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TOPIC HIGHLIGHT

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Biological subtypes of breast cancer: Prognostic and therapeutic implications

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Abstract

Breast cancer is a heterogeneous complex of diseases, a spectrum of many subtypes with distinct biological features that lead to differences in response patterns to various treatment modalities and clinical outcomes. Traditional classification systems regarding biological characteristics may have limitations for patient-tailored treatment strategies. Tumors with similar clinical and pathological presentations may have different behaviors. Analyses of breast cancer with new molecular techniques now hold promise for the development of more accurate tests for the prediction of recurrence. Gene signatures have been developed as predictors of response to therapy and protein gene products that have direct roles in driving the biology and clinical behavior of cancer cells are potential targets for the development of novel therapeutics. The present review summarizes current knowledge in breast cancer molecular biology, focusing on novel prognostic and predictive factors.

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Core tip: Breast cancer is a heterogeneous disease with many subtypes that have different treatment responses and clinical outcomes. The present review summarizes current knowledge in breast cancer molecular biology, focusing on novel classification, prognostic and predictive factors.

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INTRODUCTION

Breast cancer is a heterogeneous complex of diseases, a spectrum of many subtypes with distinct biological features that lead to differences in response patterns to various treatment modalities and clinical outcomes. Traditional classification systems regarding biological characteristics, such as tumor size, lymph node involvement, histological grade, patient's age, estrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor receptor 2 (HER2 or c-erbB2) status, may have limitations for patient-tailored treatment strategies. Furthermore, the histological appearance of the tumors may not be sufficient to establish the underlying complex genetic alterations and the biological events involved in cancer development and progression. Tumors with similar clinical and pathological presentations may have different behaviors. Therefore, recent studies have

focused on defining more detailed biological characteristics to improve patient risk stratification and to ensure the highest chance of benefit and the least toxicity from a specific treatment modality. Global gene expression profiling (GEP) studies have provided evidence for classifying breast cancer into distinct biological classes associated with patient survival, based on gene expression patterns[1,2].

Population based screening programs have resulted in a significant shift to early stage disease and increased the interest in studying biological prognostic and predictive factors[3]. Novel molecular studies have opened a broad field in cancer research that allows basic and translational researchers to look for new potential targets. Analyses of breast cancer with new molecular techniques now hold promise for the development of more accurate tests for the prediction of recurrence. Gene signatures have been developed as predictors of response to therapy and protein gene products that have direct roles in driving the biology and clinical behavior of cancer cells are potential targets for the development of novel therapeutics[4]. The present review summarizes current knowledge in breast cancer molecular biology, focusing on novel classification, prognostic and predictive factors.

IDENTIFICATION OF BREAST CANCER SUBTYPES BY GEP STUDIES

Gene expression microarray studies have identified distinct molecular tumor classes based on simultaneous expression analyses of thousands of genes in a single experiment. Perou *et al*[5] first analyzed gene expression patterns in grossly dissected normal or malignant human breast tissues in 65 tumor samples from 42 individuals with locally advanced breast cancer treated with neoadjuvant doxorubicin, using complementary microarrays representing 8102 human genes. The authors selected 496 genes based on the criteria of significantly greater variation in expression between different tumors and minimum variation between paired samples from the same patient and these genes were termed the intrinsic gene subset. Samples and genes were aggregated according to the similarity to each other (unsupervised clustering). Subset cluster analysis revealed a dendogram with two main branches that were clinically described as *ER-positive* and *ER-negative*. The tumors in the *ER-positive* group were characterized by the relatively high expression of many genes expressed by breast luminal cells (ER-responsive genes, luminal cytokeratins and other luminal associated markers), so they were termed the *luminal* group. The *ER-negative* group was further divided into *basal-like*, *ErbB2-positive* and *normal-like* subclasses. *Basal-like* tumors expressed many of the characteristics of breast basal epithelial cells that did not express ER and showed staining with basal keratins. Another cluster of tumors was characterized by the expression of high levels of HER2 oncogene, which also showed low levels of ER expression and other genes associated with ER expression. Eventu-

ally, the authors identified four groups of samples using the intrinsic gene set that might be related to different molecular features of mammary epithelial biology and they named them *ER-positive luminal-like*, *basal-like*, *ErbB2-positive* and *normal-like*. These results were confirmed in follow up experiments using larger numbers of cases[6].

Subsequent studies revealed that similar molecular subtypes of breast cancer could be identified in multiple cohorts of breast cancers and that *luminal* cancers could be subclassified into 2 or 3 groups and different molecular subtypes were shown to have distinct clinical outcomes. Sørlie *et al*[7] investigated the clinical relevance of gene expression profiles in 78 breast carcinoma patients. Of these patients, 51 were part of a prospective study with locally advanced (T3-T4 and/or N2) tumors and had received doxorubicin based chemotherapy before the surgery. The authors showed a highly significant difference in overall survival between the subtypes. Both the *basal-like* and *ErbB2-positive* subtypes were associated with the shortest survival times. The authors subclassified the *luminal-like* breast cancer into three subclasses comprising *luminal-A*, *luminal-B* and *luminal-C* and identified *luminal-A* subgroup of *ER-positive* tumors as being associated with the best outcome. Van't Veer *et al*[8] also investigated node-negative breast cancer patients and found 231 genes significantly associated with disease outcome, as defined by the presence of distant metastasis at the 5th year. These data revealed that each breast tumor has its own unique molecular portrait, providing the basis for an improved molecular taxonomy of the disease.

