Clinical Application of Gene Expression Profiling in Breast Cancer

Joseph A. Sparano, MD[a,*], Melissa Fazzari, PhD[b], Paraic A. Kenny, PhD[c]

KEYWORDS

• Gene expression profiling • Breast cancer
• Multiparameter assay • Prognostic factor • Predictive factor

Genomics is defined as the study of all of the nucleotide sequences in an organism (see **Table 1** for definition and glossary of other terms). Sequencing of the human genome in tumors is technically daunting, but is currently being performed as part of the Human Cancer Genome Atlas project.[1] One report describing an analysis including 11 breast cancers concluded that the genomic landscape of breast cancer is characterized by a handful of commonly mutated gene mountains and a larger number of gene hills that are mutated at a low frequency.[2] In addition to mutation of individual genes, it has recently become apparent that the genomes of breast tumors harbor many more somatic genomic rearrangements than had previously been identified, suggesting that novel fusion genes found at these translocations may also play a role in disease progression.[3] These methods are being used to identify specific genetic changes that may contribute to the pathogenesis of breast cancer and that may be targeted with specific therapeutic interventions, similar to targeting mutated c-KIT with imatinib in gastrointestinal stromal tumor.[4] These methods are not yet

Supported in part by grants from the National Institute of Health (P30-13330), Susan G. Komen for the Cure (KG091136), and the Department of Defense CDMRP Breast Cancer Research Program (W81XWH-09-46-0680).
[a] Department of Medicine and Oncology, Albert Einstein College of Medicine, Montefiore Medical Center, 1825 Eastchester Road, Bronx, NY 10461, USA
[b] Department of Epidemiology & Population Health, Albert Einstein College of Medicine, 1301 Morris Park Avenue, Bronx, NY 10461, USA
[c] Department of Development and Molecular Biology, Albert Einstein College of Medicine, 1301 Morris Park Avenue, Bronx, NY 10461, USA
* Corresponding author. Department of Medicine and Oncology, Albert Einstein College of Medicine, Montefiore Medical Center, 1825 Eastchester Road, Bronx, NY 10461.
E-mail address: jsparano@montefiore.org

Surg Oncol Clin N Am 19 (2010) 581–606
doi:10.1016/j.soc.2010.03.008 **surgonc.theclinics.com**
1055-3207/10/$ – see front matter

Table 1
Glossary of terms commonly used in describing microarray studies

Term	Definition
Gene Expression Analysis	
Genomics	Study of all of the nucleotide sequences, including structural genes, regulatory sequences, and noncoding DNA segments, in the chromosomes of an organism
DNA microarray	A glass slide or silicon chip with DNA sequences complementary to thousands of genes arrayed at precise locations
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction: method used for quantitative RNA expression in RNA extracted from specimens, including degraded RNA extracted from formalin-fixed paraffin-embedded tissues
Analysis of Gene Expression Data	
Hierarchical clustering	Commonly used method for performing unsupervised analysis of gene expression data
PAM or SAM	Prediction analysis of microarray or significance analysis of microarray: commonly used methods to analyze gene expression data
Centroid	Average gene expression profile defining a classifier
Regulation of Gene Expression Assays	
CLIA	Clinical Laboratory Improvement Amendments: regulations that cover approval of diagnostic tests, including multiparameter assays
IVDMIA	In vitro diagnostic multivariate index assay: term used by the FDA to describe certain types of multiparameter assays that are regulated as medical devices

510(k) clearance	Regularly approval by the FDA for medical devices characterized as an IVDMIA
Standards	
MAQC	Microarray quality control: effort initiated by the FDA to standardize methods for clinical application of microarray and other genomic assays
REMARK Guidelines	Reporting recommendations for tumor marker prognostic studies: standard criteria for reporting publications about tumor markers, including multiparameter gene expression assays
MIAME	Minimal information about a microarray experiment: set of standards for release of gene expression ion
GEO	Genomic Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo): publicly available repository for gene expression data
Interpretation of Published Literature	
Hazard ratio	Relative risk of an event in a high- versus low-risk population
Sensitivity	Proportion of actual positives which are correctly identified as such (TP/TP+FN)
Specificity	Proportion of negatives which are correctly identified (TN/TN+FP)
Positive predictive value (precision)	Proportion of patients with positive test results who are correctly diagnosed (TP/TP+FP)
Negative predictive value	Proportion of patients with negative test results who are correctly diagnosed (TN/TN+FN)
Accuracy	Proportion of true results (positive and negative) in a population (TP+TN/TP+PF+FN+TN)
Receiver operator curve	Graphical plot of the sensitivity versus (1 − specificity) for binary classifier as its discrimination threshold is varied (fraction of TP versus fraction of FP)

Abbreviations: FN, false-negative; FP, false-positive; TN, true-negative; TP, true-positive.

available for routine clinical application, however. For the most part, genomic profiling has focused on the evaluation of gene expression, or the translation of the information encoded in genomic DNA into an RNA transcript. RNA transcripts include messenger RNAs (mRNAs), which are translated into proteins, and various other RNAs (eg, transfer RNA, ribosomal RNA, micro RNA, and noncoding RNA) that have important biologic functions. For the most part, gene expression profiling in breast cancer has focused on the evaluating expression of mRNA. However, the same principles may be applied to the study of the epigenome,[5,6] micro RNAs,[7] proteins,[8] or integrative approaches that evaluate combinations of profiling methods.[9]

Substantial technical advances within the past decade have facilitated high-throughput analysis of clinical specimens for gene expression, a process that has been referred to as genomic profiling, although gene expression profiling is the more accurate term.[10] There have likewise been important advances in bioinformatics that permit analysis and interpretation of the huge of amount of data generated by expression profiling.[11] By combining high-throughput specimen evaluation and sophisticated bioinformatics analysis, one can identify distinctive patterns of expression that correlate with clinical behavior or response to specific therapies. Some have referred to these distinctive expression patterns as molecular portraits[12] or signatures,[13] and the assays used to detect these patterns as multiparameter assays; the latter term has been used because rather than relying on expression of a single gene or protein, these assays typically incorporate information from measuring expression of multiple genes by using mathematical algorithms to derive a qualitative (eg, high vs low risk) or quantitative (eg, score) test result.[14] These assays may also be categorized as a tumor marker, a clinical assay that serves as a surrogate for defining clinical end points, such as disease response or progression, or predicting clinical end points, such as prognosis or response to therapy.[14,15] Although the term tumor marker in the past has usually referred to a substance released from a tumor into the blood or other body fluids (eg, CA27-29, CEA, PSA), it more recently has been defined more broadly to include tissue-derived markers including multiparameter assays.

The promise and pitfalls in developing multiparameter assays have been reviewed elsewhere,[16–19] and specific criteria have been proposed for the level of evidence required to define and support their clinical usefulness.[15] Several multiparameter assays are currently approved for clinical use, including some which have been recommended by expert panels for clinical decision making.[14,20] This article focuses on principles of gene expression profiling, and multiparameter assays that have been developed for breast cancer. The term multiparameter assay is used interchangeably with the terms assay, tumor marker, and marker.

PROGNOSTIC AND PREDICTIVE MARKERS

A tumor marker is valuable only if it provides information above and beyond that provided by classic clinicopathologic features. A prognostic marker is one that is associated with clinical outcome, usually irrespective of the treatment given. Examples of prognostic markers include tumor size, number of positive lymph nodes, and tumor grade. A predictive marker is one that predicts clinical benefit from a specific therapy. Examples include estrogen receptor (ER) expression (predictive of benefit from endocrine therapy) and HER2/neu overexpression (predictive of benefit from anti-HER2 directed therapies). Some predictive markers are also prognostic, particularly when the therapy predicted to be beneficial is not used (eg, ER, HER2 expression). Predictive markers are more difficult to develop and validate, but are of

intrinsically greater value because they are essential in selecting patients for beneficial therapies, more difficult to identify, and fewer.

