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REVIEW ARTICLE

Biological markers in breast cancer prognosis and treatment

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Summary

In this review we will provide a synopsis of the biological markers used in the care of breast cancer patients with emphasis on clinical application. The advent of molecular technology has incorporated new biomarkers along with the older immunohistochemical and serum ones. Serum tumor markers are proteins shed from breast cancer cells. Their levels have long been used as a measure of tumor burden and disease progression or recurrence. However, limitations exist that should be known to those involved in breast cancer management. Historically, immunohistochemical markers have been used to guide treatment decisions. These markers reveal characteristics of the cancer cells and have been used both as prognostic and predictive factors. Molecular markers give information on the expression of certain genes in tumor tissues related to proliferation, invasion, and metastasis and researchers try to correlate them with the use of mathematical modeling with clinical outcomes, hence those markers exhibit prognostic and predictive significance. All these tools can guide personalized treatment by estimating patient prognosis and risk of relapse and tailor accordingly therapeutic approaches.

Key words: biomarkers, breast cancer, predictive, prognostic

Introduction-Biological markers in breast cancer

Breast cancer is the most common cancer affecting women worldwide and the second cause of cancer death. Mortality rates are decreasing and this has been attributed to better screening and more effective treatment of early disease [1]. Although surgery remains the cornerstone of treatment for early disease, a substantial proportion of women experience relapses which lead to progressive disease and death. Clinicians are relying on the use of prognostic factors that will help identify high risk patients and offer them adjuvant treatment.

With the advance in molecular diagnostics it is possible to identify 4 distinct breast cancer subtypes [2] which differ in prognosis and treatment and may result from different carcinogenesis pathways. These subtypes are Luminal A (mostly hormone receptor positive, HER-2 negative), Luminal B, HER-2 positive and basal-like (not expressing hormone receptors or HER2). Whereas molecular markers are being gradually incorporated in the management of breast cancer, older −more or less robustly validated markers− are still being used. Immunohistochemical and serum markers are the most favored and the majority of oncologists utilize them as a tool to define prognosis and tailor treatment. In this review we will provide a synopsis of the biological markers used in the care of breast cancer patients with emphasis on clinical application.

Tumor markers

Any molecule that can indicate the presence of occult malignancy or can predict its biological behavior and/or response to therapy can be considered as a tumor marker. The marker could be detected in any biologic specimen and can be cancer-or tissue-specific. Tumor markers before they can be implemented in

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clinical practice they should exhibit significant and independent predictive value in well-designed prospective clinical trials. Unfortunately that is not the case for the majority of cancer markers as they are commonly identified on the basis of retrospective data analysis.

An ideal tumor marker should posses certain characteristics. It should be tumor-specific and its levels should correlate with the tumor bulk. Furthermore, it should be sensitive enough to detect micrometastatic disease and predict disease progression before it is clinically visible. The marker must be under a cutoff value in healthy individuals and should not fluctuate independently of tumor burden. Finally, the test should be feasible, reproducible, widely available and cost-effective. Such a tumor marker could be used for risk assessment, early detection, differential diagnosis, cancer subclassification and disease monitoring. It could also serve to define prognosis and prediction of sensitivity to certain management options. Sadly, the ideal tumor marker is still a utopia. Nevertheless, considerable progress has been made. The older tissue-based tumor markers are being complemented with newer serum-based and more recently by molecular markers. Whether the latest development in breast cancer markers will displace the well-established immunohistochemical markers remains to be seen.

Serum markers

Serum markers are commonly used to aid diagnosis, monitor disease recurrence and treatment efficacy although their use in the community clinic is not evidence-based. They can detect preclinical recurrent disease with a lead time of 2-9 months [3] (Table 1).

MUC1-related markers (CA 15-3 & CA 27.29)

MUC 1 codes for a mucin glycoprotein (polymorphic epithelial mucin/ PEM) which is expressed in most glandular epithelial cells. In breast cancer mucin glycoprotein may be overexpressed and excess mucin is shed in the circulation and detected using either CA 15-3, which is a sandwich assay or CA 27.29, which is a competitive assay. These 2 types of assays measure slightly different parts of MUC1 tandem-repeats [4]. Serum marker levels correlate with tumor burden and are increased in 30-50% in primary breast cancer and 50-70% in metastatic breast cancer [5]. The prognostic significance of MUC-1 in breast cancer is supported by several studies [6-8]. However, there is no established role for screening and diagnosis of breast cancer since elevated levels of CA 15-3 and CA 27.29 can be found in several benign and malignant conditions. Increased levels of CA 15-3 can be found in pregnancy and lactation, benign breast or ovary disease, endometriosis, pelvic inflammatory disease and hepatitis. Cancers of the ovary, lung and prostate may also raise CA 15-3 levels [9]. MUC-1 assays can detect occult micrometastatic disease a few months before it becomes clinically apparent (Figure 1). The clinical significance of this is however unknown