SUBCLASSIFICATION OF LUMINAL LIKE BREAST CANCER

Approximately 75% of breast cancers are positive for ER and/or PR. The *ER-positive* tumors express ER, PR, ER responsive genes and other genes that encode typical proteins of luminal epithelial cells so they are termed the *luminal* group. Characterization of *luminal-like* breast cancer varied between various studies, probably due to the identification and use of distinct intrinsic gene sets for cluster analysis. Hu *et al*[9] evaluated an intrinsic gene set derived from three independent studies (Sørlie *et al*[7], 2001; Van't Veer *et al*[8], 2002; Sotiriou *et al*[10], 2003), joined them together into a combined data set and identified two main *luminal-like* subclasses corresponding to *luminal-A* and *luminal-B*. Most subsequent studies have supported the concept of two *luminal-like* subclasses[10-12].

Luminal-A
The *luminal-A* is the most common subtype and represents 50%-60% of all breast cancers. These tumors frequently have low histological grade, low degree of nuclear pleomorphism, low mitotic activity and include special histological types (*i.e.*, tubular, invasive cribriform, mucinous and lobular) with good prognosis. *Luminal-A* is characterized by higher levels of ER and lower levels of proliferation related genes. It is characterized by the

expression of luminal epithelial cytokeratins (CK) 8 and 18, other luminal associated markers including ER1, genes associated with ER function such as *LIV1 (zinc transporter ZIP6 or SLC39A6; solute carrier family 39 zinc transporter, member 6), hepatocyte nuclear factor 3 alpha (FOXA1), X-box binding protein 1 (XBP1), GATA binding protein 3 (GATA3), B cell lymphoma 2 (BCL2), erbB3* and *erbB4*[13]. *Luminal-A* subtype is defined as ER-positive and/or PR-positive tumors with negative HER2 and low Ki67 (proliferating cell nuclear antigen) index by immunohistochemistry[14].

Patients with *luminal-A* breast cancer have a good prognosis; the relapse rate is significantly lower than the other subtypes. Recurrence is common in bone, whereas liver, lung and central nervous system metastases occur in less than 10% of patients and treatment is mainly based on hormonal therapy[15,16].

Luminal-B

Luminal-B tumors comprise 15%-20% of breast cancers and have a more aggressive phenotype, higher histological grade, proliferative index and a worse prognosis[17]. This subtype has a higher recurrence rate and lower survival rates after relapse compared to *luminal-A* subtype[18].

The main difference between both *luminal* subgroups is increased expression of proliferation-related genes such as *avian myeloblastosis viral oncogene homolog (v-MYB), gamma glutamyl hydrolase (GGH), lysosome-associated transmembrane protein 4-beta (LAPTMB4), nuclease sensitive element binding protein 1 (NSEP1)* and *cyclin E1 (CCNE1)* in *luminal-B* breast cancers. *Luminal-B* tumors also demonstrate increased expression of growth receptor signaling genes[19]. Approximately 30% of *HER2-positive* tumors defined by immunohistochemistry are assigned to the *luminal-B* subtype[20].

It should be noted that expression levels of proliferation related genes in ER-positive disease form a continuum; therefore, the cutoffs to define *luminal-A* and *luminal-B* cancers are set in an arbitrary manner rather than emerging from a bimodal distribution of these genes' expression levels[21]. Various studies conducted to differentiate *luminal-A* and *luminal-B* subtypes defined more pragmatic criteria that can be broadly applied to clinical practice. The Ki67 index is suggested as a potential proliferation marker that could successfully differentiate *luminal-B* tumors from *luminal-A* in clinical practice. Cheang *et al*[22] studied 357 breast cancer subtypes by using microarray based gene expression profiling and the Ki67 hormone receptor and HER2 status by immunohistochemistry. The authors determined the Ki67 cut off point (14%) that distinguishes *luminal-A* from *luminal-B* tumors, then applied it to an independent microarray series of 4046 breast cancers and concluded that the two subtypes could be distinguished by the Ki67 index. However, Ki67 immunohistochemistry has known limitations, such as low intra- and inter-laboratory reproducibility, arbitrary selection of optimal antibodies for testing and different methods of cell counting (manual

vs automated) in addition to potential problems resulting from tumor heterogeneity[23]. There is also an urgent need to standardize the Ki67 expression analysis and validate its clinical utility.

