

Autoantibody profiles as biomarkers of breast cancer

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Abstract. Breast cancer is the second leading cause of cancer death in women in the United States. While mammography and breast magnetic resonance imaging (MRI) improve detection of early disease, there remains an unmet need for biomarkers for risk stratification, early detection, prediction, and disease prognosis. Sera from patients with breast cancer contain specific autoantibodies (AAb) to tumor antigens that develop as part of the natural immune response to cancer-associated changes in protein structure and expression. The recent development of proteomic tools for AAb detection, including protein microarrays, reverse-phase protein immunoblots, and phage display have identified a number of potential AAb biomarkers for clinical development. Immune response signatures have been identified that are highly specific, but with variable sensitivities for cancer detection. This review focuses on the detection and application of AAb signatures as biomarkers for breast cancer detection and monitoring.

1. Introduction

Invasive breast cancer remains a common cancer and significant public health problem, with over 192,370 new cases annually in the United States [40] and 1.15 million worldwide [62]. In 2009, it is estimated that over 40,170 people in the U.S. will die of breast cancer. Biomarkers for early detection, disease monitoring, and prognosis could have great potential clinical impact. With the advent of molecularly-targeted therapeutics, biomarkers that can describe biological subtypes of cancer may be useful for predicting responses to therapeutic interventions and side effect profiles. Proteomics-based approaches to distinguish cancer-bearing patient sera from healthy control sera have been challenged by the difficulty in identifying small quantities of protein fragments within complex protein mixtures, protein instability, and natural variations in protein content within patient populations [3, 65,105].

Antibodies to tumor antigens have advantages over other serum proteins as potential cancer biomarkers as they are highly specific, easily purified from serum, and are easily identified with high quality secondary reagents. The role of antibodies directed at self antigens, referred to as autoantibodies (AAb), and disease diagnosis is most commonly used in autoimmune rheumatic diseases (reviewed in [51,74]). The autoantigens detected (for example: Jo-1, La/SSB, RNP, Ro/SSA, Scl-70 and Sm) are intracellular antigens complexed with nucleic acids [74]. Epitopes can be linear or conformational, and may be post-translationally altered by acetylation, glycosylation, methylation or phosphorylation [21,22]. Overall, the pathophysiology that underlies the development of AAb is poorly understood, but may relate to antigen mutation, overexpression, altered antigen folding and degradation, as well as genetic factors.

Antibodies to tumor antigens have been detected in early stages of multiple types of cancer and may correlate with disease progression. In melanoma, endogenous antibodies to TA90 antigen have been encountered in patients with node negative melanoma, and were inversely correlated with risk of subsequent relapse [54]. In addition, 46% of patients with colon cancer pro-

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duced antibodies for one of 13 antigens [78]. In patients with both stage III and stage IV neuroblastoma, 10% have detectable AAb to the NY-ESO-1 antigen, but the antibody was not detectable in the sera of patients in clinical remission [73].

Tumor specific antibodies have also been identified in the sera of patients prior to cancer diagnosis and cancer relapse. In lung cancer, AAb levels against Annexin I, 14-3-3 theta and LAMR1 were significantly higher in cases whose sera were collected up to 1-year prior to diagnosis compared with high risk controls, and increased closer to disease diagnosis [66]. NY-ESO-1 AAb titers also correlate with tumor burden [37,39]. In prostate cancer, the frequency of NY-ESO-1 antibodies increases from early- to late-stage disease, which may reflect a higher frequency of antigen expression in later stages of disease [28]. TA90 AAb have been detected up to 19 months prior to the development of clinical relapse and correlate with survival in patients with early-stage melanoma [45]. P53-AAb have been detected in the sera months to years before clinical detection of angiosarcoma or lung cancer [94,95], and p53 AAb titers decrease after resection of colorectal cancer [88]. While provocative, these few studies have been limited to antigens with higher frequencies of antibodies in cancer (such as NY-ESO-1). Technical challenges of antigen identification, expression, purification, and individual assay development and validation have prevented more comprehensive studies on other antigens.

2. Methods for autoantibody detection

The advent of proteomics-based technologies has markedly expanded the detection of antigen-specific antibodies. Assays such as protein microarrays, reverse phase protein arrays, phage display, and glycan arrays have all been used to discover cancer-associated AAb. These assays are excellent discovery tools, approaching the ultimate goal of proteome-wide immune monitoring. However, multiplexed assays for clinical validation studies have lagged behind in development, so that the majority of AAb have not yet undergone phase III or later clinical validation studies [63].