PROCESS FOR DEVELOPMENT OF A MULTIPARAMETER ASSAY

There are several steps in the development of a marker, and a typical roadmap is summarized in **Box 1**. Steps included in the process may be broadly classified as (1) conceptualization, (2) clinical development, (3) technical development, (4) validation, and (5) application. For the purpose of maker validation, prospective trials may be performed by retrospectively evaluating samples from completed clinical trials with mature clinical outcomes. Models have been proposed for appropriate strategies to validate markers prospectively in either newly initiated clinical trials, or in completed clinical trials.[21] A critical issue is to ensure that there is sufficient sample size to conduct training and validation studies, and in particular a sufficient number of patients with the clinical event of interest (eg, recurrence).[17] Development of an accurate marker is largely a function of the interplay between sample size and classification difficulty.[22] It is not uncommon to find that several statistically equally good predictors can be developed for any given classification problem.[23] In the postdevelopment process, there is potential for the assay to be less accurate and informative as a result of bias in clinical application of the assay. For example, clinicians may be more apt to use an assay in patients with intermediate clinical features, and not to use it in those with good or poor risk clinical features.[24]

METHODS FOR ANALYZING GENE EXPRESSION

There are several methods for analyzing gene expression, which have been reviewed extensively elsewhere and which are illustrated in **Fig. 1** and summarized in **Table 2**.[25,26] Irrespective of the analysis platform used, messenger RNA (mRNA) is first extracted from the tissues of interest (see **Fig. 1**A–C). Because mRNA is highly vulnerable to degradation, sample handling at this step is critical; surgical specimens should be frozen as soon as possible when used for analysis, or placed in appropriate preservative (eg, RNA Later, Qiagen, Valencia, CA, USA). Following mRNA purification, several platforms exist for gene expression profiling. The first gene expression microarray technology to enter widespread use was the 2-color microarray (see **Fig. 1**D). mRNA from 2 samples (an experimental sample and a reference sample) is converted to fluorescently labeled cDNA. Each sample is labeled with a different color (red or green) and the samples are pooled at a 1:1 ratio and hybridized to the same microarray. For all microarrays, each spot on the microarray represents 1 gene and the fluorescence intensity at each spot is proportional to the expression level of that gene in the sample. In a single-color array (see **Fig. 1**E), each tumor sample is labeled with the same fluorescent dye and hybridized to its own microarray. For both array types, after removal of nonhybridized material by washing, images are obtained using laser scanning, which detects the relative fluorescent intensity of the hybridized probe at each spot. Before statistical analysis, the data must be normalized (see **Fig. 1**H) to compensate for variation in labeling, hybridization, and fluorescent detection, and filtered using specific criteria to reduce the likelihood of detecting noise. In the example shown (see **Fig. 1**H), array 3 had a higher average signal intensity (red) than array 1, which, in turn, was higher than array 2. Mathematical correction by normalization results in each array having the same average signal intensity, thereby largely eliminating variation caused by technical issues and allowing detection of biologically relevant differences in gene expression between samples. When many samples are analyzed, it is often convenient to summarize the data in a heatmap (see **Fig. 1**I). In this format, the patient samples are

Box 1
Roadmap for development of a multiparameter marker or other markers

1. Conceptualization: identify clinical need and how marker addresses the clinical need
   - Identify clinical problem
   - Identify treatment options and costs of misclassification using standard clinicopathologic criteria, and potential costs of misclassification with standard criteria (eg, over treatment, under treatment)
   - Identify potential clinical relevance of information provided by marker (prognostic, predictive, or both)
2. Clinical development: marker developed (trained) in an appropriate population, typically referred to as a training set
   - Population sufficiently homogeneous and receiving uniform treatment
   - Perform internal validation of classifier to assess whether it seems sufficiently accurate relative to standard prognostic factors that it is worth further development
3. Technical development: establish technical specifications of marker to ensure reproducible performance in clinical samples
   - Establish reproducibility and reliability of assay
   - Identify and minimize sources of preanalytic variability that may occur in sample collection and processing in the clinic
   - Identify and minimize sources of analytical variability in the laboratory that is conducting the assay
4. Validation: validation of marker in other independent data sets in prospectively planned studies
   - Identify appropriate subject population for marker validation

     Population appropriate for prognostic (no treatment or uniform treatment) or predictive assay (2 or more treatment regimens administered with differing therapeutic outcomes)

     Sufficient sample size and sufficient number of events of interest
   - Identify relevant clinical end point

     Distant recurrence, local recurrence, organ-specific recurrence, all recurrences

     Other clinically relevant end points (eg, specific toxicities)

     Death (eg, from primary cancer, other cancers, toxicity, or other causes)
   - Establish reliability of marker in correlating with clinical end points of interest in different populations
5. Application: establishment or confirmation of clinical usefulness of the assay
   - Prospective testing of marker in data sets that are independent of data sets used for initial validation
   - Postmarking experience

     Evaluate potential biased used of assay in clinical practice (eg, use preferentially in patients with intermediate-grade tumors)

     Evaluate how test information influences clinical decision making

Adapted from Simon R. Roadmap for developing and validating therapeutically relevant genomic classifiers. J Clin Oncol 2005;23:7332.

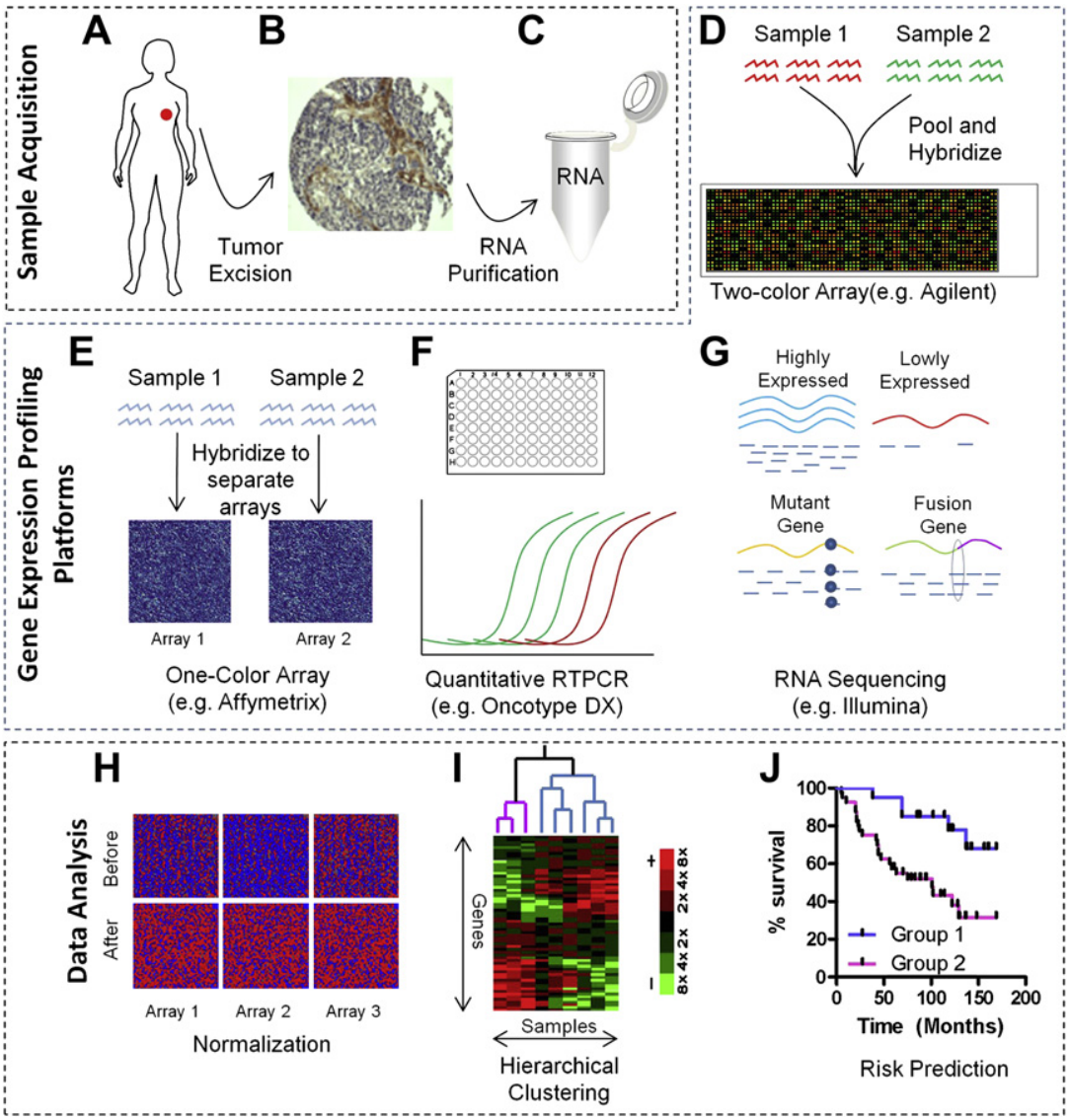

Fig. 1. Summary of steps involved in sample preparation and analysis using high-throughput genomic technologies (*A–J*).

typically represented in columns, and genes in rows. Genes that are expressed at levels above the median are colored in red, close to the median in black, and below the median in green. Other color schemes are also commonly used (eg, blue-yellow), which may be especially helpful for individuals with red-green color blindness. By comparing the expression level of a large number of genes in each of the samples, the technique of hierarchical clustering can be used to determine which samples are most similar in gene expression. For example, in **Fig. 1**I, the samples from 2 distinct clusters are indicated by the blue and purple branching in the dendrogram at the top of the heatmap. These groups may correspond to samples with different biologic properties (eg, ER-positive vs ER-negative tumors, or high-grade vs low-grade tumors) or groups with different clinical outcomes (see **Fig. 1**J).