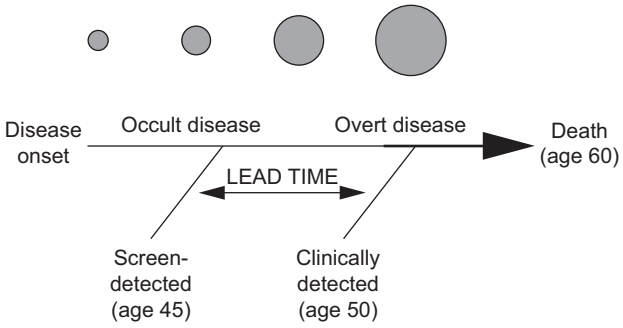

Figure 1. Lead time bias is the bias that occurs when two tests for a disease are compared, and one test (the new, experimental one) diagnoses the disease earlier, but there is no effect on the outcome of the disease - it may appear that the test prolonged survival, when in fact it only resulted in earlier diagnosis when compared to traditional methods. It is an important factor when evaluating the effectiveness of a specific test.

Table 1. Principles of the "ideal" marker in correlation with the most widely serum markers used in the past

Marker characteristic	*CEA*	*CA15-3*	*CA549*	*CA M26*	*CA M29*	*MCA*
Negative test in health or benign disease			+			
Produced by tumor cells						
Present frequently in targeted malignancy		+				
Detectable in occult disease						
Marker's degree of expression can reflect tumor burden & prognosis	+	+	+	+	+	+
Correlation with therapeutic results	+	+	+	+	+	+

since it has not been shown that earlier institution of therapeutic maneuvers can result in improved outcome of breast cancer patients [10]. Therefore, the American Society of Clinical Oncology (ASCO) guidelines do not recommend the use of MUC-1 assays for early detection of relapse. In addition, although levels of CA 15-3 and CA 27.29 are frequently used to monitor treatment response there are no evidence-based data to support this practice and one should keep in mind that spurious rises in both CA 15-3 and CA 27.29 can occur in the first few weeks following the beginning of chemotherapy. On the other hand, in case there is RECIST-responding disease it is pointless to evaluate any marker rate [3].

Carcinoembryonic antigen (CEA)

Carcinoembryonic antigen was first identified in 1965 by Gold and Freedman in human colon cancer tissue extracts [11]. It is a glycoprotein that functions as an intercellular adhesion molecule. It is produced during fetal development and is markedly reduced after birth. CEA is detected at very low levels in healthy individuals and increased levels are associated with smoking, ulcerative colitis, pancreatitis and cirrhosis. Neoplastic conditions associated with increased CEA levels are colorectal, gastric, pancreatic, lung, breast and medullary thyroid carcinomas.

CEA levels are less sensitive than MUC-1 assays for breast cancer. There are no robust data to support the use of CEA measurement for screening, diagnosis, staging, surveillance or response to treatment in breast cancer patients.

HER-2 extracellular domain (HER-2- ECD)

HER-2/neu oncogene is located on chromosome 17q and encodes for a transmembrane tyrosine kinase protein of the EGF family of receptors. Its overexpression has been associated with worse prognosis and more aggressive disease [12]. The receptor consists of an extracellular domain, a transmembranic domain and an intracellular tyrosine kinase domain. The glycosylated extracellular domain (ECD) that has a molecular mass of 97 to 115 kDa is cleaved from the full-length receptor by the catalytic activity of matrix metalloproteinases and shed to biological fluids [13]. HER-2 ECD can be detected by ELISA and has been correlated with clinical endpoints such as disease stage [14,15] and poor prognosis [16]. Detectable levels of ECD/HER-2 have been found both in the presence and absence of HER2 tissue overexpression. It has been proposed that HER-2 receptor dimerization and/or HER-2 ECD cleavage induces activation of the intracellular tyrosine kinase domain and signal transduction [17]. Cleavage of the extracellular domain of HER2 leaves a membrane-bound phosphorylated p95, which can activate signal-transduction pathways. Binding of trastuzumab to a juxtamembrane domain of HER2 reduces shedding of the extracellular domain, thereby reducing p95 [18]. HER-2 cleavage is inhibited by trastuzumab [19], possibly because antibody binding to extracellular HER-2 results in a conformational alteration that makes HER-2 ECD cleavage site inaccessible to metalloproteinases and reduces the levels of activated HER-2 p95 fragments.