2.1. Phage-based expression assays

The technologic development of serologic expression cloning (SEREX) of tumor antigens by screening phage libraries with sera derived from cancer patients has resulted in the identification of many tumor

antigens from multiple solid and hematologic tumor types [26,30,31,36,38]. The SEREX assay relies on immunoblotting epitopes expressed by phage, so that the identification of SEREX antigens may be restricted to linear epitopes and those gene products that can be expressed in bacteria. Because the phage libraries are produced from cDNA, genes corresponding to abundant mRNA species (often housekeeping genes) are over-represented. Despite these limitations, numerous candidate tumor antigens have been discovered using this approach, with a bias towards nuclear and intracellular antigens, many of which are upregulated, mutated, or specifically expressed in tumors. SEREX screening has led to the identification of many cancer/testis (CT) antigens, differentiation antigens, amplified/overexpressed gene products, mutated gene products, splice variants and cancer-related autoantigens [38,58,61]. Through a database established by the SEREX collaborative group, sequences from 2316 clones (1545 genes) have been deposited as of October 2009 [16] (<http://ludwig-sun5.unil.ch/CancerImmunomeDB/Intro.php>). Although many candidate tumor antigens have been identified by SEREX, relatively few have been examined in detail in studies comparing sera from cancer patients with normal controls, in part because of the technical challenges associated with protein production, purification, and ELISA assay development.

A number of modifications of the original SEREX design have been introduced to improve antigen selection [11]. Phage display is emerging as a powerful approach for the identification of tumor antigens [27, 82] and phage-displayed antigen microarrays have been used to monitor autoantibody profiles in cancer [14, 53,100]. By expressing antigens as fusions with phage proteins, antigens can be identified with a small quantity of serum. Several groups have successfully applied phage display with variations for cancer AAb discovery, including breast cancer [58], ovarian cancer [14], prostate cancer [100] and lung cancer [15]. Yeast display systems are also being investigated [59].

2.2. Cellular fractionation and reverse-phase protein arrays

An alternative to phage display is the reverse-phase protein microarray and two-dimensional (2-D) immunoblot. In this approach, tumor cell lines or tissue lysates are chromatographically fractionated, and each final fraction of the lysates is printed in microarray format and probed with sera [7,67] or immunoblotted by 2-D gel format [52]. Because the final fraction is a mix-

ture of antigenic molecules, further investigation, such as mass spectrometry, is required to identify the specific antigen. These approaches incorporate immunogenic variations in post-translational modifications that occur in cancer cells, and have been used to demonstrate antibodies in the sera of lung and prostate cancer patients [7,67].

2.3. Protein microarrays

Protein microarrays [55,110] and glycan arrays [99] offer emerging platforms to present tumor antigens to screen for immune responses. In comparison to traditional ELISAs, protein microarrays are capable of presenting and assessing hundreds of tumor antigens simultaneously. The responses are rapidly identified because the address of each protein is known in advance and there are no representation issues; all proteins, even rare ones, are represented equally (usually in duplicate). The proteins are arrayed on a single microscope slide and require only a few microliters of serum per assay. Known tumor antigens as well as predicted tumor antigens can be included to generate a comprehensive protein tumor antigen array. High density recombinant protein arrays have been applied to profile ovarian cancer immune response leading to the discovery of novel tumor antigens [33].

Despite their potential, protein microarrays are not yet widely used. In part, this may be due to the labor and technical issues associated with the high-throughput (HT) production, purification, and quality control of proteins for spotting on the array. Traditional production of tumor antigen microarrays, for example, requires the purification of hundreds of different human proteins for spotting on the array. The expression and purification of mammalian proteins is tedious and costly and often unsuccessful in heterologous systems. In addition, the use of these proteins on a microarray for clinical research would require significant quality control during the protein preparation and the printing of the arrays. Moreover, because proteins are notoriously labile, concerns arise about the stability of proteins on the array during storage. Commercial human proteome arrays are expensive, limiting cancer immune profiling that requires large sample sizes to identify low prevalence biomarkers.

As an alternative for printed protein microarrays, programmable protein microarrays (NAPPA) substitutes using purified proteins with cDNAs encoding the target proteins at each feature of the array. The proteins are then transcribed and translated by a cell-free system

and immobilized *in situ* using epitope tags fused to the proteins [69,70]. This approach eliminates the need to express and purify individual proteins. This chemistry also has the advantage that mammalian proteins can be expressed in a mammalian milieu (rabbit reticulocyte lysate) to increase the efficiency of expression and to encourage natural folding of the proteins [9,102].