Two nonmicroarray-based technologies are also finding applications in this area. In quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR), RNA from the tumor is converted to cDNA and arrayed in different wells of a multiwell plate, each well containing specific PCR primers for a particular gene (see **Fig. 1**F). qRT-PCR analysis can then be used to rapidly and accurately quantify the expression level of each gene of interest within the sample. Unlike microarray analysis, which

Table 2
Commonly used methods for measuring gene expression

Method	Description	Advantages	Disadvantages
Spotted cDNA microarray (eg, Agilent)	Glass slides robotically spotted with purified cDNA clones, PCR products from clones, or oligonucleotides	Ability to design custom arrays	Operator dependent, labor intensive, not always reproducible, requires fresh or frozen tissue
Photolithography (eg, Affymetrix Gene Chips)	DNA probes directly synthesized on silicon chips	Ability to design custom arrays	Requires frozen tissue or placement in RNA preservative media
Real-time reverse transcriptase polymerase chain reaction (RT-PCR)	Generate DNA copies of RNA by reverse transcription, amplify DNA by PCR, quantify DNA product using specific fluorescent reagents	May be performed using RNA extracted from paraffin tissue	Requires development and validation of probes, technical limitations in number of genes that may be assayed
RNA sequencing	Massively parallel sequencing of all mRNAs in a sample	Can detect mutations, splice variants, and fusion genes in addition to changes in gene expression level	Expensive to run the experiments and time consuming to perform the analysis

interrogates tens of thousands of genes, the qRT-PCR technology is more appropriate when a limited group of genes is being investigated, although this method may still allow analysis of several hundred genes simultaneously.

The recent advent of high-throughput massively parallel sequencing machines has opened up an entirely new possibility for gene expression profiling, termed RNA sequencing. It is now feasible to profile samples by direct sequencing of cDNAs derived from many millions of RNA transcripts in a given sample. In addition to providing absolute expression levels (as the precise number of transcripts can be counted), this technology also allows the identification of alternatively spliced isoforms, mutations, and novel transcripts arising from fusion genes. In the example shown (see **Fig. 1**G), the blue gene is more highly expressed than the red gene, resulting in many more sequencing reads. Furthermore, mutations and fusion genes may be detected using this technology, which is not possible using the microarray platforms or qRT-PCR. In this example, the yellow gene has a mutation that is detected in the sequencing reactions, whereas the other transcript results from the fusion of 2 independent genes (green and purple) and is recognized from sequencing reads spanning the boundary between the 2 genes. Recent studies in breast, prostate, and leukemic cell lines show the potential of this approach.[27,28]

QUALITY CONTROL OF GENE EXPRESSION ANALYSIS METHODS

There are multiple sources of error in clinical application of multiparameter assays, including preanalytical, analytical, and postanalytical. Approval requires meeting specific technical requirements regarding performance, reliability, and reproducibility.

The Microarray Quality Control (MAQC) project was organized by the US Food and Drug Administration (FDA) to improve current and next-generation molecular profiling technologies and foster their proper applications in discovery, development, and review of FDA-regulated products (http://www.fda.gov/ScienceResearch/BioinformaticsTools/MicroarrayQualityControlProject/default.htm). The effort includes multiple stakeholders, including multiple centers within the FDA and other federal agencies, major providers of microarray platforms and RNA samples, academic laboratories, and others. In MAQC-I, 2 human reference RNA samples were evaluated, and differential gene expression levels between the 2 samples were calibrated with microarrays and other technologies (eg, qRT-PCR). The resulting microarray data sets were used for assessing the precision and cross-platform/laboratory comparability of microarrays, and allowing individual laboratories to more easily identify and correct procedural failures.[29–34] In MAQC-II, teams developed classifiers for 13 end points from 6 relatively large training data sets, and produced more than 18,000 models that were tested by independent and blinded validation sets generated for MAQC-II. In MAQC-III, also called sequencing quality control, the technical performance of next-generation sequencing platforms is being evaluated by generating benchmark data sets with reference samples and evaluating advantages and limitations of various bioinformatics strategies in RNA and DNA analyses.

METHODS FOR BIOINFORMATICS ANALYSIS OF GENE EXPRESSION

The method by which gene expression data are analyzed depends on the objectives of the analysis, which may be broadly classified as class comparison, class prediction, or class discovery, as described by Simon and colleagues.[19] A description of the statistical analytical methods is beyond the scope of this article, but has been reviewed by others.[35] Class comparison involves determining differences in expression profiles associated with a specific known clinical characteristic (eg, BRCA mutation-associated cancer) or outcome (eg, recurrence or organ-specific recurrence). The primary goal of this type of analysis is to find an informative set of genes and to estimate corresponding population parameters, such as the individual effect of increased expression in each gene on the probability of recurrence. Given the multiplicity of testing at the gene level, gene importance is inferred by ranking all of the genes measured on the array by statistical significance, summarized by the magnitude of the test statistic, corresponding *P*-value or adjusted *P*-value.[36,37] Extending the univariate approach, modeling approaches such as linear or logistic regression may be used to adjust for other genes of interest or known clinical factors such as tumor stage, age, or treatment modality. Model building must take relationships between predictors (included in and omitted from the model) into account to produce precise estimates of effect as well as valid inferences. Statistical association does not confer predictive ability; in classification, for example, a marker exhibiting an odds ratio as high as 3.0 is a poor classification tool.[38] Similar to, but distinct from class comparison, class prediction involves developing a gene expression–based algorithm that accurately predicts group membership of a particular sample. It is well understood that high correlation between predictors in the model does not preclude a good fit; therefore less emphasis is placed on the interpretability of the final model in favor of highly accurate predictions. To this end, the error rate or mean squared error is of primary importance in assessing model performance. Predictive ability is first assessed through internal cross-validation approaches.[39] However, testing the model in independent and more heterogeneous data sets is vital to properly evaluate its true predictive value.

Class comparison and prediction fall into the category of top-down approaches, in which gene expression data from cohorts with known clinical outcomes are compared with genes that are associated with prognosis without any a priori biologic assumption. After this unbiased evaluation, it has become standard to test the subset of important genes from the ranked list or model for enrichment of specific molecular pathways using tools such as Ingenuity IPA (Ingenuity Systems, Redwood City, CA, USA).[40] Alternatively, a bottom-up approach may be used, in which gene expression patterns that are associated with a specific biologic phenotype or deregulated molecular pathway are first identified and then subsequently correlated with the clinical outcome.[26] Class discovery may also be based on unsupervised statistical clustering algorithms such as hierarchical or k-means clustering. An analysis of 4 validated gene expression signatures developed either by the class discovery or bottom-up approach (intrinsic gene set, wound-response signature) or class comparison or top-down approach (70-gene assay, 21-gene assay) showed significant agreement in their outcome predictions for individual patients, suggesting that they are tracking similar biologic phenomena despite being developed by differing methodologies.[41] Although proliferation is the strongest parameter predicting clinical outcome in the ER-positive/HER2-negative subtype and the common denominator of most currently available prognostic gene signatures, immune response and tumor invasion are predominant molecular processes associated with prognosis in the triple negative and HER2-positive subgroups, respectively.[42]

VALIDITY, REPRODUCIBILITY, AND REPORTING OF MICROARRAY STUDIES

Several steps are typically involved in properly developing a gene expression signature, including identifying the signature in a training set, and then validation of the signature in other data sets. There are methods for internally cross-validating the signature in the same data set, but external validation is always necessary in other data sets. Failure to properly adhere to these fundamental principles has led some to challenge the validity and reproducibility of microarray-based studies.[43] Dupuy and Simon[44] specifically evaluated the quality of 42 microarray-based breast cancer studies published in 2004 and found that at least 50% contained at least 1 fundamental methodological flaw. The most common design flaws included inadequate control for multiple testing, a spurious claim for class discovery using outcome-related genes, and biased estimation of predictive accuracy by improper cross-validation. All of these deficiencies could potentially contribute to false discovery. The investigators proposed guidelines including dos and don'ts that are essential reading for anyone engaged in the clinical development of gene expression signatures.

Criteria have been developed for assessing and reporting tumor markers, including multiparameter assays, called the REMARK guidelines (reporting recommendations for tumor marker prognostic studies). These guidelines were developed by an expert panel to address methodological deficiencies that were commonplace in most reports evaluating tumor markers, including multiparameter gene assays on recommendation of the first meeting held in 2000 of the National Cancer Institute-European Organization for Research and Treatment of Cancer (NCI-EORTC) First International Meeting on Cancer Diagnostics. The guidelines provide relevant information about the study design, preplanned hypotheses, patient and specimen characteristics, assay methods, and statistical analysis methods. The guidelines are intended to encourage transparent and complete reporting of the relevant information for the scientific community. Most peer-reviewed journals require that reports describing tumor markers, including multiparameter assays, follow the REMARK guidelines to be

considered for publication. In addition, standards termed minimal information about a microarray experiment (MIAME) for reporting the data have been established by the Microarray Gene Expression Data Society,[45] and most journals require that the gene expression data described in the publication be deposited in a publicly available database (eg, Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo)).