The association between HER-2 ECD and clinical outcome has been investigated in a systematic review in over 6500 patients [20]. The authors found a large variation of serum HER-2 ECD and a correlation of its levels with tumor burden and poor prognosis. Elevated levels of HER-2 ECD could predict poor response to chemotherapy and hormonal treatment. On the contrary, they were associated with benefit when patients were treated with trastuzumab. They also demonstrated a correlation of HER-2 ECD levels and appearance of relapse. However, ASCO is not convinced that the available data are robust enough to recommend the use of HER-2 ECD measurement in the routine clinical setting [21].

Tissue markers and early breast cancer prognosis

The first and still most powerful prognostic factors identified in breast cancer are the size of the primary tumor and the number of the involved lymph nodes [22]. A few years later grading was also correlated to prognosis [23] and in 1982, the Nottingham prognostic Index (NPI) was developed, representing the first prognostic tool for breast cancer [24]. NPI uses 3 factors identified as independently significant in multivariate analysis. Tumor grade, number of lymph nodes involved and size of the tumor. Using a mathematical model, an index score is identified and correlated to prognosis [25]. However, the most familiar prognostic tool available is adjuvant online website [26]. This program was developed by Ravdin et al. [27] in 2001 using data from the Surveillance, Epidemiology and End Results (SEER) program of the National Cancer Institute and from the analysis of the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) data in 2000. A patented mathematical formula is used to estimate breast cancer patient prognosis and the benefit of adjuvant systemic treatments applied based on age, comorbidities, grade, estrogen receptor (ER) status, tumor size and number of involved lymph nodes. The model has recently been

validated [28] in population-based dataset. A newer version incorporating results of the Oncotype Dx Recurrence Score is going to be available soon.

Hormone (estrogen and progesterone) receptors

The importance of estrogens [29] and their receptors in breast cancer is well established [30]. The elucidation of the hormone receptor biology led to better understanding of the hormonal pathways and the development of targeted therapies. Both ER and progesterone receptors (PR) are located in the cytoplasm and upon binding to their ligands they change their conformation to reveal DNA binding elements. They translocate to the nucleus where they bind to estrogen response elements and promote the expression of target genes. PR is an estrogen-regulated gene and its expression is thought to indicate a functioning estrogen receptor pathway [31]. Indeed, tumors that appear PR-positive respond better to tamoxifen [32,33].

Estimation of ER and PR expression is essential in pathologic diagnostics of breast cancer and has both predictive [30,32] and prognostic [34,35] significance. Immunohistochemistry is the method of choice for measuring receptor expression, since it has shown excellent correlation with response to hormonal therapies [36].

Human epidermal growth factor receptor 2 (HER-2)

HER-2 is a member of the epidermal growth factor receptor (EGFR) family [37]. The family consists of 4 receptors and several ligands. Engagement of the ligand to the receptor induces receptor homo- or heterodimerization with other family members. The dimerization results in phosphorylation of intracellular tyrosine kinases which ultimately leads to the activation of various signaling pathways promoting cell proliferation, survival, increased motility and invasiveness. HER-2 has no known ligand but its importance lies on the fact that it comprises the preferred heterodimerization partner of the other family members [38].

In breast cancer, HER-2 amplification on chromosome 17 occurs in 20-25% of the cases, leading to a marked increase in the expression levels of HER-2 on the surface of breast cancer cells. This overexpression is associated with an increased risk of relapse and death for patients with early-stage breast cancer [39]. HER-2 status is also prognostic for response to systemic treatment and in particular predicts for response to trastuzumab [40].

Trastuzumab is a monoclonal antibody against the extracellular domain of HER-2 and has substantial activity in both the adjuvant and metastatic setting as monotherapy and in combination with cytotoxic agents. Indeed, trastuzumab has changed the natural history of HER-2-overexpressing breast cancer, abrogating the negative effect of HER-2 overexpression on survival [41]. HER-2 status should be assessed in every breast cancer specimen in order to guide the use of trastuzumab.