2.4. Validation assays: High-sensitivity ELISAs and Bead-Arrays

Once AAb's are identified, moving these potential biomarkers forward into blinded, multicenter clinical testing requires clinical-grade diagnostic assays. For clinical AAb diagnostics, the most common method, ELISA, uses purified recombinant proteins that are adhered on the surface of plates [18,109]. However, the use of bacterial recombinant proteins or peptides may result in loss of conformational epitopes or lack of mammalian post-translational processing [85,107]. Newer assays, such as Luminex bead arrays [44,104] and electrochemiluminescence (ECL) assays [56], have been developed to perform multiplexed AAb detection in clinical platforms.

3. Autoantibody biomarkers in breast cancer

A number of tumor-specific AAb have been detected in the sera of breast cancer patients (Table 1), but only a few of these antigens (TP53, Her2/neu, MUC1) have been examined in detail as potential diagnostic or prognostic markers in early stage breast cancer. Many of these antigens are also detected in other cancers, limiting their potential clinical applications.

3.1. Cancer-testis antigens

Cancer testis (CT) antigens are a class of immunogenic tumor antigens with tissue expression restricted to the testis and to multiple cancers (including breast cancers), but not in adult somatic tissues. SEREX and phage display microarray screening of breast cancer and testicular libraries has identified a number of CT-specific AAb in breast cancer, including NY-ESO-1 [37], SSX-2 [37,42], SCP-1 [38], NY-BR-1 [38], MAGE-D [77], MAGE-3 [77], and MAGE-6 [77]. However, in general, these AAb are detected in few patients with early stage breast cancer. AAb to NY-ESO-1 are found in 4% [35], SCP-1 in 6% [38], and SSX2 in only 1% [86] of sera from breast cancer patients.

Table 1
Breast cancer autoantibodies

Analysis	Function	Marker	Cases/controls	Sensitivity	Specificity	Reference
Single Markers	Apoptosis	SURVIVIN	46/10	24%	–	[2, 106]
		LIVIN	46/10	33%	–	
		P53	165/230	7%–21%	95%–100%	[2,99, 29,99, 50,99, 68,99, 97,99]
			165/0			
	Growth	ANNEXIN XIA	144/242			
			90/51	19%(PBC)	98%	[25]
				(60%DCIS)		
	Signaling	RPA32	801/65	11%	100%	[93]
		SPAG9	94	80%	–	[43]
	Adhesion	HER2	107/200	11%	100%	[5, 20]
AAb Panels	Other	CYCLOPHILIN	28/21	86%	52%	[89]
		GLOBO H	58/47	58%	73%	[99]
		FXBP52	142/93	–	95%	[18]
		PPIA				
		PRDX2				
		HPS0				
		MUC1				
		ASB-9	87/87	80%	100%	[109]
		SERAC1				
		RELT				
		BRCA1	133/94	64%(PBC)	85%	[12]
		C-MYC		45%(DCIS)		
		HER2				
		MUC1				
		NY-ESO1				
		P53				
		BRCA2				
		P53 mutants	55/8	16%	100%	[4]
		C-MYC	64/82	77%–92%	85%–91%	[46]
		CYCLIN B1				
		IMP1				
		KOC				
		P53				
		P62				
		SURVIVIN				

PBC: Primary Breast Carcinoma; DCIS: Ductal Carcinoma In Situ.

Sperm-associated antigen 9 (SPAG9) is a CT antigen that is overexpressed in 88% of breast cancers, and AAb to SPAG9 were detected in 80% of patients with SPAG-9 expressing tumors. SPAG9 is also associated with a higher risk of tumor recurrence [43].

3.2. TP53

Tumor protein p53 is an example of a mutated tumor antigen (reviewed in [83]). Originally identified in 1979 [17], p53 AAb are correlated with p53 mutations and are thought to result from accumulation of mutant p53 in the cell nucleus [10,103]. Antibodies to p53 have been studied in patients with a wide variety of cancers, which has demonstrated a strong correlation between AAb in serum and cancer [83]. P53-AAb are more frequently detected in cancers associated with p53 mutations, including head and neck carcinoma

(32%), esophageal carcinoma (30%), colorectal carcinoma (24%), and carcinoma of the uterus (23%) [81].

p53-specific AAb have been detected in 10–15% of early-stage breast cancer [2,29,76]. As with other cancers, p53-AAb in breast cancer are associated with p53 mutations in the primary tumor [96]. The titer of anti-p53 AAb has been shown to specifically decrease after neoadjuvant chemotherapy [2] and increase prior to disease relapse [71]. P53 AAb are variably associated with prognosis [50,57]. However, current ELISA assays for p53 AAb have poor concordance, which may explain the different clinical findings [97].