From the immunohistochemical point of view, *luminal-B* subtype is defined as ER-positive, HER2-negative and Ki 67 high or ER and HER-2 positive tumors, but this definition does not include all *luminal-B* tumors as up to 6% of them are negative for both ER and HER2. Moreover, the Ki67 cut off point to distinguish *luminal-A* and *-B* has not been standardized[24].

Overall survival in untreated *luminal-B* breast cancers is similar to the *basal-like* and *HER2-positive* subtypes which are widely recognized as high-risk tumors[9]. *Luminal-B* tumors have poorer outcomes with hormonotherapy. Several studies have suggested that *luminal-B* breast cancer was relatively insensitive to endocrine therapy compared to *luminal-A* breast cancer and to paclitaxel- and doxorubicin-containing preoperative chemotherapy compared with *HER2-positive* and *basal-like* breast cancers. However, *luminal-B* breast cancer responds better to neo-adjuvant chemotherapy than luminal-A subtype, achieving higher pathological complete response rates[25-29]. Increased relapse rates observed in *luminal-B* tumors are limited to the first 5 years after diagnosis[30].

Recent evidence suggests that certain alternative growth factor pathways, such as *fibroblast growth factor receptor 1 (FGFR1), HER1, phosphoinositide 3 kinase (PI3K) catalytic alpha polypeptide*, and *sarcoma proto-oncogene (Src),* may contribute to the higher proliferation and poorer prognosis of *luminal-B* breast cancer and related therapeutic agents are in active clinical development[31].

In breast cancer, changes to fibroblast growth factor signaling are considered important for oncogenesis, mainly through amplification of *FGFR1* and *FGFR2. FGFR1* is amplified in 10% of all breast cancers. Recent data suggest that the *luminal-B* subtype is enriched for *FGFR1* gene amplification[32]. Studies suggested that *FGFR1* gene amplification might be a contributor to the poor prognosis observed in *luminal-B* breast cancer through increased proliferation and resistance to endocrine therapy. Several antibodies and small molecule inhibitors of *FGFR* are currently under clinical study processes.

In breast cancer, the PI3K pathway is frequently activated. Amplification of upstream receptors such as HER2, loss of negative regulators such as PTEN, amplification of downstream targets such as protein kinase B (PKB or Akt) and activating mutations or genetic amplification of the alpha catalytic subunit of PI3K have all been described in breast cancer. Targeting the PI3K pathway appears promising, although more extensive studies are required[33].

HER2-positive

Human epidermal growth factor receptor-2 is a member of the family of four membrane tyrosine kinases. The HER2 receptor is encoded by the HER2 gene, which is a proto-oncogene mapped in chromosome 17q21. Upon

ligand binding to their extracellular domains, HER proteins undergo dimerization and transphosphorylation of their extracellular domains. HER2 does not have a ligand and relies on heterodimerization with another family member or homodimerization with itself to be activated when expressed at very high levels. These phosphorylated tyrosine residues interact with numerous intracellular signaling molecules, leading to activation of downstream second messenger pathways and crosstalk with other membrane signaling pathways. Transcription factors activated by the pathway regulate many genes involved in cell proliferation, survival, differentiation, angiogenesis, invasion and metastasis[34-37].

HER2-positive cancer accounts for 15-20% of breast cancer subtypes. HER2 positivity confers more aggressive biological and clinical behavior. These tumors are characterized by high expression of the HER2 gene and other genes associated with the HER2 pathway and/or HER2 amplicon located in the 17q12 chromosome. Morphologically, these tumors are highly proliferative, 75% have a high histological and nuclear grade and more than 40% have p53 mutations[38]. Nearly half of *HER2-positive* breast cancers are positive for ER but they generally express lower ER levels.

The immunohistochemical profile of *ER-negative* and *HER2-positive* does not correspond perfectly with the intrinsic subtype since only 70% of HER2 tumors by microarray have the protein overexpressed on immunohistochemistry. Conversely, all tumors with HER2 amplification or overexpression are not included in the HER2 cluster by microarray analysis[39,40].

Staaf *et al*[41] identified three separate subtypes of *HER2-positive* tumors, one with a clearly poor prognosis with a 12% 10 year survival compared to the 50-55% survival in the other two groups using HER2 derived prognostic predictor (HDPP) gene analysis. The HDPP was not directly related to the expression of proliferation gene and HER2 pathway but was mostly associated with genes related to immune response to tumor invasion and metastasis.

In the absence of treatment, *HER2-positive* tumors have a poor prognosis. They have increased sensitivity to certain cytotoxic agents such as doxorubicin, relative resistance to hormonal agents and a propensity to metastasize to the brain and visceral organs. Doxorubicin sensitivity is possibly due to coamplification of the topoisomerase-2 gene which is near the HER2 locus on chromosome 17 and is the target of this drug[42,43]. Advances in translational science have led to the development of a large spectrum of HER directed therapies.