REGULATORY APPROVAL OF MULTIPARAMETER ASSAYS

Approval of multiparameter breast cancer assays had been regulated in the past under the provisions of the Clinical Laboratory Improvement Act of 1988 (CLIA), which provided the basis for approval of the Oncotype DX assay.[46] The regulations apply to laboratories that examine human specimens for the diagnosis, prevention, or treatment of any disease or assessment of health. The FDA released a guidance document in 2007 indicating that approval of multiparameter assays falls under regulatory jurisdiction of the agency under regulations governing approval of medical devices (http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm079163.htm#1). A gene expression profiling test system for breast cancer prognosis was defined as a device that measures the RNA expression level of multiple genes and combines this information to yield a signature (pattern or classifier or index) to aid in prognosis. Approval provided by this mechanism is commonly known as 510(k) clearance. There are currently 4 approved multiparameter assays, which are summarized in **Table 3** and described in greater detail later.

CLASSIFIERS DEVELOPED BY SUPERVISED ANALYSIS

Several classifiers have been developed by comparing gene expression profiles from relapsing versus nonrelapsing cancers, including a 70-gene profile,[47,48] 76-gene profile,[49,50] 21-gene profile,[51,52] and 2-gene HOXB13/IL17BR ratio.[53–56] The characteristics of the populations used for the external validations studies and key results are shown in **Table 4**.

70-Gene Assay

A 70-gene assay associated with prognosis was first identified in a test set of breast tumors derived from 117 women treated at the Netherlands Cancer Institute in Amsterdam.[63] RNA extracted from frozen tumor specimens was analyzed using the Rosetta Hu25K microarrays for expression of nearly 25,000 genes, and supervised classification was applied to identify a gene expression signature strongly predictive of a short interval to distant metastases (poor prognosis signature) in patients with lymph node–negative disease, and a signature that identified tumors of BRCA1 carriers. The poor prognosis signature consisted of genes regulating cell cycle, invasion, metastasis, and angiogenesis. The signature was then prospectively validated in a separate cohort of 295 consecutive patients treated at the same institution less than 53 years of age with stage I to II primary breast carcinoma associated with negative (n = 151) or positive (N = 144) axillary lymph nodes, of whom 180 (61%) had a poor prognosis signature and 115 (39%) had a good-prognosis signature.[48] The probability of remaining free of distant metastases was 51% and 85% for the high- and low-risk signatures, respectively. The estimated hazard ratio (HR) for distant metastases 5.1 (95% confidence interval [CI] 2.9–9.0) for the high- compared with the low-risk signature, which remained significant when adjusted for clinical covariates in a multivariable Cox regression analysis. The 70-gene signature subsequently was externally validated in a separate validation set consisting of 307 patients 60 years of age or less with primary breast cancers measuring 5 cm or less associated with negative axillary lymph

Table 3
Multiparameter assays for breast cancer approved by regulatory agencies

Assay (Company)	Method	Tissue Type	Approval	Patient Population	Prognosis/Prediction
Mammaprint 70-gene assay (Agendia)	DNA microarray	Fresh or frozen	Europe and United States (FDA)	ER-positive/negative stage I–II breast cancer	Prognostic for distant recurrence
Oncotype DX 21-gene assay (Genomic Health)	qRT-PCR	FFPE	Europe and United States (CLIA)	ER-positive stage I–II breast cancer	Prognostic for distant recurrence Predictive of chemotherapy benefit if RS high
Theros 2-gene ratio (Biotheranotics)	qRT-PCR	FFPE	United States (CLIA)	ER-positive, lymph node–negative breast cancer	Prognostic for distant recurrence
MapQuantDX 5-gene molecular grade (Ipsogen)	DNA microarray	Fresh or frozen	Europe	ER-positive, grade 2 tumors	Prognosis: reclassification of tumors from grade 2 to grade 1 or 3

Abbreviation: FFPE, formalin-fixed paraffin-embedded.

nodes who received no adjuvant systemic therapy, including endocrine therapy (71% had ER-positive disease).[47]

76-Gene Assay

A 76-gene assay associated with prognosis was first identified in a test set derived from 286 patients with lymph node–negative breast cancer who received no adjuvant systemic therapy treated at the Erasmus Cancer Institute in Rotterdam, the Netherlands, of whom 73% had ER-positive disease.[50] In contrast to the populations in which the 70-gene assay was validated, this analysis did include patients 60 years or older, or who had T3 to T4 tumors (only 3% for the latter). RNA extracted from frozen tumor specimens was analyzed using the Affymetrix Human U133a GeneChips for expression of 22,000 transcripts. In a training set of 115 tumors, a 76-gene signature consisting of 60 genes for ER-positive tumors and 16 genes for ER-negative tumors was identified. An external validation study was subsequently performed in 180 patients of any age with tumors less than 5 cm in diameter and negative axillary nodes who received no systemic adjuvant therapy, including endocrine therapy.[49] The HR for distant metastasis within 5 years was 7.4 (95% CI 2.6–20.9), and was 11.4 (95% CI 2.67–48.4) when adjusted for clinical covariates. For distant metastases at 5 years, the sensitivity was 90% and specificity was 50%. The positive and negative predictive values were 38% (95% CI 29%–47%) and 94% (95% CI 86%–97%), respectively.

2-Gene HOXB13/IL17BR Ratio

Ma and colleagues developed a 2-gene ratio training set which included 60 women with ER-positive breast cancer. An expression signature predictive of disease-free survival was reduced to a 2-gene ratio, HOXB13/IL17BR.[61] The investigators also evaluated the biologic function by ectopic expression of HOXB13 in MCF10A breast epithelial cells, and showed that transfection enhanced motility and invasion in vitro. High HOXB13/IL17BR ratio was subsequently evaluated in a separate validation set including 206 women with ER-positive breast cancer and was significantly associated with inferior disease-free survival (HR 2.38, 95% CI 1.30–4.36) and overall survival (HR, 2.48, 95% CI 1.22–5.06) in 130 patients with axillary lymph node–negative disease, but not in the 76 patients with lymph node–positive disease. A separate validation study was performed in 619 patients with operable ER-positive breast cancer treated with adjuvant tamoxifen, and 193 patients with metastatic breast cancer receiving first-line tamoxifen therapy for metastatic disease. An increased HOXB13/IL17BR ratio was inferior disease-free survival for node-negative patients only. For patients with metastatic disease when adjusted for clinical covariates, a high ratio was the strongest predictor in multivariate analysis for a poor response to tamoxifen therapy (odds ratio = 0.16; 95% CI 0.06–0.45) and a shorter progression-free survival (HR 2.97; 95% CI 1.82–4.86).

Genomic Grade Index

Sotiriou and colleagues[64] compared the gene expression profile of tumors associated with poor and good histologic grade, and applied the grade signature to those with intermediate grade to predict outcomes in those with an intermediate grade. Microarray data from 64 ER-positive breast cancers derived from 3 published gene expression data sets were analyzed for differential gene expression in tumors associated with a poor versus good histologic grade, which identified 97 genes that were differentially expressed, most involved in cell cycle regulation and proliferation. In an independent validation data set including 597 tumors, the gene expression grade index was strongly associated with histologic grade. However, among tumors with

Table 4
Results of pivotal validation studies for selected multiparameter markers developed by class comparison/prediction or top-down approaches

Assay and Reference	Patient Population	Treatment	No. of Patients	Key Findings
70-Gene Assay (Mammaprint)				
Van de Vijver et al[48]	Stage I–II breast cancer < 53 years	Chemo (38%), endocrine therapy (14%), or no adjuvant therapy	295	70-gene signature (good vs poor risk) prognostic for distant recurrence
Buyse et al[47]	Stage I–II breast cancer < 61 years	No adjuvant therapy	302	70-gene signature adds independent prognostic information to clinicopathologic risk assessment
76-Gene Assay				
Foekens et al[49]	Lymph node–negative	None	180	76-gene signature (good vs poor risk) prognostic for distant recurrence
21-Gene Assay (Oncotype DX)				
Paik et al[52]	ER-positive, lymph node–negative breast cancer	Tamoxifen	668	RS is prognostic for risk of distant recurrence as a categorical (low, intermediate, or high) or continuous variable
Habel et al[51]	ER-positive, lymph node–negative	Tamoxifen	790	External population-based validation study shows RS predictive of breast cancer death in tamoxifen-treated patients with ER-positive, lymph node–negative breast cancer