3.3. Her2/neu

The epidermal growth factor receptor Her2/neu encodes a transmembrane receptor tyrosine kinase that

induces cellular proliferation [79]. In 20–25% of breast cancers, genetic amplification leads to overexpression of the HER2 oncogene and a poorer clinical prognosis. AAb to Her2/neu have been detected in the sera of 20% of Her2+ patients with early stage breast cancer and in 7% of late stage breast cancer patients [19,20]. These antibodies can function to block Her2 phosphorylation [60], but the impact on clinical outcome is not known.

3.4. Apoptotic proteins

Many proteins associated with the apoptotic pathway are highly immunogenic. AAb and T cells to ML-IAP have been detected after melanoma vaccination [80]. Among the 6 known inhibitors of apoptosis, survivin and livin are both highly expressed in breast cancer, and expression of survivin is associated with poor survival [84]. AAb to survivin are detected in 11 of 46 (24%) and AAb to livin are detected in 15/46 (33%) of breast cancer patient sera [106].

3.5. Carbohydrate antigens expressed on the surface

Cancer-associated carbohydrate antigens expressed the surface of cancer cells may be shed into the serum, and alterations in glycan processing can lead to glycan- and glycoprotein-specific AAb. AAb to MUC1 [98] have been associated with a reduced risk for disease progression in patients with breast cancer [34]. Using a glycan microarray of Globo H and its analogs, AAb to Globo H were specifically detected in breast cancer patient sera [99].

3.6. Other markers

SEREX approaches have identified a number of other tumor antigens that are detected by breast cancer patient sera, including fibulin-1, lactate dehydrogenase-A (LDH-A), lactate dehydrogenase-B (LDH-B), thyroid hormone-binding protein (THBP), and TPD52 [26,77]. AAb to the signal transduction related protein replication protein A (RPA32) have been detected in 87/801 patients with invasive breast cancer and 4/39 patients with ductal carcinoma in situ (DCIS) [93]. Using a novel approach of directly cloning monoclonal antibodies representing the native B cell repertoire from breast cancer patients, GIPC1 was identified as a breast cancer AAb [75].

Using 2-D separation of breast cancer cell lines and immunoblotting with breast cancer patient sera, AAb to

a novel protein that regulates RNA–protein interaction (designated RS/DJ-1) was identified [48]. Similarly, using 2-D separation of breast cancer tumor tissue and LC-MS/MS analysis, AAb to cyclophilin A (peptidyl-prolyl cis-trans isomerase A), triosephosphate isomerase and ubiquitin-conjugating enzyme E2N were detected [89]. Immunoblotting of urine from breast cancer patients and MALDI-TOF, AAb to the alpha 2-HS glycoprotein (AHSG) were identified. AHSG AAb were detected in 64 of 81 breast cancer patients (79.1%) and in 7 of 73 controls (9.6%) [108].

4. Multiplexed autoantibody signatures for breast cancer detection

One of the limitations for most of the tumor antigens identified to date is that a relatively small fraction of patients respond to each antigen. Thus, a biomarker test based on a single antigen would demonstrate poor sensitivity. In order to increase the predictive value of tumor-specific antibodies for use as immunodiagnos-tics, several groups have begun testing multiple anti-gens in parallel [2,94,41,94,72,94,87,94,92,94]. This approach offers a greater chance that any patient will reveal a response to at least one of the antigens, and may distinguish between different tumor types. In a lung cancer study of a panel of seven tumor antigens (p53, c-myc, HER2, NY-ESO-1, CAGE, MUC1 and GBU4-5), sera from 76% of lung cancer patients were shown to have elevated autoantibody levels against at least one of the seven antigens at a specificity of 92% [13]. Similarly, using a panel of seven tumor-associated antigens (c-myc, cyclin B1, p62, IMP-1, Koc, p53, and survivin), sera from patients with different cancers could be distinguished from each other and from healthy donor sera [46]. Of these 7 antigens, AAb to c-myc, Koc, and cyclin B1 were identified in the breast cancer patient sera. Sensitivities ranged from 0.77–0.92 and specificities ranged from 0.85–0.91. With a panel of six tumor antigens (p53, c-myc, HER2, NY-ESO-1, BRCA2 and MUC1), elevated levels of AAb were seen in at least one of the six antigens in 64% of primary breast cancer patient sera and 45% of patients with ductal carcinoma *in situ* (DCIS) at a specificity of 85% [12]. Using phage display, a 12-antigen biomarker panel (annexin XI-A, Ku, RP-S6, and several unknown antigens) discriminated sera from both invasive and in situ breast carcinomas and controls [24]. A combined analysis of five tumor antigens (FKBP52, PPIA, PRDX2, HSP60 and MUC1) discriminated primary breast cancer (AUC =