Basal-like

The *basal-like* subtype represents from 8% to 37% of all breast cancers, depending on the proportion of poorly differentiated G3 cases included in the population studied[44]. *Basal-like* cancers are associated with high histological and nuclear grade, poor tubule formation and the presence of central necrotic or fibrotic zones,

pushing borders, conspicuous lymphocytic infiltrate and medullary features with exceptionally high mitotic and proliferative indices. Most of these tumors are infiltrating ductal tumors with solid growth pattern, aggressive clinical behavior and high rate of metastasis to the brain and lung[45].

Tumors belonging to the *basal-like* subgroup express high levels of basal myoepithelial markers, such as CK5, CK 14, CK 17 and laminin, and do not express ER, PR and HER2, hence referred to as *triple-negative*. They also overexpress P-cadherin, fascin, caveolins 1 and 2, alpha-beta crystallin and epidermal growth factor receptor (EGFR). *Basal-like* cancers present with frequent mutations in the *tumor protein 53 (TP53)* gene, evidence of genomic instability and inactivation of the *retinoblastoma (Rb)* pathway. Deregulated integrin expression has also been detected and may contribute to aggressive cell behaviors and progression in this subtype[45].

It is important to clarify that the terms *triple-negative* and *basal-like* are not completely synonymous and there is approximately 20%-30% discordance across studies. The term *triple-negative* refers to the immunohistochemical classification of breast tumors lacking ER, PR and HER2 protein expression, whereas the *basal-like* subtype is defined *via* gene expression microarray analysis. The *basal-like* classification is available only in the research setting to date and thus the *triple-negative* phenotype currently is a reliable surrogate in the clinical setting[46].

There are several reported biomarkers associated with the *basal-like* group as well as putative candidates suitable for immunohistochemical screening. However, currently there is no specific international consensus on complementary biomarkers that can define *basal-like* cancers[47].

Several genes related to the *basal-like* subtype have been implicated in promoting cellular proliferation, cell survival, cell migration and invasion. Despite the wide diversity of the involved pathways, signaling molecules, such as the mitogen activated protein kinase (MAPK), PI3K, Akt and nuclear factor kappa B (NF-κB), are commonly deregulated as seen in other breast cancer subtypes. Other alterations such as cytoplasmic and nuclear accumulation of beta catenin were also observed in *basal-like* cancers, being the marker suggested as a potential therapeutic target for this cancer[48].

Microarray and immunohistochemical analyses demonstrated that basal-like subtype constitute approximately three quarters of *breast cancer 1 (BRCA1)* gene related breast cancers. This gene, often termed the *caretaker of the genome*, is located on chromosome 17 and is related with both inherent DNA damage sensing processes and DNA repair mechanisms. Breast cancers related to BRCA1 often express *triple-negative* phenotype and are frequently positive for Ki67 basal cytokeratins, TP53, EGFR and P cadherin and X chromosome abnormalities. Outcomes for women with *basal-like* tumors and BRCA1 related breast cancers are similar, in particular for early relapse and pattern of metastatic disease[49]. *Basal-like* cancers with deficient BRCA1 pathway may respond to specific

Table 1 2013 St. Gallen - intrinsic subtypes of breast cancer

Intrinsic subtype	Clinicopathological surrogate definition	
Luminal-A	"Luminal-A-like" all of: ER and PgR positive HER2 negative Ki-67 "low"a Recurrence risk "low" based on multi-gene-expression assay (if available) b	a A level of < 14% best correlated with the gene-expression definition of Luminal A based on the results in a single reference laboratoryb PgR cut-point of ≥ 20% to best correspond to Luminal A subtype
Luminal-B	Luminal-B-like (HER 2 positive) ER positive HER2 negative and at least one of: Ki-67 "high" PgR "negative or low" Recurrence risk "high" based on multi- gene-expression assay (if available) Luminal-B (HER 2 negative) ER positive HER2 over-expressed or amplified Any Ki-67 Any PgR	
Erb-B2 overexpression	HER 2 positive (non-luminal) HER2 over-expressed or amplified ER and PgR absent	
Basal-like	Triple negative (ductal) ER and PgR absent HER2 negative	There is an 80% overlap between "triple-negative" and intrinsic "basal-like" subtype

therapeutic regimens such as poly-ADP ribose polymerase (PARP) enzyme inhibitors. Also, BRCA1 deficient cells have defects in DNA double strand break repair mechanisms that could render them particularly sensitive to therapeutic agents that generate DNA double strand breaks such as PARP enzyme inhibitors[50]. As often overexpressed in *basal-like* cancer, EGFR may also be another potential therapeutic target. Dong *et al*[51] identified notch pathway as one of the mechanisms of resistance to EGFR inhibition in *basal-like* breast cancer as valuable information to overcome this resistance. Dual pathway inhibition may be a viable clinical strategy in *basal-like* cancers.

As one of the *triple-negative* subtypes, *claudin-low* breast cancer was described by Herschkowitz *et al*[52]. This subtype is characterized by low expression of genes involved in tight junctions and cell-cell adhesions including claudins 3, 4 and 7, occludin and E cadherin showing high expression of epithelial to mesenchymal transition genes and stem cell features. Currently, it has been reported that patients with *claudin-low* tumors also have poor clinical outcomes like other *triple-negative* tumors.