Topoisomerase IIα

Topoisomerases are proteins that regulate the uncoiling of DNA for transcription by guiding the unknotting of DNA and creating transient breaks in the DNA. Topoisomerase II is the target of anthracyclines and is located on chromosome 17q12 in close proximity with the HER-2 gene. These genes are co-amplified in one third of the cases. Patients whose tumors show amplification of the topoisomerase II gene derive the greatest benefit from anthracyclines [42-44]. Topoisomerase IIα can therefore be used as a predictive (rather than a prognostic) tool. A FISH assay has recently been approved by FDA for testing topoisomerase II amplification (TOP2A FISH pharmDx TM; DAKO, Glostrup, Denmark).

Urokinase and PAI-1

Urokinase (uPA) is a serine protease originally isolated from human urine but it can be found normally in other tissues. It converts plasminogen to the active molecule plasmin which in turn triggers a cascade that leads either in thrombosis or extracellular matrix degradation depending on the surrounding microenvironment. Levels of uPA and its inhibitor PAI-1 have been linked with invasion, angiogenesis and metastasis [45]. Both factors can be measured by ELISA on a minimum of 300 mg of fresh or frozen breast cancer tissue. Overexpression of uPA and/or PAI-1 has been consistently related to poor prognosis in early-stage breast cancer [46,47]. Low levels of both markers are associated with a sufficiently low risk of recurrence and gain minimal benefit from chemotherapy while CMF-based adjuvant chemotherapy provides substantial benefit in patients with high risk of recurrence as determined by high levels of uPA and PAI-1[48].

Molecular markers

The advances on genomics expanded our knowledge of genes and their contribution to breast carcinogenesis. A number of prognostic multigene expression assays have emerged and seek their role in breast cancer

management and have already been compared with traditional prediction tools. These assays have been developed in an endless effort to improve prognosis and spare over-or under-treatment of our patients (Table 2).

Oncotype Dx

Oncotype Dx is a real-time polymerase chain reaction (RT-PCR) - based assay. It was developed from Paik and colleagues after studying breast cancer recurrence in 447 patients enrolled in the National Surgical Adjuvant Breast and Bowel Project clinical trial B-14[7]. Initially 250 genes were chosen from gene expression profile experiments by published literature. From these candidate genes, 16 cancer-related and 5 reference genes were selected. In RNA extracted from fresh-frozen paraffin-embedded (FFPE) tissue, the level of expression of these candidate genes is calculated as a recurrence score using a prospectively derived mathematical algorithm. Each sample is given a score between 0 and 100 with the higher score indicating greater chance of recurrence. Each patient is classified in 3 predefined categories: low risk (recurrence score, less than 18), intermediate risk (recurrence score 18 or higher but less than 31), and high risk (recurrence score 31 or higher). This score is positively correlated to the rate of distant recurrence at 10 years.

Oncotype Dx was originally tested in node-negative patients treated with tamoxifen but later on the method was also tested in node positive population [49,50] and predicts response to chemotherapy [51]. Recently is has been shown to perform as a prognostic tool for relapse in both node positive and node negative patients better than adjuvant online [52] and to be valid for patients who are going to be treated with aromatase inhibitors [53]. It seems that besides being considered a prognostic test, Oncotype DX turns to have predictive significance since it can predict patients who derive no extra benefit from the addition of chemotherapy to adjuvant hormonal treatment.

Table 2. Assays that address prognosis of early breast cancer, according to molecular profile

Molecular profiles assays [Ref. no.]	*Number of genes*
Intrinsic subtype [67]	496
Mammaprint [54]	71
Rotterdam [57]	76
Oncotype Dx [50]	21
Invasive gene signature [58]	186
Wound response signature [60]	512
Genomic grade index [61]	242
Mammostrat [68]	5

The Oncotype Dx assay is being prospectively tested in the TAILORx trial, where decision for adjuvant therapy will depend on recurrence score (RS). Low RS patients will receive hormonal therapy alone, high risk will additionally receive chemotherapy, while the intermediate score will be randomized between hormonal therapy and chemotherapy followed by hormonal therapy.

