

Reliable Biomarkers to Identify New and Recurrent Cancer

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ABSTRACT

Breast cancer is the most frequent cancer detected throughout both the developing and the developed world. Its incidence is on the rise in the developing world. Great strides have been made in developing biomarkers to guide therapy for women diagnosed with breast cancer. Far fewer advances have occurred with biomarker development for the early diagnosis of breast cancer. Standard screening for new and recurrent breast cancer involves clinical breast exam and breast imaging. There are no Food and Drug Administration (FDA) approved noninvasive body fluid tests for the early detection of new or recurrent breast cancer. Promising biomarker approaches include multianalyte testing of tissue for individuals diagnosed with breast cancer and body fluid analysis for both at risk women and to monitor individuals after treatment.

Keywords: Breast cancer, biomarkers, carbohydrates, proteins, lipids

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Introduction

The breast is the most frequent site of cancer among women in both developed and in developing countries (1). Breast cancer is the most frequent cause of cancer death in women from less developed countries and second most frequent (after lung cancer) among women in developed countries (1). It is increasing in incidence in the developing world due to increased life expectancy, increased urbanization and the adoption of western lifestyles (2). According to the World Health Organization (WHO), "Early detection in order to improve breast cancer outcome and survival remains the cornerstone of breast cancer control" (2).

In 2017 approximately 41,070 U.S. women are projected to die from breast cancer (3). It is estimated that in 2011 508,000 women worldwide died of breast cancer (2). Although incidence rates are lower in developing regions than in Western Europe and North America, almost 50% of breast cancer cases and 58% of deaths occur in less developed countries (2). Breast cancer survival rates range from over 80% in many developed countries to below 40% in low-income countries (4). The low survival rates in less developed countries are due to later stage at disease presentation, as well as by the lack of adequate diagnosis and treatment facilities.

Early detection is crucial to improving the survival of individuals with breast cancer, as survival is inversely related to disease stage (5). An invasive needle or surgical biopsy must be performed when an area of suspicion is identified in order to confirm the presence of malignancy, and most of these invasive biopsies will turn out to be benign (6). The development of non-invasive biomarkers that would distinguish between women with or without breast cancer is highly desirable.

Twenty-one percent of all breast cancer deaths worldwide have been attributed to alcohol use, high body mass index (BMI), and physical inactivity (7). Thus, while some breast cancers may be prevented through lifestyle modification strategies, most cannot. Currently known risk factors address only a subset (up to half) of risk for breast cancer. Biomarkers hold promise to aid breast cancer assessment. A highly predictive biomarker is stable, reproducible, sensitive, specific and easy to detect. Biomarkers can be assessed in tissue and/or body fluids.

Whereas tissue biomarkers are fine for women from whom breast tissue has been collected for diagnostic purposes, body fluid analysis is more desirable when evaluating healthy individuals.

Breast Cancer Markers in Clinical Use

Physical Examination

Breast self-examination (BSE) has not been shown to be an effective screening strategy, but does raise awareness of the disease and therefore is recommended by the WHO for this purpose (7). The U.S. Preventive Services Task Force (USPSTF) recommends against teaching BSE (8). The American Cancer Society (ACS) advises the use of BSE as an optional screening tool. The WHO suggests that CBE has been demonstrated effective both in affluent and less affluent countries, whereas the USPSTF has concluded that current evidence is insufficient to determine if CBE adds additional benefit to screening mammography in women 40 years or older (7). The ACS does not recommend BSE or CBE for average risk women (9).

Breast Imaging

Mammography: The sensitivity of mammography is inversely related to breast density such that higher density decreases sensitivity. Anything that may increase breast density, such as fibrosis related to prior surgery and/or breast irradiation, decreases mammographic sensitivity. For average risk women, the USPSTF currently recommends biennial mammographic screening for women 50-74 years of age, indicating that the decision to begin mammography screening earlier than age 50 is an individual choice. The ACS recommends that women be given the opportunity to start annual mammographic screening at age 40, and that they should in any case start annual mammographic screening by age 45. Starting at age 55 women should be given the option to continue annual or transition to biannual screening. The ACS further indicates that women should continue mammographic screening so long as they are in good health and have a life expectancy of at least 10 years (9).