0.73; 95% CI: 0.60–0.79) and carcinoma in situ (CIS) (AUC = 0.80; 95% CI: 0.71–0.85) from healthy individuals [18]. These results demonstrate that the simultaneous assessment of antibodies to multiple tumor antigens markedly may enhance the utility of tumor antigen-specific antibodies as immunodiagnostics of cancer.

5. Impact of tumor subtype on autoantibody profiles

Breast cancers are classified into different cancer types depending on the expression of ER, PR, and Her2 [64]. Hormone-receptor negative cancers (estrogen receptor (ER) and progesterone receptor (PR) negative) and Her2+ cancers are more rapidly proliferative, have increased mortality, and occur more frequently in younger women where mammographic screening is less reliable. In addition, these subsets of breast cancers are more prevalent in minority populations. The basal-like triple-negative subset (ER-PR-HER2-) is more frequent in premenopausal African-American women (39% vs. 16%) [8], and Her2+ cancers are more frequent in premenopausal Hispanic women [47].

Since the breast cancer tumor subtypes have different patterns of gene expression, it is predicted that serum biomarkers will have different sensitivities of detection for the different biologic types. Early data suggests that AAb biomarkers may be subtype-specific. Antibodies to p53 have been shown to be preferentially detected in ER- cancers, consistent with the increased rate of p53 mutations in those cancers [2,29,76,101]. Antibodies to survivin were selectively detected in Her2+ cancers [2], and AAb to Her2/neu are specifically detected in Her2+ cancers [19]. Clinical development of these biomarkers will need to take into account the relative sensitivities of detection and rates for these different breast cancer subtypes, with an emphasis on the detection of cancers that are more frequently missed by mammographic screening.

6. Validation studies of autoantibody biomarkers

Currently, the only serum biomarkers in use for breast cancer are CEA, CA27.29, and CA 15-3, which are used for monitoring response to therapy for patients with advanced disease [32]. Despite promising early studies, no serum AAb biomarkers have been demonstrated in phase III or later validation studies [63] for

early detection, prediction, or prognosis of early-stage disease. This is likely due to several factors.

First, assay development for the clinical detection of AAb biomarkers requires thorough evaluation of each AAb to determine their individual sensitivities and specificities. The predictive value of each antigen must be considered in the context of the other antigens, as it is possible that antigens do not all behave independently, but rather reveal concordant responses. Most proteomic-based discovery platforms are not technically amenable to large-scale multicenter serologic screening for clinical validation studies. These studies require both tightly controlled coefficients of variation and cost-effective assay development. Even traditional ELISA assays would require large scale production of protein for each antigen, usually in bacteria or baculovirus. Subtle changes in protein conformation or post-translational modifications of recombinant protein may markedly change immunogenicity. Clinical-grade platforms designed for multiplexed detection of selected antigens using limited sera or plasma need to be developed and validated.

Second, most biomarker discovery projects rely on single-institution serum sets. Unlike proteomic-based detection assays, AAb levels are relatively stable and are less sensitive to sample collection variation, but may be dependent on age, gender, or other clinical factors. Cases and controls must be collected with identical sample handling protocols, in comparable time frames, ideally in the exact clinical setting for which the biomarker is designed (i.e. screening mammography). Cases should be collected prior to clinical diagnosis and biopsy, since the biopsy itself has the potential to alter biomarker levels. Controls should also include benign breast disease controls, including both proliferative and non-proliferative lesions. Potential confounding variables include clinical autoimmunity, hormone use, menopausal status, immunosuppressive medications, and parity. The National Cancer Institute Early Detection Research Network (NCI EDRN) is developing a multi-center Breast Cancer Reference Set of plasma and sera for investigator use, which will be an invaluable resource for blinded validation studies of potential AAb biomarkers.