MammaPrint 70-Gene profile

MammaPrint is the first commercially available microarray assay. It was developed in the Netherlands by comparing the difference in gene expression profiles in patients who recurred vs. those who remained disease free [54] in a population of node-negative breast cancer patients younger than 55 years of age. The comparison led to the identification of a 70-gene signature that could classify patients in low and high risk (for relapse) groups. The assay was validated by the same group [55] and independently [56] and led to the clearance of the assay by the FDA for determining breast cancer patient prognosis in conjunction with clinicopathologic parameters.

MammaPrint is being prospectively validated in the MINDACT trial (Microarray in Node Negative Disease May Avoid Chemotherapy) where the assay is being compared with the "AdjuvantOnline!" tool. Patients classified as low risk with both methods will receive hormonal therapy, those classified as high risk will additionally receive chemotherapy and discordant cases will be randomized to receive treatment based on either "AdjuvantOnline!" or MammaPrint. It is expected that the use of molecular signature will spare 10-20% of patients' chemotherapy without compromising their survival.

Additional gene expression assays

The *Rotterdam 76-gene array* is another test developed to estimate the prognosis of lymph node-negative breast cancer patients [57]. In the original study included were 286 lymph node negative patients with locoregional treatment only. Separate genes for ER positive and negative patients were selected and then combined in a single 76-gene prognostic signature. The signature was able to predict distant relapse with a sensitivity of 93% and specificity of 48%. The gene set was validated in two other cohorts of patients with comparable results.

The *invasive gene signature* is a set of genes that were identified when normal breast was compared with cancer stem cells. Cells with CD44 high CD24 low expression have shown stem cell properties in experimen-

tal models [58]. These cells were compared to normal breast epithelium and 186 genes were identified. These genes were named "the invasive gene signature" and were associated with shorter overall survival and metastases free survival [59].

Another molecular gene signature is the *wound response signature,* developed from genes whose expression was altered following activation of cultured fibroblasts with serum. This signature was validated in early breast cancer patients and patients whose tumors had a high score had worse overall survival and metastases free survival compared with those that did not [60].

Comparing the different microarray platforms

Gene expression profiling arrays used to discriminate different outcomes of breast cancer patients show minimal gene overlap. Yet they are able to stratify patients in cohorts with similar outcomes. A study tested the predictions of several of these assays in a single data set of 295 samples [61] and found high rates of concordance in their prediction despite the use of different gene sets. There is some skepticism about that but the most probable explanation is that all different molecular signatures can detect cellular phenotypes with common biological characteristics and behavior. A recent study attempted to investigate the motive for this phenomenon. They used a single dataset and analyzed it with the methodology of Van't Veer et al. (MammaPrint). In the original study the authors used a large expression library of 24,481 genes which was filtered, narrowing the number to 5,852 and from them the first 70 genes were correlated with survival. The authors in this provocative study [62] found that they can separate good and bad signature tumors even if they used the next set of 70 genes in the row and for the next 7 classifier gene sets. The same results were obtained if the tumor was categorized using genes 1-70, 71-140, 141-210 and so on. It seems that many genes are related to survival and their differences are small, so one only needs to detect similar cancer phenotypes irrespective of the methodology used.

Microarrays as predictive tools

Apart from being used as a tool to estimate prognosis, microarrays have also been utilized to predict sensitivity to various pharmaceutical agents used in the treatment of breast cancer. Response to docetaxel in the neoadjuvant setting has been evaluated [63] and a set of 92 genes has been identified that could detect docetaxel sensitivity with great specificity and sensitivity. Another set of 44 genes is used to identify response to tamoxifen [64] in patients with advanced breast cancer. Prediction of benefit from tamoxifen in early-stage disease was reported with an assay that uses RT-PCR to define the ratio of two genes [65]. This method uses FFPE tissue and is commercially available (AviaraDx H/I TM; AviaraDx, Carlsbad, CA). Another group using quite the same method has found predictors of platinum sensitivity in triple negative breast cancer tumors [66].

Conclusions

A large variety of prognostic markers has emerged in an effort to better characterize an individual patient's risk of relapse. The majority of them, however, has not been accepted by regulatory bodies and has not reached clinical practice. It seems that we are gradually moving from traditional pathological markers to more modern molecular assays to offer individualized prognosis for each patient. However, the use of established clinical factors cannot be substituted, at least till these molecular tests are validated in larger number of patients in community-based practice. We need to stay up-to-date, having full knowledge and making good use of the tools that cancer research is providing us. On the other hand we should keep distances from the commercial competition this new technology brings along. Only wise use of all the clinical and molecular data will provide the best service to our patients.

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