Ultrasound: In average risk women undergoing asymptomatic screening, ultrasound is generally used as a supplement for those with dense breast tissue. For women with a sign or symptom of breast disease, according to the American College of Radiology (ACR) Practice Guidelines (2016 revision), breast ultrasound is appropriate for 1) the evaluation and characterization of palpable masses and other breast related signs and/or symptoms; 2) evaluation of suspected or apparent abnormalities detected on other imaging studies, such as mammography or magnetic resonance imaging (MRI); 3) initial imaging evaluation of palpable masses in women under 30 years of age who are not at high risk for the development of breast cancer, and in lactating and pregnant women. 4) evaluation of problems associated with breast implants, 5) guidance for breast biopsy and other interventional procedures, 6) treatment planning for radiation therapy, and 7) identification of and biopsy guidance for abnormal axillary lymph nodes (10).

Magnetic Resonance Imaging: Breast MRI is not recommended as the initial tool to screen average risk woman except as part of a scientific study. Current indications for breast MRI based on American Society of Breast Surgeons 2017 recommendations include screening high risk patients, screening the contralateral breast in women newly diagnosed with breast cancer who have associated clinical or conventional indeterminate imaging findings suspicious for malignancy, assessing extent of disease in women with *in situ* or invasive carcinoma both before and after surgery, assessment for eligibility and response to neoadjuvant endocrine therapy or chemotherapy before, during, or af-

ter treatment, for evaluation of suspected breast implant rupture, and in assessing breast cancer recurrence when clinical, mammographic or ultrasound findings are inconclusive (11).

Biomarkers

There has been considerable effort to identify novel biomarkers that might offer clinical utility. Relatively few identified candidates have been subjected to rigorous validation. Validation requires the analysis of hundreds and sometimes thousands of samples to adequately survey the variability in biomarker expression that is present in patient samples.

The evaluation of changes in both DNA and proteins in body fluids and tissue shows considerable promise in the diagnosis and management of breast cancer, but analysis of body fluids is preferred for diagnosis because sampling is minimally invasive and ongoing assessment is practical. Analysis of fluids also has promise, either alone or in combination with tissue analysis, for determining if breast cancer will recur.

Despite the potential already demonstrated, researchers have not delivered validated biochemical markers that can be used to optimally diagnose and manage breast cancer. Only four of the Food and Drug Administration (FDA)-approved markers (CA 15-3, CA 27.29, HER-2/neu, and circulating tumor cells analysis of EpCAM, CD45, CK8, 18, 19) can be measured and assessed longitudinally. Notably, there are no FDA approved biomarkers for breast cancer diagnosis or screening (12).

Biomarkers in tissue

1. Hormone receptor status: Cancers that express the estrogen receptor (ER) are estrogen dependent, whereas cancers that do not express ER are estrogen independent. Two thirds of invasive breast cancers express ER and are classified ER+ (13). The progesterone receptor (PR) becomes activated when it interacts with the hormone progesterone. Approximately 65% of breast cancers that are ER positive are also PR positive. Expression of both ER and PR are measured via immunohistochemical (IHC) assay. Tumors that are ER+ and/or PR+ generally respond to antihormonal therapy with tamoxifen or an aromatase inhibitor, whereas those that are ER/PR negative do not.

2. Cytokeratin (CK): IHC expression of CK7, CK8, CK18 and CK19 is observed in more than 90% of all breast carcinomas (14). Expression of CK5/6, CK14 and CK20 correlate with high tumor grade.