Normal breast-like
These tumors account for about 5%-10% of all breast carcinomas. They are poorly characterized and have been grouped into the classification of intrinsic subtypes with fibroadenomas and normal breast samples. They express gene characteristics of adipose tissue presenting an intermediate prognosis between *luminal* and *basal-like* cancers and usually do not respond to neoadjuvant chemotherapy. As they lack the expression of ER, PR and

HER2, these tumors can also be classified as *triple-negative* but they are not considered to be *basal-like* cancers as they are negative for CK5 and EGFR. There are few studies on this subtype and their clinical significance remains undetermined. There are doubts about their existence as a breast cancer subtype and some researchers believe they could be a technical artifact from high contamination with normal tissue during the microarrays[53]. In fact, in a large series of samples where the neoplastic cells were isolated by microdissection, no cases of *normal breast-like* subtype were found, supporting this hypothesis.

The implications of the molecular classification in the therapeutic era have been accepted by international panels. In the 2011 and the latest 2013 St. Gallen International Breast Cancer Conferences, the expert panel members agreed that therapeutic decisions should be made based on the recognition of the intrinsic subtypes of breast cancer. Panel members agreed that the different breast cancer subtypes can be defined only by genetic array testing but approximation to this classification can be made by immunohistochemistry[54,55] (Table 1).

Although molecular taxonomy of breast cancer has attracted great attention, to date, actual practical adaptation seems limited. Certain critical issues have been raised, such as validation, reproducibility and clinical utility. The four main molecular classes frequently reported can be considered an oversimplification of a novel molecular classification system and add little to our understanding of the biology and behavior of breast cancer. Sub classification of the largest *luminal* class remains unresolved. Most *luminal* tumors are hormone receptor positive and can be identified in routine practice using immunohisto-

chemistry. Hormone receptor expression in *luminal* phenotype is recognized as a validated predictor to hormonal treatments. The difference between *basal-like* and *triple-negative* is disputed, with triple negativity in clinical practice providing a more practical and routinely applicable classification. Similarly, strongly *HER2-positive* breast cancer patients by immunohistochemistry are likely to be offered anti-HER2 therapy, especially if their tumors show evidence of HER2 gene amplification, regardless of their molecular classification. Furthermore, the *normal breast-like* class is not well defined and the proportion of some classes defined by GEP varies substantially. Finally, the contribution of this molecular taxonomy to current clinical practice is just the modification of treatment protocols related to ER, PR, HER2 and Ki67 status of breast cancer. Molecular classification based on combination of the classical well-defined immunohistochemical markers can be considered a simpler and more practical approach and it is expected to remain as such unless novel target molecules driving individual classes are identified.

BIG 1-98 is a randomized, phase Ⅲ study that compared five years of tamoxifen or letrozole and their sequences in post-menopausal women with ER positive early breast cancer. Metzger *et al*[56] updated benefit of endocrine treatment among *Luminal* subgroups in this trial. ER positive subtypes were defined as *Luminal-A* (ER+ and/or PR+ HER2- and Ki67 < 14%) or *Luminal-B* (ER+ and/or PR+, HER2- and Ki67 ≥ 14%). In the invasive ductal carcinoma subset, 1436 (44%) and 1163 (36%) were classified as *Luminal-A* and *Luminal-B*, while in the invasive lobular carcinoma subset, 237 (59%) and 87 (22%) were classified as *Luminal-A* and *Luminal-B*, respectively. In lobular carcinoma patients, disease free survival hazard ratios for letrozole *vs* tamoxifen were 0.51 (95%CI: 0.33 to 0.79) for *Luminal-A* and 0.35 (95%CI: 0.21 to 0.56) for *Luminal-B* subtypes. The disease free survival hazard ratios for letrozole *vs* tamoxifen were 0.93 (95%CI: 0.74 to 1.77) for invasive ductal carcinoma *Luminal-A* and 0.64 (95%CI: 0.52 to 0.78) for invasive ductal carcinoma *Luminal-B*. A greater reduction in risk of a disease free survival event was shown in women with Luminal B for both invasive ductal carcinoma and invasive lobular carcinoma[56].

Currently, the available molecular tests have offered the opportunity to challenge the molecular complexity of breast cancer but do not provide sufficiently robust information to modify established treatment schemes. These tests require validation in large series and comparison with traditional classification systems in the context of comprehensive clinical trials.