Paik et al[57]	ER-positive, lymph–node negative	Tamoxifen ± CMF	651	RS is predictive of benefit from adjuvant CMF chemotherapy (beneficial only if RS $\geq$ 31)
Goldstein et al[58]	ER-positive, 0–3 positive lymph nodes	Tamoxifen + doxorubicin and cyclophosphamide or docetaxel	465	RS is prognostic in patients treated with adjuvant endocrine therapy plus chemotherapy and provides information complementary to classic clinicopathologic features
Albain et al[59]	ER-positive, lymph node–positive and postmenopausal	Tamoxifen ± CAF	367	RS is predictive of benefit from adjuvant CAF chemotherapy (beneficial only if RS $\geq$ 31)
Dowsett et al[60]	ER-positive, lymph node–negative or –positive	Anastrozole versus tamoxifen ± chemotherapy	1308	RS is prognostic in anastrozole treated patients
2-Gene Ratio				
Goetz et al[61]	ER-positive, lymph node–negative or –positive	Tamoxifen	211	2-gene *HOX13/IL17B* ratio prognostic in node-negative but not node-positive disease
Jansen et al[62]	ER-positive lymph node–negative or –positive	No adjuvant therapy	468	2-gene *HOX13/IL17B* ratio prognostic in node-negative disease
	ER-positive metastatic disease	Tamoxifen	193	2-gene *HOX13/IL17B* ratio associated with progression-free survival

intermediate histologic grade, the index spanned the values for histologic grade 1 to 3 tumors, and a high molecular grade gene expression grade index was associated with a higher risk of recurrence (HR 3.61, 95% CI 2.25–5.78). The 97-gene genomic grade index (GGI) was subsequently reduced to a 5-cell cycle-related genes to molecular grade index (MGI) and evaluated in 2 publicly available microarray data sets including 410 patients, followed by development of a real-time RT-PCR assay that was tested in 2 additional cohorts including 323 patients. MGI performed as consistently as the more complex 97-gene GGI.[55] In addition, the analysis showed that in patients treated with endocrine therapy, MGI and HOXB13/IL17BR modified each other's prognostic performance; high MGI was associated with significantly worse outcome only in combination with high HOXB13/IL17BR ratio, and likewise, high HOXB13/IL17BR was significantly associated with poor outcome only in combination with high MGI.

21-Gene Assay (Oncotype DX Recurrence Score)

The 21-gene assay was developed using different methods from those previously described. The assay includes 16 tumor-associated genes and 5 reference genes, with the result expressed as a computed recurrence score (RS).[52] The genes selected for incorporation in the model were derived from a panel of 250 candidate genes that was assembled by searching the published literature, genomic databases, pathway analysis, and microarray-based gene expression profiling experiments performed in fresh frozen tissue to identify genes likely to be associated with prognosis. The gene panel was tested in formalin-fixed paraffin-embedded primary tumor samples from 447 patients with breast cancer.[52,65] Genes were tested in a heterogenous group because it was hypothesized that the genes most highly correlated with recurrence would survive evaluation across diverse patients and treatments. Genes that were consistently significant across multiple studies provided the basis for developing of the RS model. The tumor-related genes included in the algorithm include genes involved in ER signaling (*ESR1*, *PGR*, *BCL2*, *SCUBE2*), proliferation (Ki67, *STK15*, *Survivin*, *CCNB1*, *MYLB2*), Her2 signaling (*HER2*, *GRB7*), invasion (*MMP-11*, *CTSL2*), and other genes involved in immune function (*CD68*), drug metabolism (*GSTM1*), and apoptosis (*BAG1*). Relative expression levels of the 16 genes are measured in relation to average expression levels of 5 reference genes (the latter are not included in the RS calculation). A score is generated for each gene ranging from 0 to 15, with each integer of 1 corresponding to a 2-fold increase in RNA expression level. For each of the gene groups, the mean of the values from each gene group is obtained. The mean expression value for each of the 4 gene groups plus the expression level for each of the 3 individual genes are each multiplied by a coefficient. RS is calculated using the sum of the adjusted RNA expression values for each gene group or gene. Greater relative weight is reflected by the higher coefficient values derived for the proliferation, HER2, and ER-related genes. Higher expression levels for favorable genes (ER group, *GSTM1*, *BAG1*) result in a lower RS (as a result of a negative coefficient in the algorithm), whereas higher expression of unfavorable genes (proliferation group, HER2 group, invasion group, and *CD68*) contribute to a higher RS (as a result of a positive coefficient in the algorithm). The analytical and operational performance specifications defined for the Oncotype DX assay allow the reporting of quantitative RS values for individual patients with a standard deviation of within 2 RS units on a 100-unit scale.[66]

Several pivotal studies have been performed evaluating the 21-gene assay, which are summarized in **Table 4**. The assay was first tested in a prospective validation study that was performed in patients with ER-positive, node-negative breast cancer who received a 5-year course of tamoxifen in the National Surgical Adjuvant Breast and Bowel Project (NSABP) trial B-14.[52,67–69] The study included 668 of 675 patients for whom tumor

blocks were available and who had sufficient tumor for analysis. When evaluated as a categorical variable, the proportions of patients categorized as having an RS defined as low (<18), intermediate (18–30), or high (>31) risk by the RT-PCR assay were 51%, 22%, and 27%, respectively. The Kaplan-Meier estimates of the rates of distant recurrence at 10 years in the low-, intermediate-, and high-risk groups were 7%, 14%, and 31%, respectively. In a multivariate Cox model, RS predicted distant recurrence independent of age and tumor size, and was also predictive of overall survival. The prognostic usefulness of the 21-gene assay was subsequently validated in other data sets including patients with ER-positive disease, including a population-based study including lymph node–negative patients treated with tamoxifen,[51] lymph node–positive or –negative patients treated with adjuvant anastrozole,[60] or patients with up to 3 positive axillary nodes treated with adjuvant tamoxifen plus doxorubicin-containing chemotherapy.[58] Subsequent evaluation in other data sets indicated that high RS was associated with benefit from adjuvant cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) chemotherapy in patients with ER-positive, lymph node–negative breast cancer, and from cyclophosphamide, doxorubicin, and 5-fluoroucacil (CAF) in postmenopausal patients with node-positive breast cancer.[59]

MULTIPARAMETER ASSAYS COMPARED WITH INTEGRATED CLINICAL INFORMATION

Most studies evaluating multiparameter assays have evaluated the assay in models adjusted for clinical covariates to show that the assay provides additional information beyond that provided by clinical features.[26] Some reports have described models integrating molecular and clinical data into an algorithm that is more accurate in predicting clinical outcomes than either alone.[70] Some reports have described a comparison of risk stratification predicted by the assay compared with treatment guidelines (eg, St Gallen critieria[20]), indices based on clinical factors (eg, Nottingham Prognostic Index [NPI][71]), or Adjuvant! Online (http://www.adjuvantonline.com), a web-based tool that predicts 10-year breast cancer outcomes with and without adjuvant systemic therapy that has been validated in a population-based study.[72] Patients were also assigned to the clinicopathologic low-risk group if their 10-year survival probability, as estimated by Adjuvant! software,[72] was greater than 88% for ER-positive tumors or 92% for ER-negative tumors.

Buyse and colleagues[47] evaluated the 70-gene profile in 307 patients who received no adjuvant systemic therapy. The prognostic information provided with the 70-gene profile was compared with risk categories assigned by the St Gallen criteria, NPI, or Adjuvant!. Ten-year survival estimated by Adjuvant! was defined as low risk if 92% or higher for ER-positive disease, and 88% for ER-negative disease; a lower threshold was used for ER-positive disease because it was estimated that the use of endocrine therapy would result in a 4% absolute improvement in survival at 10 years. The gene signature exhibited comparable sensitivity for distant recurrence when compared with clinical features as assessed by Adjuvant! Online (87%), NPI (79%), or St Gallen criteria (96%). On the other hand, the specificity of the high-risk signature for distant metastases (42%) appeared better than some clinical predictors, such as the St Gallen criteria (10%) or Adjuvant! Online (29%), but not others such as the NPI (48%). Other studies have shown that the 21-gene assay does not correlate with Adjuvant! Online, suggesting that the assay provides complementary information.[58,73]

CLASSIFIERS DEVELOPED BY OTHER METHODS

Several markers that have been developed by other methods are summarized in **Table 5**, including the intrinsic gene set or PAM50 developed using what would be

Table 5
Results of pivotal validation studies for selected multiparameter markers developed by other methods

Assay and Reference	Patient Population	No. of Patients	Key Findings
Intrinsic Gene Set			
Sorlie et al[74,75]	Locally advanced breast cancer (publicly available data sets previously reported by Perou and Geisler)	115	Expression pattern of 535 intrinsic genes analyzed by hierarchical clustering confirmed identification of distinct subtypes including basal-like, HER2-overexpressing, and 2 luminallike, and 1 normal breast cancer subtypes with differing response to chemotherapy and prognosis
Parker et al[70]	Stage I–II breast cancer (publicly available data sets including data sets previously reported by Loi, Wang, and Ivshima)	761	PAM50 developed in training set using panel of 10 genes to define 4 distinct breast cancer subtypes, including basal-like, HER2-enriched, luminal A, and luminal B subtypes; normal expression pattern indicative not of distinct subtype but rather insufficient tumor tissue in specimen Breast cancer subtypes identified by PAM 50 associated with distant prognosis
Wound Expression			
Chang et al[76]	Stage I–II breast cancer (publicly available data set reported by van de Vijver)	295	Wound-response signature identified by evaluating transcriptional response to normal serum Wound-response signature associated with an adverse prognosis
Invasive Gene Signature			
Liu et al[77]	Stage I–II breast cancer (publicly available data sets reported by van de Vijver and Wang)	581	Differential gene expression profile of CD44+CD24 −/low tumorigenic breast cancer cells compared with normal breast epithelium used to identify a 186 IGS IGS associated with recurrence and survival

best characterized as an unsupervised method, and the wound-response signature and invasive signature developed using a bottom-up approach.