3. Heregulin (HER)2: Also known as epidermal growth factor receptor (EGFR) II, HER2 is overexpressed in 20% of breast cancers, most often due to HER2 gene amplification (15). HER2 overexpression upregulates the cell signaling pathway leading to uncontrolled cell growth. HER2 positive breast cancers are generally aggressive and patients with HER2 overexpression have a worse prognosis than patients whose breast cancers do not overexpress HER2 (16). FDA approved agents targeting HER2 include trastuzumab, lapatinib, T-DM1 (ado-trastuzumab emtansine) and pertuzumab.

4. Ki67: This protein is a marker of cell proliferation for many types of cancer. The fraction of cells that stain positive for this protein reflects the fraction of cells in G1, S, G2 or mitosis, but not those that are in G0. Higher levels of Ki-67 correlate with more rapid tumor growth and tumor aggressiveness (17).

5. Oncotype Dx: This is a 21 gene expression biomarker panel that uses formalin fixed tissue. The test is valid for women with hormone

sensitive breast cancer. It is most often used for women with early stage (DCIS, stages I and II node negative) disease. Results provide a statistical inference of chemotherapy benefit and likelihood of recurrence (18).

6. Mammaprint: This 70 gene biomarker panel uses fresh or fixed tissue to determine the likelihood of recurrence of breast cancer within 10 years of diagnosis and response to treatment with chemotherapy. Mammaprint can be used to analyze both ER (-) and ER+ early stage (i.e. stage I or II) node negative (U.S. criteria; international criteria allow up to three positive nodes) invasive cancers (19).

7. Prosigna: The 50 gene assay, formerly called the PAM50 test, analyzes the activity of certain genes in node-negative (stage I or II) or node positive (stage II), hormone receptor-positive breast cancer patients. It provides individualized assessment of a patient's risk of recurrence at 10 years if given endocrine therapy alone (20).

Biomarkers in body fluids

The most common body fluid currently in use for biomarker detection is blood or its components. Three breast specific fluids that are being evaluated for predictive biomarkers are nipple aspirate fluid (NAF), breast milk and ductal lavage (DL). Each fluid contains proteins, carbohydrates and lipids, DNA and RNA. NAF, collected neat through breast massage +/- suction on the breast nipple, has a relative paucity of cells, but concentrated proteins, carbohydrates and lipids. Breast milk contains high concentrations of certain proteins, including α -lactalbumin, lactoferrin, secretory IgA, lysozyme and albumin, whose primary function is feeding an infant, but there are also less abundant proteins, carbohydrates and lipids with potential cancer biomarker usefulness. DL is collected through the insertion of a micro catheter into the nipple, providing irrigating fluid, and analyzing the effluent. There are more cells in DL than NAF. The irrigant dilution factor for analysis of proteins, lipids and carbohydrates is somewhat uncertain, as often not all of the irrigant is collected.

Biomarkers in body fluids: serum

1. CA15-3 and CA 27.29: For women diagnosed with breast cancer, assessment of the expression of both CA 15-3 (MUC1) and CA 27.29 in serum are FDA approved to monitor patients. American Society of Clinical Oncology guidelines recommend using these tumor markers in conjunction with imaging and clinical examination to assess treatment response/failure. A confirmed increase of $\geq 25\%$ has been suggested as clinically significant (21).

2. HER2/neu: Reports suggest that the sensitivity of HER2/neu tissue testing may be enhanced by evaluating the external fragment of the HER2 protein, which is shed into the bloodstream. This assessment is referred to as the serum HER2 (sHER2) test (22). An increasing level of circulating HER2 is an early indicator of progression, particularly in HER2-positive patients. The rise and fall parallels the clinical course of disease, independent of therapy. HER2 status of the primary tumor may not accurately reflect the HER2 status of recurrent disease. As such, elevated serum HER2 levels may be an early signal of a HER2-positive metastatic tumor and therefore alert the physician to re-assess HER2 status using a tissue test (22).

3. Circulating tumor cells (CTCs): The CellSearch system was FDA approved in 2004 to detect the presence of CTCs and monitoring disease progression based on CTC level in patients with metastatic breast, colorectal, and prostate cancer. The CellSearch test has not yet been established as a means of selecting therapies for these patient popula-

tions, hampering its incorporation into treatment guidelines (23).