CLINICAL GENE EXPRESSION BASED ASSAYS

Although up to 70% of patients with early breast cancer currently receive adjuvant chemotherapy, only a specific subgroup of these patients derive benefit from this treatment. Therefore, in parallel with the advances in the molecular sub classification of breast cancer, several multigene predictors of outcome have been developed (Table 2). It was conceived that microarray based gene signatures were able to identify a subgroup of patients sufficiently with a good prognosis that would not be treated with adjuvant chemotherapy. Currently, many classifiers have been generated by using various technologies such as cDNA and oligonucleotide arrays and multiplex polymerase chain reaction (PCR) analysis. These genomic tests assess expression of different but sometimes overlapping sets of genes. Despite differences in candidate genes in each of the assays, most of them can quite reliably predict the biology of tested tumors. In fact, when some of these tests were compared with each other, they were found to be quite similar in their abilities to predict metastases-free and overall survivals. Five different prognostic signatures were shown to have a high correlation, even among tests utilizing expression of very few genes in common. One important finding from analyses of various genomic tests is the fact that they assign almost all patients with hormone receptor negative disease as high risk. Therefore, most of these tests are more applicable to patients with ER-positive cancers who constitute a more heterogeneous group for prognosis and probability of response to chemotherapy. Given this distinction, the utility of these tests in practice will still depend on clinical and histological assessments to identify specific patients who would then be appropriate for additional testing with gene expression signatures.

PAM 50
PAM 50 is a 50 gene expression assay based on microarray and quantitative real time (qRT)-PCR that was developed by analyzing 189 breast tumor samples to separate them into four molecular breast cancer subtypes (*luminal-A, luminal-B, HER2-positive* and *basal-like*)[57].

PAM 50 assay can provide a risk of relapse score that predicts relapse-free survival for node-negative breast cancer patients who had not received adjuvant systemic therapy. The validation study revealed that patients with *luminal-A* subtype had better prognosis in contrast to the other types and were less responsive to chemotherapy[58].

The most well described, albeit investigational, classifier for the intrinsic subtypes that can be performed on the fixed tissue available in most pathology laboratories is the PAM 50 assay; however, this assay requires further validation for routine clinical practice[59].

MammaPrint
MammaPrint is a microarray based gene expression profiling assay that was developed after analyzing data from 78 patients with ER-positive, node-negative breast cancer patients who had not received adjuvant systemic therapy. Of the 78 patients, 34 developed distant metastasis and 44 were disease free at the 5th year. The tumors' mRNA was extracted for reverse transcription into cDNA, which was tested on microarray containing 25000 human genes. Seventy genes that had the strongest association with

Table 2 First generation gene expression signatures

Gene signature	MammaPrint	OncotypeDX	MapQuantDX	Breast cancer index	PAM 50 assay
Starting material	FF or stabilized RNA, FFPE	FFPE	FFPE, FF	FFPE	FFPE
Analytical platform	Microarray, RT-PCR	qRT-PCR	Microarray, qRT-PCR	qRT-PCR	nCounter
Number of genes	70	21	97/9	7	50
Indications	Stage I / II, 5 cm, ER (+), Node (-)/[1-3 Node (+)]	ER(+), Node (-)	ER (+), G2	ER (+)	All, Node (-) untreated
Application	Clinical outcome	Clinical outcome, benefit from chemotherapy	Molecular grading prediction of response to TMX	Clinical outcome, prediction of response to TMX	Subtype definition, risk of relapse without treatment
FDA approved	Yes	No	No	No	No
ASCO and NCCN recommendation	No	Yes	No	No	No

FF: Fresh frozen; FFPE: Formalin fixed paraffin embedded; G: Grade; TMX: Tamoxifen.

outcome *i.e.*, predicted good and poor risk disease were accurately selected[60]. The genes that comprise the MammaPrint assay are proliferation genes and genes associated with invasion and angiogenesis. This test is based on microarray results and hence requires high quality RNA from freshly collected tissues[61]. The expression of the selected genes defines a prognostic classification of patients as having a good or poor prognosis. This test was approved by Food and Drug Administration (FDA) for lymph node-negative breast cancer patients younger than 61 years of age with tumors smaller than 5 cm in size.

The microarRAy-prognoSTics-in-breast-cancER (RASTER) study is the first study designed to prospectively evaluate the performance of the 70-gene signature. 427 patients with cT1-3N0M0 breast cancer were treated based on the Dutch CBO 2004 guidelines, the 70-gene signature and doctors' and patients' preferences. Five year distant recurrence-free interval probabilities were compared between subgroups based on the 70-gene signature and Adjuvant! Online. Fifteen percent (33/219) of the 70-gene signature low risk patients and 81% (169/208) of the 70-gene signature high risk patients received adjuvant chemotherapy. The 5 year distant recurrence-free interval probabilities for 70-gene signature low risk (n = 219) and high risk (n = 208) patients were 97.0% and 91.7%. The 5 year distant recurrence-free interval probabilities for adjuvant online low risk (n = 132) and high risk (n = 295) patients were 96.7% and 93.4% respectively. For 70-gene signature low risk adjuvant online high risk patients (n = 124), of whom 76% (n = 94) had not received adjuvant chemotherapy, 5 year DRFI was 98.4%. In this prospective community-based observational study, the 5 year distant recurrence-free interval probabilities confirmed the additional prognostic value of the 70-gene signature to clinicopathological risk estimations[62].

