed metastases (poor prognosis) with patients who remained metastasis free (good prognosis), those genes that are associated with the development of metastases will emerge. A profile that can correctly classify patients who will either develop metastasis or who will remain free of metastases should ideally be developed in patients untreated with adjuvant therapy allowing an undisturbed look at the true natural history of the disease. By including patients who have received adjuvant treatment, the profile will also likely contain genes that predict response to adjuvant treatment instead of simply being a reliable prognosis profile influenced exclusively by tumor biology.

The challenge of developing such prognostic profiles on intact genomic specimens is that only limited patient cohorts are available that are untreated, have long clinical follow-up and for whom frozen tumor samples are available. Also, the survival rates of patients from these cohorts are lower than patients diagnosed today as both local and systemic treatment regimens have improved patient outcomes dramatically. Thus, even though the prognosis profile is developed and validated in multiple patient cohorts, the survival rates of these studies do not reliably indicate a current patient's prognosis.

MINDACT and TAILORx

Two large prospective randomized trails are currently undertaken to prospectively determine clinical utility of multigene assays, MINDACT for MammaPrint and Tailorx for Oncotype DX.
The MINDACT trial (Microarray in Node-negative Disease may Avoid ChemoTherapy) is being conducted by the European TRANSBIG Breast International Group, a research network of 39 institutions in 21 countries. To date, ~3000 patients have been enrolled from almost 70 cooperating institutions. This prospective, randomized phase III study will compare risk assessment using MammaPrint with risk assessment using common clinical-pathologic criteria (Adjuvant! Online) in selecting patients for adjuvant chemotherapy in node-negative breast cancer and patients with up to 3 positive lymph nodes. The goal is to study 6000 women using this design. If both the gene signature and the clinical assessment are ''high risk'' ($n = 3300$), patients will be randomized to one of two chemotherapy regimens. If both are ''low risk'' ($n = 780$), then no chemotherapy will be administered. If the two forms of risk assessment are discordant ($n = 1920$), then patients will be randomized to therapy, based either on the clinical assessment or the gene expression signature. Hormone receptor positive disease will be randomized to one of two hormonal regimens. The results will help show how best to use the clinico-pathologic and the gene signature tests together as the true benefit to gene expression profiling must be additive to be clinically useful and cost-effective.
The TAILORx trial (Trial Assigning IndividuaLized Options for Treatment), which is currently recruiting subjects, will help prospectively determine the value of chemotherapy in patients with an intermediate RS. The goal is to study 10,000 patients. In this trial, patients with a recurrence score higher than 25 will receive chemotherapy plus hormonal therapy, patients with a recurrence score lower than 11 will receive hormonal therapy alone and patients in the intermediate RS group will be randomly assigned to receive adjuvant hormonal therapy with or without chemotherapy. The intermediate group as defined for TAILORx (women with an RS between 11 and 25, almost 45% of all trial subjects) is different from the definition of intermediate risk group for the currently available commercial test, which runs from 18 to 31.

Ideally, physicians would like to be able to provide the patient with personalized treatment advice. In this way, the patient who has a ''good'' prognosis signature can safely have chemotherapy withheld and the patient with a ''poor'' prognosis profile can be offered adjuvant chemotherapy.

Conclusion

The goal of any prognostic and/or predictive assay is to augment the clinician's ability to make meaningful treatment decisions that influence patient outcomes. This level of evidence generally requires completion of a prospective trial wherein the result of the test in question is the critical variable being examined. Such a trial is MINDACT, the multi-institution EU study designed to determine if patients are better served by having their therapy prescribed by MammaPrint or by Adjuvant! Online, an internet-based clinico-pathologic risk assessment tool. MINDACT is designed to accrue 6000 patients of whom ~50% are already registered [49]. While definitive answers await study completion and analysis in 2015, published data and recent affirmation by the St Gallen international consensus panel [50] suggests an important role for 'validated multigene assays' in the management of patients with early stage breast cancer.

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Conflict of interest statement

Emiel Rutgers declares no financial and personal relationship with other people or organizations that could inappropriately influence his work. Richard Bender reports to be an employee of Agendia Inc. Annuska Glas and Femke de Snoo report to be employees of Agendia BV.

Authorship

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