Breast Cancer Biomarkers Being Evaluated

Biomarkers in tissue

1. p53 is the most commonly mutated gene in human cancers. Individuals who have germline mutations in *TP53* have Li-Fraumeni syndrome. Li-Fraumeni syndrome is rare, with approximately 400 known families. Patients with Li-Fraumeni syndrome are at high risk for early-onset breast cancer. The primary limitation of performing screening for germline p53 mutations is their rarity (24).

3. Ataxia Telangiectasia (AT) is caused by mutations in the AT mutated (*ATM*) gene which leads to the generation of defective AT protein (25). The normal AT protein detects DNA strand breaks, recruits proteins to fix the break, and prevents a cell from making new DNA until the repair is finished. People with AT are at an increased risk of multiple cancers, including lymphoma, leukemia, and breast cancer. Compared to the general population, women who are heterozygous or homozygous for AT have double the risk of developing breast cancer (26). The relative infrequency of the mutation limits its justification for screening the general population to identify individuals at increased risk.

4. Mutations in the Phosphatase and Tensin (PTEN) gene can contribute to the development of a variety of cancers, including breast cancer (26) Approximately 50% of breast cancers have loss of PTEN expression, which is associated with lymph node metastases and poor survival (27). Individuals with Cowden's disease, who have germline mutations in PTEN, have a 25-50% lifetime risk of developing breast cancer (28).

5. Multiple gene analyses: There are a variety of hereditary breast cancer syndromes which have genetic mutations associated with them, and confer an increased risk of developing breast +/- other malignancies (29). These include Hereditary Breast and Ovarian Cancer syndrome, with mutations in *BRCA1* or *BRCA2*, Li-Fraumeni, with mutations in *TP53*, Cowden's syndrome, involving *PTEN*, Hereditary Diffuse Gastric Cancer syndrome, involving *CDH1*, Peutz-Jeghers, involving *STK11*, Lynch syndrome, involving *MLH1*, *MSH2*, *MSH6*, or *PMS2*, and Fanconi anemia, involving *PALB2*. Lifetime risk of breast cancer is over 20% for mutation carriers of these syndromes, ranging up to 80% for *BRCA1* mutation carriers (29). Gene panels have been developed to evaluate patient samples for alterations in some or all of these genes.

a. BROCA: This panel from the University of Washington evaluates mutations in genes involved with a variety of human cancers. BROCA is most useful for analyzing patients with a suspected cancer predisposition. An advantage of the BROCA gene panel is that specific gene testing can be selected or the investigator can opt for the entire panel. The number of genes in the panel changes over time based on new information (30).

b. BreastNext: This 17 gene panel developed by Ambry Genetics is very similar to the BROCA panel in that it analyzes cancer risk and is best suited for patients with a suspected hereditary predisposition to breast or ovarian cancer. Like BROCA, this panel offers the option of specific gene testing or analysis of the entire panel. A further advantage to BreastNext is that it includes duplication and deletion gene analysis (30).

c. BRCAPLUS: This 6 gene panel developed by Ambry Genetics performs next generation sequencing (*BRCA1/2*, *CDHI*, *PALB2*, *PTEN*, *TP53*). Each of the genes analyzed is linked to hereditary cancer syndromes and has published management guidelines (31).

d. Breast/Ovarian cancer panel: This 20 gene panel developed by GeneDx evaluates genes that have been linked to an hereditary disposition to breast and/or ovarian cancer (30).

e. The Myriad myRisk® Hereditary Cancer test is a 28-gene panel that identifies an elevated risk for eight cancers (breast, ovarian, gastric, colorectal, pancreatic, melanoma, prostate, and endometrial) (30).

Gene panels have also been developed by Ambry Genetics, Breast-HealthUK, Centogene, the Emory Genetics Laboratory, Fulgent Diagnostics, Invitae, Quest Diagnostics, and Prevention Genetics (29).