MammaPrint has not yet been sufficiently evaluated as a predictive tool. MINDACT (microarray in node negative and 1 to 3 lymph node-positive disease may avoid chemotherapy) is a large prospective randomized trial designed to document when chemotherapy can be omitted if genomic information and conventional clinical risk

assignment system are discordant[63].

Oncotype DX
Oncotype Dx is the most widely used prognostic and predictive clinical 21 gene qRT-PCR based assay for women with hormone receptor positive, node-negative breast cancer[64]. The test is based on qRT-PCR technology that utilizes short and homogeneous amplicons. This method accurately measures gene expression even in the presence of mRNA fragmentation that occurs in archived formalin fixed paraffin embedded tissues. The test is based on 21 selected genes essentially related to proliferation, ER and HER2 signaling and was developed and validated through a retrospective analysis of formalin fixed paraffin embedded materials from three independent clinical trials[65,66]. The gene expression pattern was translated into a quantitative recurrence score used as a continuous variable to estimate the probability of recurrence. Recurrence score divided patients into 3 groups as low, intermediate and high risk categories. The 21 gene signature has been subsequently evaluated in other cohorts of breast cancer patients and was shown to be an independent prognostic parameter in patients with ER-positive tumors with up to 3 positive nodes receiving adjuvant chemotherapy and in postmenopausal patients with ER-positive tumors treated with anastrozole[67].

Multiple retrospective validation studies in various clinical settings established the prognostic and predictive accuracy of Oncotype Dx assay. Examination of the genes of the 21 gene profile by intrinsic subtype suggests that virtually all *luminal-B* tumors would have high recurrence scores, whereas 29% of *luminal-A* tumors would have high recurrence scores due to relative endocrine resistance[68]. A high recurrence score is able to predict poorer outcome among hormone receptor positive tumors despite endocrine therapy and also predicts sensitivity to a variety of adjuvant cytotoxic regimens[69]. For this reason, the recurrence score is thought to predict general chemosensitivity in hormone receptor positive breast cancer and is a reasonable assay for decision making on chemotherapy, particularly in the node-negative popula-

tion.

Oncotype DX is suggested by the American Society of Clinical Oncology and the National Comprehensive Cancer Network for the decision of adjuvant chemotherapy in ER-positive, node-negative breast cancer patients[70,71]. Tailor X is a large prospective randomized trial set to validate OncotypeDx in clinical practice by better defining the intermediate risk stratum[72].

GENOMIC GRADE INDEX

MapquantDx is a predictor test that defines the tumoral histological grade by gene expression features, used to assign a grade index to ER-positive breast cancers in attempt to refine their molecular classification. It was derived by identifying 97 genes from grade 1 and 3 breast tumors. The test was able to classify grade 2 tumors into low and high genomic grades with a statistically significant difference in relapse free survival[73,74]. Most of the genes in this signature are involved in cell cycle regulation and proliferation. Genomic grade index (GGI) was strongly associated with recurrence risk among patients with grade 2 tumors. This assay is microarray based and requires freshly prepared tissues.

BREAST CANCER INDEX

The breast cancer index (BCI) prognostic assay provides an assessment of the likelihood of distant recurrences in patients diagnosed with ER-positive, node-negative breast cancer. This assay has been developed from the combination of two indices: the ratio of HOXB13/IL17BR genes, which predicts distant recurrence in ER-positive patients treated with tamoxifen, and a proliferation related five gene molecular grade index, which discriminates grade 1 from grade 3 disease. The test is based on qRT-PCR using RNA from paraffin embedded tissues[75,76].

The biological roles of the genes included in most of these tests are not completely understood and it is often unclear which clinical or tumor characteristics are being measured. Although proliferation related genes are essential components of most classifiers, there is little overlap and instabilities exist among different gene series.

These prognostic profiles have been far better examined in the node-negative population as estimating the risk according to signature may be more difficult in node-positives. The expression patterns of hormone receptors and Ki67 may especially show differences in the tumor cells at the lymph nodes and the primary lesion, probably due to tumor cell heterogeneity in parallel to increased tumor burden[77].

Although research results indicate that these multi-gene molecular assays can reclassify some breast cancer patients who are ranked as high risk using the traditional classification systems into low risk (*i.e.*, reducing the number of patients who might unnecessarily undergo chemotherapy) and vice versa, available data are insufficient to challenge classical classification systems and to justify withholding chemotherapy for high risk patients if classified as low risk using multigene assays. However, it should be realized that these assays can potentially provide important prognostic information in clinically indeterminate subgroups and, in such situations, combining these tests with conventional predictors may yield valuable information. For instance, high grade but small (10 mm) sized, node-negative breast cancer may be offered systemic therapy if it is classified as high risk using multigene assays as staging information in such cases may be insufficient to reflect the behavior of these early detected tumors.