7. Applications for clinical development

Serum-based biomarkers have a tremendous potential for early breast cancer detection, but the application of these markers will need to incorporate radiograph-

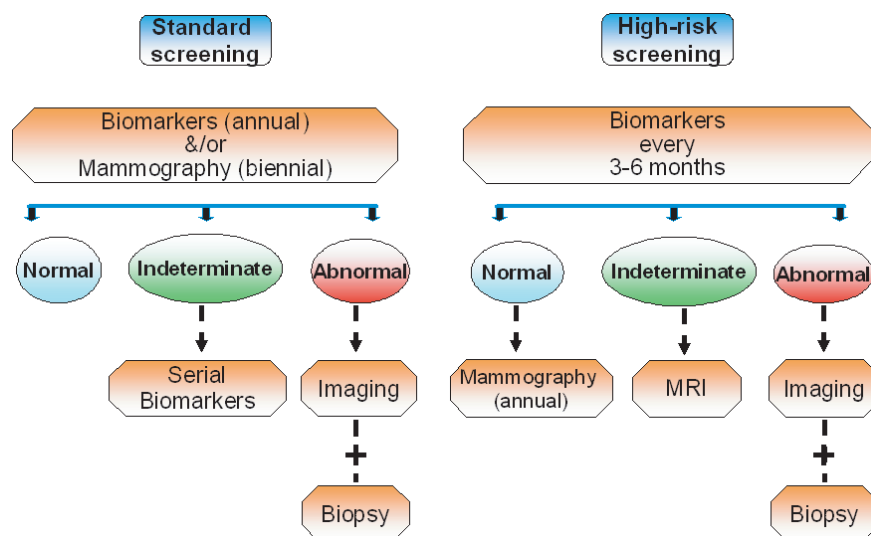


Fig. 1. Tailored biomarker-based detection for breast cancer. Left. Serum-based biomarkers can be used as an adjunct (or possibly replacement) for routine screening mammography to identify higher-risk patients. Changes in biomarker levels would prompt further imaging (ultrasound and/or MRI) for tumor localization. Right. Higher-risk patients (identified by epidemiologic and/or genetic factors) would undergo more intensive serum biomarker screening as well as imaging studies based on level of risk.

ic screening (mammography, ultrasound, and/or MRI) for tumor localization. Recent guidelines from the U.S. Preventive Services Task Force recommend biennial mammographic screening for average-risk women between ages 50 and 74 [1]. The National Comprehensive Cancer Network (NCCN) guidelines recommend annual mammography and breast MRI screening for high-risk women ($> 20\%$ lifetime risk), based on epidemiologic risk, genetic risk (BRCA1/2 carriers), or prior radiation [49]. Statistical modeling by the Cancer Intervention and Surveillance Modeling Network (CISNET) has estimated that the proportion of the total reduction in the rate of death from breast cancer attributed to screening is 28 to 65 percent (median, 46 percent) [6].

The use of mammography as a primary screening tool has limitations. High-grade, rapidly-proliferative cancers that are associated with higher mortality frequently present as interval cancers, and are not detected by routine screening. In the neoadjuvant I-SPY clinical trial of locally advanced breast cancers, 85% of the malignancies were interval cancers (not detected with routine screening) [23]. Increased breast density both obscures the detection of breast cancers by mammography and is one of the strongest predictors of breast cancer risk [90,91]. Women in the highest category of mammographic density have a four- to six-fold increased risk of breast cancer as compared with women in the lowest category of risk. Mammographic density is higher

in premenopausal than in postmenopausal women. The mechanism by which mammographic density increases breast cancer is unclear, although it may represent increased cellular proliferation and cumulative exposure to steroid hormones.

There are several potential clinical applications for breast cancer biomarkers for early detection (Fig. 1). Blood-based biomarkers could be used to 1) replace mammography, 2) enhance detection by mammography, or 3) risk stratification for further imaging, such as MRI screening. Targeted screening approaches that rely on the identification of higher-risk populations by clinical, epidemiologic, and genomic data are likely to have the greatest clinical impact [23]. More intensive screening of these high-risk populations may involve frequent AAb biomarker screening (i.e. every 3–6 months) as an adjunct to yearly mammographic detection.

8. Summary and future directions

Proteomic-based detection has led to the identification of multiple cancer-specific and breast cancer-specific autoantibodies that develop due to cancer-induced changes in protein or glycan expression and structure. Serologic immune profiling, using panels of tumor antigens, shows promise as potential biomarkers of breast cancer, but blinded validation studies us-

ing well-annotated sera cohorts are required for further clinical applications. These AAb profiles have the potential for use in early detection, as well as the prognosis of breast cancer.

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