Intrinsic Breast Cancer Subtypes and PAM50

Perou and colleagues[12] first reported the intrinsic breast cancer subtypes by evaluating variation in gene expression patterns using hierarchical clustering in a set of 65 breast cancers from 42 individuals using complementary DNA microarrays representing 8102 human genes. When a panel of 534 intrinsic genes selected from the microarray were tested in other data sets, the findings were recapitulated.[74,75] It was hypothesized that these subtypes had distinctive gene expression profiles because they originated from different cell types, including luminal epithelial cells (the cells that line the duct and give rise to most breast cancers) and basal epithelial cells of the normal mammary gland (characterized by expression of cytokeratins 5/6 and 17). The intrinsic gene panel was subsequently reduced to a panel of 50 genes, using microarray and qRT-PCR, with 10 genes selected for each centroid used to define each of 4 intrinsic subtypes, including luminal A, luminal B, basal, and HER2-enriched, plus a fifth category defined as normal, which indicates a sample that had insufficient tumor material to permit accurate classification.[70,78] This assay has been referred to as the PAM50, because it was developed using the prediction analysis of microarray method, and because it include 50 genes; each tumor analyzed by this method is assigned to gene expression centroid representing the specific subtype it is most similar to. After development of the assay in a training set including 189 prototype samples, its prognostic usefulness was subsequently validated in test sets from 761 patients who received no systemic adjuvant therapy, and its predictive usefulness was validated in 133 patients for prediction of pathologic complete response (pCR) to a neoadjuvant taxane and anthracycline regimen. With regard to prognosis, the intrinsic subtypes showed prognostic significance and remained significant in multivariable analyses that incorporated clinical covariates. In addition, a prognostic model for node-negative breast cancer was developed using intrinsic subtype and clinical information; the C-index estimate for the combined model (subtype and tumor size) was significantly better than either clinicopathologic model or subtype model alone. The intrinsic subtype model predicted neoadjuvant chemotherapy efficacy with a negative predictive value for pCR of 97%.

WOUND-RESPONSE SIGNATURE

Chang and colleagues[76] hypothesized that features of the molecular program of normal wound healing might play an important role in cancer metastasis and identified consistent features in the transcriptional response of normal fibroblasts to serum, which they characterized as the wound-response signature. This signature was found to be associated with distant metastases-free survival and overall survival in a data set including 295 patients with early breast cancer. The signature was subsequently validated in separate data set and found to reliably differentiate normal and malignant breast tissue,[79] and to be significantly associated with local recurrence.[80]

INVASIVE GENE SIGNATURE

Liu and colleagues[77] hypothesized that a subpopulation of breast cancer cells exhibited greater tumorigenic capacity that could be identified by expression of high expression of CD44 with low or undetectable expression of CD24 (CD44+CD24-/low), they developed a 186-gene invasive gene signature (IGS) by comparing the gene expression profile of CD44+CD24−/low tumorigenic breast cancer cells with

that of normal breast epithelium. There was a significant association between the IGS and overall and metastasis-free survival that was independent of established clinical and pathologic variables, and was more significant when combined with the wound-response signature. The IGS was also associated with the prognosis in other cancer types, including medulloblastoma and carcinoma of the lung and prostate.

PROSPECTIVE CLINICAL TRIALS EVALUATING MULTIPARAMETER ASSAYS

Two clinical trials are prospectively evaluating multiparameter assays in clinical practice.[26] In the TAILORx trial (Trial Assigning Individualized Options for Treatment), patients 75 years of age or younger with ER-positive, HER2-negative, axillary node–negative breast cancer who meet established National Comprehensive Cancer Network (NCCN) guidelines for adjuvant chemotherapy have the treatment assigned or randomized from the 21-gene assay results (NCT00310180). Patients with a low RS are directed to endocrine therapy and those with high RS are directed to chemoendocrine therapy, whereas those with an indeterminate midrange RS (11–25) are randomized to receive chemoendocrine therapy (the standard treatment arm) or endocrine therapy alone (the experimental arm).[81] In the MINDACT trial (Microarray in Node-Negative Disease May Avoid Chemotherapy) trial (NCT00433589), patients with stage I to II breast cancer undergo risk assessment by the 70-gene assay and Adjuvant! Online and are assigned to endocrine therapy alone if genomic and clinical criteria are concordant for low-risk disease and chemoendocrine if concordant for high-risk disease; patients whose risk is discordant by genomic and clinical features are randomized to treatment assigned by clinical or genomic criteria.

Several gene expression signatures have been identified that are predictive of response to neoadjuvant chemotherapy.[23,82–84] An ongoing prospective clinical trial is evaluating whether a multiparameter is more accurate in predicting response to therapy than clinical criteria (NCT00336791).

CLINICAL USEFULNESS OF MULTIPARAMETER ASSAYS AND CONCLUSIONS

Clinical usefulness is defined by whether a marker informs clinical decision making, and whether patients benefit from that information. Two expert reviews have indicated that evidence supporting the clinical usefulness of gene expression profiles for breast cancer is insufficient.[85,86] On the other hand, expert panels convened by the American Society of Clinical Oncology (ASCO) and NCCN concluded that certain multiparameter assays such as the 21-gene assay show clinical usefulness and recommend their use in specific clinical scenarios.[14,87] These recommendations are based largely on validation studies reporting a benefit from chemotherapy in high-risk subjects as identified by multiparameter gene expression assay.[57] It is biologically plausible that high-risk populations identified by other assays might also benefit from chemotherapy, because poor-risk signatures are driven by high expression of proliferation-associated genes that predict response to chemotherapy (particularly in ER-positive, HER2-negative disease).[42]

Information provided by markers may influence clinical decision making in several ways summarized in **Table 6**, including treatment sparing, selection, direction, and confirmation. Markers have been used thus far predominantly in patients with ER-positive disease; in this clinical scenario, although the risk of recurrence is reduced by adjuvant endocrine therapy, there may be a considerable residual risk of recurrence that typically results in a recommendation for adjuvant chemotherapy according to expert-based guidelines to further reduce the risk of recurrence.[14,20] The residual risk of recurrence after endocrine therapy, and the potential benefits of adding

Table 6
Potential influence of multiparameter assay in clinical decision making

Influence of Marker on Treatment	Therapeutic Recommendation Based on Clinical Features	Therapeutic Recommendation Based on Molecular Markers	Clinical Usefulness/ Benefit
Sparing	Yes	No	Reduce unnecessary therapy
Selection	No	Yes	Reduce recurrence risk
Direction	Uncertain	Yes or no	Provide therapeutic recommendation
Confirmation	Yes or no	Same as recommendation based on clinical features	Reinforce therapeutic recommendation
No influence	Uncertain	Uncertain	None

chemotherapy, may be estimated by using validated algorithms such as Adjuvant! Online that integrate clinicopathologic and treatment information.[72] Although adding chemotherapy reduces the risk of recurrence on average by about 25% to 30%, the absolute benefit for an individual patient is small, ranging from 1% to 5%.[69] However, many patients are willing to accept adjuvant chemotherapy even if the likelihood of benefit is small.[88] Therefore, many subjects with low-risk disease (eg, ER-positive, node-negative) are overtreated with chemotherapy, because most would have been cured with endocrine therapy alone. In treatment sparing, the clinical features indicate a need for chemotherapy, but the molecular marker is discordant by indicating a favorable prognosis without therapy, thereby resulting in sparing of adjuvant chemotherapy. In treatment selection, the clinical features indicate a favorable prognosis, but the molecular marker is discordant by indicating an unfavorable prognosis with endocrine therapy alone, thereby resulting in selection of chemotherapy in an individual for whom it otherwise would not have been recommended. In treatment direction, there is a position of therapeutic equipoise regarding a recommendation for adjuvant chemotherapy based on clinical criteria, and the marker provides direction toward a clear treatment path. Treatment confirmation occurs when a marker confirms a therapeutic recommendation that was made on clinical features alone, which may be of intangible value to the patient and the clinician. However, only treatment sparing and selection, and arguably treatment direction, seem to meet the definition of demonstrable clinical usefulness. It has also been clearly established that equipoise or uncertainty may remain even after the results of a molecular marker are available. Several reports have indicated that application of the 21-gene assay or the 70-gene assay results in a change in therapeutic recommendations in approximately 20% to 25% of patients, usually in the direction of treatment sparing.[89–91]