NEXT GENERATION SEQUENCING

Gene expression profiling and microarray analysis led to new molecular classification systems in breast cancer. In recent years, research has moved from gene expression profiling to a more detailed overview through the biological mechanism of carcinogenesis and tumor progression by mutational profiling. Technological advances such as array comparative genomic hybridization (array-CGH), single nucleotide polymorphism (SNP) and high throughput screening (HTS) are applied to further *in vitro* and *in vivo* research in order to improve knowledge on breast cancer biology and understand the complex process of metastasis[78].

Next generation sequencing is based on deep sequencing which produces billions of short sequences at a time. It is quantitative and can analyze the entire genome at base repair resolution without the limitations of microarrays[79].

Sanger sequencing was the first approach for sequencing the genome but it was both expensive and time consuming. Next generation sequencing (NGS), known as massive parallel sequencing, can be applied to study the whole genome (exons, introns and intergenic regions for about 22000 genes) more specifically to whole exome or to the 200-400 potentially targetable exons. High sensitivity of this technique allows the evaluation of single nucleotide variants, small insertions, deletions, copy number alternations (gain and losses) and structural variations (translocations, inversions). NGS can also be applied to the RNA for expression level analysis and to alternative splicing, RNA editing and fusion transcripts. NGS can be applied to the tumor to identify somatic mutations compared to normal tissues or to the peripheral blood samples to investigate germ line alterations. The study of germ line aberrations may give more information about germ line actionable mutations, toxicity susceptibility, drug metabolism and familial disease susceptibility[80].

Application of NGS has led to the extension of knowledge to produce a comprehensive catalogue of likely genomic drivers of the most common breast cancer subtypes. The Cancer Genome Atlas Network analyzed more than 800 primary breast cancers using all the cutting edge technologies. They demonstrated four main breast

cancer classes, each of which shows significant molecular heterogeneity. They showed that somatic mutations in only three genes (TP 53, PIK3A and GATA 3) occurred at 10% incidence across all breast cancers. There were numerous subtype associated novel gene mutations, including the enrichment of specific mutations in GATA3, PIK3CA and MAP3K1 with the *luminal-A* subtype[81].

Although NGS creates a massive amount of information, each mutation/alteration is not a good candidate to become a target for specific therapeutics. Molecular Taxonomy of Breast Cancer International Consortium (METABRICK) study revealed ten different subtypes, each characterized by common genetic alterations such as PPR2A, MAP2K4 and MTAP deletions that are potentially targetable and linked to survival[82]. Alterations in the gene expression landscape can also be useful to guide treatments with conventional and experimental therapeutics.

Recently, the prospective multicenter molecular screening trial SAFIR 01 (High Throughput Technologies to Drive Breast Cancer Patients to Specific Phase Ⅰ / Ⅱ Trials of Targeted Agents) analyzed 423 patients with metastatic breast cancer. Metastatic sites were biopsied and profiled using the copy number changes array and Sanger sequencing PIK3CA (exon 10/21) and AKT1 (exon 3). A targetable genomic alteration was identified in 204 patients. The most frequent genomic alterations were PIK3CA mutations, CCND1, FGF4 and FGFR1 amplifications. In this study, 46 out of 277 (17%) patients with genomic analyses received a targeted therapy matched to the genomic alteration, covering twelve different targets[83].

The clinical applications of NGS have many difficulties. Searching for every single gene alteration or pathway abnormality is uncertain. There are biological issues due to tumor heterogeneity, clonal evaluation and the difficulty of discriminating between driver and passenger mutations. There are also some technical problems in terms of tumor tissue availability, stromal interference and laboratory reproducibility of the results.

CONCLUSION

One of the main contributions of the breakthrough in cancer research is the integration of molecular studies into clinical trials. Advances in molecular biology of breast cancer over the past decade have led to the classification of the disease from a molecular point of view. Incorporation of multigene molecular classifiers to conventional breast cancer classification systems seems more realistic and practical to support more effective tailoring of therapy. These multigene classifiers can complement traditional methods through provision of additional biological prognostic and predictive information by identifying important, clinically relevant, biological processes better than that determined using morphological factors or individual molecular markers.

New molecular techniques hold promise for improv-

ing diagnosis and sub typing, better assessment of recurrence risk, careful selection of therapy and identification of targets involved in carcinogenesis and function of tumor cells, leading to the discovery of selective drugs. Understanding the pathways regulating the processes involved in neoplastic development helps in the design of clinical trials aimed at patients with specific characteristics that are candidates to benefit from specific treatments. Protein gene products that have direct roles in driving the biology and clinical behavior of cancer cells are potential targets for the development of novel therapeutics. Research efforts have focused on the investigation and identification of new molecular factors, which can improve the predictability of risk of metastasis and the likelihood of response to therapies.

Probably in the near future, the tumoral key mechanisms of regulation will be identified individually and treatments will be more specific and effective, with minimal toxicity. Numerous agents targeting various biological pathways are currently under clinical development to achieve an ideal, personalized medical therapeutic approach in breast cancer.

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