Current Biomarker Challenges

The accurate assessment of biomarker expression is influenced by many things. Three of the most important factors are tumor heterogeneity, treatment effect, and whether the tumor is new or recurrent. Because of tumor heterogeneity, expression of tumor markers can differ in a patient with newly diagnosed, untreated breast cancer between core biopsy and surgical resection specimens. Additionally, treatment can lead to changes in expression, with resistant subclones which survive becoming predominant after treatment. Tumor DNA, which is inherently unstable, can change over time, leading to changes in primary vs. recurrent/persistent tumors. Moreover, some breast tumors contain both basal and luminal clones with distinct genetic alterations (32). The molecular phenotype of primary vs. recurrent tumors can differ due to treatment of the primary and the innate instability of tumor DNA.

Heterogeneous tumors

Immunohistochemical analysis of a newly diagnosed breast cancer generally includes ER, PR and HER2, and is most often performed on the core biopsy specimen. Because of intratumoral heterogeneity, the smaller the core biopsy, the more likely it is to not accurately represent the ER, PR or HER2 status of the entire tumor (33). Moreover, expression may differ between a primary and recurrent tumor as well as between the primary tumor and its metastasis/metastases (33).

There is heterogeneity in tumor cell division between diagnostic needle or core biopsy and surgical resection specimens as demonstrated in the proliferation marker Ki67. The authors propose this the average proliferation difference was 3.9%, with biopsy specimens having a higher proliferation rate than surgical excision specimens and with the assessment of 800 additional (total 1000) cells, the difference was no longer present. The authors proposed this was due to hot spot sampling in the core specimens. Treatment can also contribute to acquired heterogeneity because it can alter the tumor phenotype and change the ER/PR and HER2 status of the original tumor. These findings suggest that overall under sampling of the primary tumor may be occurring.

The effects of treatment

Both endocrine and chemotherapeutic cancer treatment can change tumor phenotype. Investigators evaluating postmenopausal women with ER+ breast cancer who were receiving neoadjuvant endocrine therapy found that the ER downregulator fulvestrant decreased Ki67, ER and PR expression after 16 weeks of treatment in a dose dependent fashion (34). In a separate study of women who received neoadjuvant chemotherapy for breast cancer, tumor grade changed in 35%, ER, PR and HER2 expression in 43, 55 and 27% of cases, respectively (35).

Whether the tumor is new or recurrent

Primary vs. recurrent tumors can differ both due to treatment of the primary and the inherent instability of tumor DNA. If recurrent disease is in a different location from the primary tumor, a particular alteration may have selected for tumor spread to the new location. In a study evaluating patients with biopsy proven relapsed breast cancer, using a similar approach to assess expression in both primary and relapsed specimens, the molecular phenotype of the original tumor and the tumor metastasis, there was 19% discordance in the ER/PR or HER2 status between the primary and relapsed lesion (36). A second study evaluating patients with biopsy proven relapsed/metastatic disease observed discordance for ER, PR and HER2 of 32, 41 and 15%, respectively (37). In the second study there was an overall survival advantage among women with stable ER+ tumors vs. those whose tumors changed to ER- at relapse.

Consequences of Current Biomarker Limitations

Standard screening for breast cancer involves physical examination and breast imaging, generally mammography with or without breast ultrasound. These tools alert the treating provider to the presence of a lesion which is palpated and/or an abnormality which is visualized. The diagnosis of breast cancer requires demonstration of visual changes in the nuclei in a cytologic or histologic preparation of breast cells. In order to obtain the cells for pathologic review, an invasive needle, core, or surgical biopsy must be performed. These procedures are subject to sampling error, and only approximately 15-20% of the procedures detect malignancy (38). Liquid biopsies have the goal of providing useful clinical information from body fluid analysis. These body fluids can be collected noninvasively, or minimally invasively. These body fluids can be either breast specific, such as nipple aspiration, ductal lavage (DL), and ductoscopy, or not, such as blood.