Although these principles currently apply primarily to the use of multiparameter markers for providing a recommendation for adjuvant chemotherapy recommendation, particularly in ER-positive disease, these same principles could be applied to in other scenarios. For example, specific signatures have been identified for organ-specific recurrence, including recurrence in bones,[92] lungs,[93] and brain[94] These signatures may be useful for selecting individuals most likely to benefit from organ-specific therapies, such as bisphosphonates to prevent bone metastases,[95] or for specific therapies more likely to penetrate the blood-brain barrier.[96] Other potential

applications include identification of cancers that may need no additional local or systemic therapy after diagnostic biopsy, locally therapeutic excision, or ablation.[97]

REFERENCES

1. Hede K. Superhighway or blind alley? The cancer genome atlas releases first results. J Natl Cancer Inst 2008;100:1566.
2. Wood LD, Parsons DW, Jones S, et al. The genomic landscapes of human breast and colorectal cancers. Science 2007;318:1108.
3. Stephens PJ, McBride DJ, Lin ML, et al. Complex landscapes of somatic rearrangement in human breast cancer genomes. Nature 2009;462:1005.
4. Joensuu H, Roberts PJ, Sarlomo-Rikala M, et al. Effect of the tyrosine kinase inhibitor STI571 in a patient with a metastatic gastrointestinal stromal tumor. N Engl J Med 2001;344:1052.
5. Hatchwell E, Greally JM. The potential role of epigenomic dysregulation in complex human disease. Trends Genet 2007;23:588.
6. Thompson RF, Suzuki M, Lau KW, et al. A pipeline for the quantitative analysis of CG dinucleotide methylation using mass spectrometry. Bioinformatics 2009;25: 2164.
7. Iorio MV, Casalini P, Tagliabue E, et al. MicroRNA profiling as a tool to understand prognosis, therapy response and resistance in breast cancer. Eur J Cancer 2008; 44:2753.
8. Gast MC, Schellens JH, Beijnen JH. Clinical proteomics in breast cancer: a review. Breast Cancer Res Treat 2009;116:17.
9. Figueroa ME, Reimers M, Thompson RF, et al. An integrative genomic and epigenomic approach for the study of transcriptional regulation. PLoS One 2008;3: e1882.
10. Hanash S. Integrated global profiling of cancer. Nat Rev Cancer 2004;4:638.
11. Simon R. Diagnostic and prognostic prediction using gene expression profiles in high-dimensional microarray data. Br J Cancer 2003;89:1599.
12. Perou CM, Sorlie T, Eisen MB, et al. Molecular portraits of human breast tumours. Nature 2000;406:747.
13. Freidlin B, Jiang W, Simon R. The cross-validated adaptive signature design. Clin Cancer Res 2010;16:691–8.
14. Harris L, Fritsche H, Mennel R, et al. American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. J Clin Oncol 2007;25:5287.
15. Hayes DF, Bast RC, Desch CE, et al. Tumor marker utility grading system: a framework to evaluate clinical utility of tumor markers. J Natl Cancer Inst 1996;88:1456.
16. Dobbin K, Simon R. Sample size determination in microarray experiments for class comparison and prognostic classification. Biostatistics 2005;6:27.
17. Dobbin KK, Simon RM. Sample size planning for developing classifiers using high dimensional DNA microarray data. Biostatistics 2007;8:101–7.
18. Ransohoff DF. Rules of evidence for cancer molecular-marker discovery and validation. Nat Rev Cancer 2004;4:309.
19. Simon R, Radmacher MD, Dobbin K, et al. Pitfalls in the use of DNA microarray data for diagnostic and prognostic classification. J Natl Cancer Inst 2003;95:14.
20. Goldhirsch A, Ingle JN, Gelber RD, et al. Thresholds for therapies: highlights of the St Gallen International Expert Consensus on the primary therapy of early breast cancer 2009. Ann Oncol 2009;20:1319.

21. Sargent DJ, Conley BA, Allegra C, et al. Clinical trial designs for predictive marker validation in cancer treatment trials. J Clin Oncol 2005;23:2020.
22. Popovici V, Chen W, Gallas BG, et al. Effect of training sample size and classification difficulty on the accuracy of genomic predictors. Breast Cancer Res 2010; 112:R5.
23. Hess KR, Anderson K, Symmans WF, et al. Pharmacogenomic predictor of sensitivity to preoperative chemotherapy with paclitaxel and fluorouracil, doxorubicin, and cyclophosphamide in breast cancer. J Clin Oncol 2006;24:4236.
24. Shak S, Baehner FL, Palmer G, et al. Relationship between proliferation genes and expression of hormone and growth factor receptors: quantitative RT-PCR in 10,618 breast cancers [abstract 6111]. Breast Cancer Res Treat 2006;100(Suppl 1).
25. Quackenbush J. Microarray analysis and tumor classification. N Engl J Med 2006;354:2463.
26. Sotiriou C, Pusztai L. Gene-expression signatures in breast cancer. N Engl J Med 2009;360:790.
27. Levin JZ, Berger MF, Adiconis X, et al. Targeted next-generation sequencing of a cancer transcriptome enhances detection of sequence variants and novel fusion transcripts. Genome Biol 2009;10:R115.
28. Maher CA, Palanisamy N, Brenner JC, et al. Chimeric transcript discovery by paired-end transcriptome sequencing. Proc Natl Acad Sci U S A 2009;106: 12353.
29. Canales RD, Luo Y, Willey JC, et al. Evaluation of DNA microarray results with quantitative gene expression platforms. Nat Biotechnol 2006;24:1115.
30. Guo L, Lobenhofer EK, Wang C, et al. Rat toxicogenomic study reveals analytical consistency across microarray platforms. Nat Biotechnol 2006;24:1162.
31. Patterson TA, Lobenhofer EK, Fulmer-Smentek SB, et al. Performance comparison of one-color and two-color platforms within the MicroArray Quality Control (MAQC) project. Nat Biotechnol 2006;24:1140.
32. Shi L, Reid LH, Jones WD, et al. The microarray quality control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. Nat Biotechnol 2006;24:1151.
33. Shippy R, Fulmer-Smentek S, Jensen RV, et al. Using RNA sample titrations to assess microarray platform performance and normalization techniques. Nat Biotechnol 2006;24:1123.
34. Tong W, Lucas AB, Shippy R, et al. Evaluation of external RNA controls for the assessment of microarray performance. Nat Biotechnol 2006;24:1132.
35. Asyali MH, Colak D, Demirkaya O, et al. Gene expression profile cllassification: a review. Curr Bioinform 2006;1:55.
36. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Ser A 1995;B57:289.
37. Storey JD, Tibshirani R. Statistical significance for genomewide studies. Proc Natl Acad Sci U S A 2003;100:9440.
38. Pepe MS, Janes H, Longton G, et al. Limitations of the odds ratio in gauging the performance of a diagnostic, prognostic, or screening marker. Am J Epidemiol 2004;159:882.
39. Molinaro AM, Simon R, Pfeiffer RM. Prediction error estimation: a comparison of resampling methods. Bioinformatics 2005;21:3301.
40. Ingenuity® Systems, Redwood City, CA, USA. Available at: http://www.ingenuity.com. Accessed March 27, 2010.
41. Fan C, Oh DS, Wessels L, et al. Concordance among gene-expression-based predictors for breast cancer. N Engl J Med 2006;355:560.

42. Desmedt C, Haibe-Kains B, Wirapati P, et al. Biological processes associated with breast cancer clinical outcome depend on the molecular subtypes. Clin Cancer Res 2008;14:5158.
43. Marshall E. Getting the noise out of gene arrays. Science 2004;306:630.
44. Dupuy A, Simon RM. Critical review of published microarray studies for cancer outcome and guidelines on statistical analysis and reporting. J Natl Cancer Inst 2007;99:147–57.
45. Ball CA, Sherlock G, Parkinson H, et al. Standards for microarray data. Science 2002;298:539.
46. Medicare, Medicaid and CLIA programs; regulations implementing the Clinical Laboratory Improvement Amendments of 1988 (CLIA)–HCFA. Final rule with comment period. Fed Regist 1992;57:7002.
47. Buyse M, Loi S, van't Veer L, et al. Validation and clinical utility of a 70-gene prognostic signature for women with node-negative breast cancer. J Natl Cancer Inst 2006;98:1183.
48. van de Vijver MJ, He YD, van't Veer LJ, et al. A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med 2002;347:1999.
49. Foekens JA, Atkins D, Zhang Y, et al. Multicenter validation of a gene expression-based prognostic signature in lymph node-negative primary breast cancer. J Clin Oncol 2006;24:1665.
50. Wang Y, Klijn JG, Zhang Y, et al. Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. Lancet 2005;365:671.
51. Habel LA, Shak S, Jacobs MK, et al. A population-based study of tumor gene expression and risk of breast cancer death among lymph node-negative patients. Breast Cancer Res 2006;8:R25.
52. Paik S, Shak S, Tang G, et al. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. N Engl J Med 2004;351:2817.
53. Jerevall PL, Brommesson S, Strand C, et al. Exploring the two-gene ratio in breast cancer–independent roles for HOXB13 and IL17BR in prediction of clinical outcome. Breast Cancer Res Treat 2008;107:225.
54. Ma XJ, Hilsenbeck SG, Wang W, et al. The HOXB13:IL17BR expression index is a prognostic factor in early-stage breast cancer. J Clin Oncol 2006;24:4611.
55. Ma XJ, Salunga R, Dahiya S, et al. A five-gene molecular grade index and HOXB13:IL17BR are complementary prognostic factors in early stage breast cancer. Clin Cancer Res 2008;14:2601.
56. Ma XJ, Wang Z, Ryan PD, et al. A two-gene expression ratio predicts clinical outcome in breast cancer patients treated with tamoxifen. Cancer Cell 2004;5:607.
57. Paik S, Tang G, Shak S, et al. Gene expression and benefit of chemotherapy in women with node-negative, estrogen receptor-positive breast cancer. J Clin Oncol 2006;24:3726.
58. Goldstein LJ, Gray R, Badve S, et al. Prognostic utility of the 21-gene assay in hormone receptor-positive operable breast cancer compared with classical clinicopathologic features. J Clin Oncol 2008;26:4063.
59. Albain KS, Barlow WE, Shak S, et al. Prognostic and predictive value of the 21-gene recurrence score assay in postmenopausal women with node-positive, oestrogen-receptor-positive breast cancer on chemotherapy: a retrospective analysis of a randomised trial. Lancet Oncol 2010;11:55–65.
60. Dowsett M, Cuzick J, Wales C, et al. Prediction of risk of distant recurrence using the 21-gene recurrence score in node-negative and node-positive postmenopausal patients with breast cancer treated with anastrozole or tamoxifen: a TransATAC study. J Clin Oncol 2010;28:1829–34.

61. Goetz MP, Suman VJ, Ingle JN, et al. A two-gene expression ratio of homeobox 13 and interleukin-17B receptor for prediction of recurrence and survival in women receiving adjuvant tamoxifen. Clin Cancer Res 2006;12:2080.
62. Jansen MP, Sieuwerts AM, Look MP, et al. HOXB13-to-IL17BR expression ratio is related with tumor aggressiveness and response to tamoxifen of recurrent breast cancer: a retrospective study. J Clin Oncol 2007;25:662.
63. van't Veer LJ, Dai H, van de Vijver MJ, et al. Gene expression profiling predicts clinical outcome of breast cancer. Nature 2002;415:530.
64. Sotiriou C, Wirapati P, Loi S, et al. Gene expression profiling in breast cancer: understanding the molecular basis of histologic grade to improve prognosis. J Natl Cancer Inst 2006;98:262.
65. Cobleigh MA, Tabesh B, Bitterman P, et al. Tumor gene expression and prognosis in breast cancer patients with 10 or more positive lymph nodes. Clin Cancer Res 2005;11:8623.
66. Cronin M, Sangli C, Liu ML, et al. Analytical validation of the oncotype DX genomic diagnostic test for recurrence prognosis and therapeutic response prediction in node-negative, estrogen receptor-positive breast cancer. Clin Chem 2007;53:1084.
67. Fisher B, Costantino J, Redmond C, et al. A randomized clinical trial evaluating tamoxifen in the treatment of patients with node-negative breast cancer who have estrogen-receptor-positive tumors. N Engl J Med 1989;320:479.
68. Fisher B, Jeong JH, Bryant J, et al. Treatment of lymph-node-negative, oestrogen-receptor-positive breast cancer: long-term findings from National Surgical Adjuvant Breast and Bowel Project randomised clinical trials. Lancet 2004;364:858.
69. Fisher B, Jeong JH, Dignam J, et al. Findings from recent National Surgical Adjuvant Breast and Bowel Project adjuvant studies in stage I breast cancer. J Natl Cancer Inst Monogr 2001;30:62–6.
70. Parker JS, Mullins M, Cheang MC, et al. Supervised risk predictor of breast cancer based on intrinsic subtypes. J Clin Oncol 2009;27:1160.
71. Balslev I, Axelsson CK, Zedeler K, et al. The Nottingham prognostic index applied to 9,149 patients from the studies of the Danish Breast Cancer Cooperative Group (DBCG). Breast Cancer Res Treat 1994;32:281.
72. Olivotto IA, Bajdik CD, Ravdin PM, et al. Population-based validation of the prognostic model ADJUVANT! for early breast cancer. J Clin Oncol 2005;23:2716.
73. Bryant J. Oncotype DX correlates more closely with prognosis than adjuvant online. 9th International Conference: Primary therapy of early breast cancer. St Gallen (Switzerland), January 25–28, 2005.
74. Sorlie T, Perou CM, Tibshirani R, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 2001;98:10869.
75. Sorlie T, Tibshirani R, Parker J, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci U S A 2003;100:8418.
76. Chang HY, Nuyten DS, Sneddon JB, et al. Robustness, scalability, and integration of a wound-response gene expression signature in predicting breast cancer survival. Proc Natl Acad Sci U S A 2005;102:3738.
77. Liu R, Wang X, Chen GY, et al. The prognostic role of a gene signature from tumorigenic breast-cancer cells. N Engl J Med 2007;356:217.
78. Cheang MC, Voduc D, Bajdik C, et al. Basal-like breast cancer defined by five biomarkers has superior prognostic value than triple-negative phenotype. Clin Cancer Res 2008;14:1368.

79. Troester MA, Lee MH, Carter M, et al. Activation of host wound responses in breast cancer microenvironment. Clin Cancer Res 2009;15:7020.
80. Nuyten DS, Kreike B, Hart AA, et al. Predicting a local recurrence after breast-conserving therapy by gene expression profiling. Breast Cancer Res 2006;8:R62.
81. Sparano JA. TAILORx: trial assigning individualized options for treatment (Rx). Clin Breast Cancer 2006;7:347.
82. Chang JC, Wooten EC, Tsimelzon A, et al. Gene expression profiling for the prediction of therapeutic response to docetaxel in patients with breast cancer. Lancet 2003;362:362.
83. Cleator S, Tsimelzon A, Ashworth A, et al. Gene expression patterns for doxorubicin (adriamycin) and cyclophosphamide (cytoxan) (AC) response and resistance. Breast Cancer Res Treat 2006;95:229.
84. Gianni L, Zambetti M, Clark K, et al. Gene expression profiles in paraffin-embedded core biopsy tissue predict response to chemotherapy in women with locally advanced breast cancer. J Clin Oncol 2005;23:7265.
85. Recommendations from the EGAPP Working Group: can tumor gene expression profiling improve outcomes in patients with breast cancer? Genet Med 2009;11:66.
86. Marchionni L, Wilson RF, Wolff AC, et al. Systematic review: gene expression profiling assays in early-stage breast cancer. Ann Intern Med 2008;148:358.
87. Carlson RW, Allred DC, Anderson BO, et al. Breast cancer. Clinical practice guidelines in oncology. J Natl Compr Canc Netw 2009;7:122.
88. Simes RJ, Coates AS. Patient preferences for adjuvant chemotherapy of early breast cancer: how much benefit is needed? J Natl Cancer Inst Monogr 2001;30:146–52.
89. Bueno-de-Mesquita JM, van Harten WH, Retel VP, et al. Use of 70-gene signature to predict prognosis of patients with node-negative breast cancer: a prospective community-based feasibility study (RASTER). Lancet Oncol 2007;8:1079.
90. Lo SS, Mumby PB, Norton J. Prospective multicenter study of the impact of the 21-gene recurrence score assay on medical oncologist and patient adjuvant breast cancer treatment selection. Biostatistics 2010;28:1671–6.
91. Sparano JA, Solin LJ. Defining the clinical utility of gene expression assays in breast cancer: the intersection of science and art in clinical decision making. J Clin Oncol 2010;10:1625–7.
92. Smid M, Wang Y, Klijn JG, et al. Genes associated with breast cancer metastatic to bone. J Clin Oncol 2006;24:2261.
93. Landemaine T, Jackson A, Bellahcene A, et al. A six-gene signature predicting breast cancer lung metastasis. Cancer Res 2008;68:6092.
94. Bos PD, Zhang XH, Nadal C, et al. Genes that mediate breast cancer metastasis to the brain. Nature 2009;459:1005.
95. Gnant M, Mlineritsch B, Schippinger W, et al. Endocrine therapy plus zoledronic acid in premenopausal breast cancer. N Engl J Med 2009;360:679.
96. Geyer CE, Forster J, Lindquist D, et al. Lapatinib plus capecitabine for HER2-positive advanced breast cancer. N Engl J Med 2006;355:2733.
97. Zahl PH, Maehlen J, Welch HG. The natural history of invasive breast cancers detected by screening mammography. Arch Intern Med 2008;168:2311.