Discovery Targets

The success of breast cancer biomarker research depends on: 1) combining newly discovered biomarkers with established methods so that cancer diagnosis can be optimized; 2) identifying biomarkers to establish response to treatment, recurrence, and survivorship; 3) the use of biomarkers to guide therapies in patient targeted medicine; and 4) the use of biomarkers to determine drug candidates for the development of new therapies (17).

There are no generally accepted body fluid biomarkers for the early detection of breast cancer. A major barrier to the development of early breast cancer detection biomarkers relates to disease heterogeneity. Biomarker studies to date have generally been small, evaluate one or a few markers in a single sample type, and enrollment and exclusion criteria vary from study to study. Some studies evaluate subjects at high risk for cancer, based on risk assessment or clinical findings, while other biomarker studies screen the general population. Finally, cancer detection biomarker studies often collect a single sample, which may not adequately account for random biomarker variation in a given individual. More progress has been made among subjects which biopsy proven breast cancer, since a patient's tumor can be phenotyped. Because of the inherent instability of tumor DNA, as well as treatment effect, changes in biomarkers can occur, as already discussed. Nonetheless, new biomarker development, both for the early detection and treatment of breast cancer, is an active area of investigation. Among the biomarkers under evaluation are autoantibodies, inflammatory response molecules, DNA methylation (i.e. CpG Islands), benign breast disease, and pregnancy associated breast cancer biomarkers.

Challenges in the identification and validation of new biomarkers

Most biomarker development studies are not completely representative due to selection bias. Biomarker assessment in healthy individuals is generally limited to body fluids, as there is no tumor to collect and ethics boards are reluctant to approve invasive approaches in healthy individuals. Successful sample collection of some body fluids such as NAF or DL requires learning the technique and practice. Inadequate sample collection can limit biomarker assessment.

Validation of an initially promising observation is often the weakest link to biomarker development. This is in part because those who made the initial observation lack the tools (adequate finances, adequate infrastructure, adequate number of subjects, etc.) to validate the findings. Validation requires high throughput and access to large cohorts of well-defined clinical samples. A variety of candidate biomarkers (e.g., peptides, proteins, carbohydrates, DNA, RNA, metabolites) analyzed simultaneously optimizes yield and provides a direct comparison of the performance characteristics of each biomarker.

Choosing the proper type of sample for biomarker validation

Samples in the blood (serum, plasma): Serum and plasma are routinely used in clinical chemistry. Sample collection is minimally invasive and in some settings blood is the most practical sample to collect. Even though a marker may be diluted markedly in serum or plasma, much larger volumes can be obtained (1-5 mL) than are commonly collected in a breast specific body fluid such as NAF. In other words, a marker detected in NAF could be diluted by a factor of 1.000 or more and still be detected in routinely collected volumes of serum (39).

Breast specific samples: Both DL and NAF are collected from the breast ducts. The ductal epithelium gives rise to the vast majority of breast cancers. Unlike blood, the samples are not diluted by biomarkers contributed by other organs in the body. DL is more cellular than NAF, and therefore better for the analysis of intracellular components such as DNA and RNA, whereas NAF has a highly concentrated protein and carbohydrate content, and therefore is well suited for the analysis of the latter two types of biomarkers (39).

Conclusion

Great strides in breast cancer biomarker development have already been made to tailor treatment to individuals with newly diagnosed breast cancer. This is because the available tumor can be phenotyped through assessment of specific gene alterations, gene panels, or whole genome sequencing, to guide hormonal therapy, biologic therapy as well as chemotherapy. Tissue assessment is generally not as practical to screen healthy individuals, or to follow individuals long term after cancer treatment to monitor for disease recurrence. For early detection, body fluids are appropriate for biomarker development. Interventions on the breast with excisional biopsy followed by radiation or mastectomy make breast specific body fluid samples primarily useful for early detection, whereas serum/plasma is the logical body fluid for serial assessment of disease response and recurrence.

Biomarker panels are coming into increasing use. This multianalyte assessment is both practical and a reasonable approach to optimize sensitivity and specificity. Validation of biomarkers is critical before general clinical use.

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