

## Humoral Immunity Directed against Tumor-Associated Antigens As Potential Biomarkers for the Early Diagnosis of Cancer

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Over the past decade, it has been demonstrated that cancer is immunogenic, and multiple tumor antigens have been identified in cancer patients. It is now possible to potentially harness the immune response elicited by cancer growth as a potential diagnostic tool. Humoral immunity, or the development of autoantibodies against tumor-associated proteins, may be used as a marker for cancer exposure. Unlike circulating proteins that are shed by bulky tumors, serum autoantibodies are detectable even when antigen expression is minimal. This paper will review the methods used for tumor antigen discovery and overview what is known about autoantibodies targeting common cancer antigens with a focus on breast cancer. Data will be presented modeling the use of tumor antigen associated autoantibodies as a breast cancer diagnostic. The endogenous humoral immune response present in cancer patients may allow the identification of individuals exposed to the malignant transformation of somatic cells.

**Keywords:** breast cancer • serum antibody • autoantibody • cancer diagnosis • biomarker

### Introduction

Many solid tumors are potentially curable if diagnosed at an early stage when the cancer can be completely surgically removed. Novel methods to aid in early diagnosis of cancer are sorely needed. A case in point is the need for early diagnosis in patients with breast cancer. Breast cancer is the most commonly diagnosed cancer in women.<sup>1</sup> Despite the availability of routine screening with mammography, about 40% of breast cancers, when first diagnosed, are not localized.<sup>1</sup> The development of new biomarkers that may help in the early detection of breast cancer will greatly facilitate the clinical management of the disease. Early detection by novel methods is critically important in younger premenopausal women whose mammograms may be compromised by increased breast density. The development of a serum-based assay that could indicate cancer exposure would be of great benefit. The detection of tumor-shed proteins in serum may be challenging due to the abundance of nonspecific serum proteins such as albumin and the requirement for larger tumor bulk to be able to detect the circulating shed protein. A promising alternative approach is to identify immune response markers, that is, serum autoantibodies that are generated in response to tumor-associated antigens (TAAs).

Tumors can express aberrant levels of mutated or modified forms of proteins that are associated with malignant growth. Such proteins can be immunogenic and stimulate cellular and humoral immune responses.<sup>2-4</sup> A number of TAAs, which elicit

humoral immunity, have been identified in cancer patients, particularly breast cancer.<sup>2,5,6</sup> Autoantibody responses to TAAs are currently being investigated as potential diagnostic tools in multiple cancer types and are associated with several characteristics which would facilitate assay development. Serum antibody is stable, and can be readily detected with well-validated secondary antibodies.<sup>7</sup> Furthermore, B cells can produce specific antibodies in large amounts after stimulation by a small amount of tumor antigen.<sup>8</sup> As a result, TAA-specific serum antibodies can be detected at high titer in patients with early stages of cancer.<sup>2</sup>

### Methods To Identify Serum Autoantibodies As Potential Diagnostic Biomarkers

The successful identification of serum antibody markers is dependent on the development of high-throughput screening assays. The recent advances in proteomic technologies such as mass spectrometry and protein array have greatly facilitated the discovery of new antibody markers in cancer patient serum.<sup>9</sup> Both DNA- and protein-based techniques have been useful in identifying autoantibody biomarkers.

A powerful technique that has resulted in the identification of over 2000 immunogenic TAAs is Serological Screening of cDNA Expression Library (SEREX). SEREX was first developed by Sahin et al.<sup>10</sup> about 10 years ago. In this approach, a cDNA library is constructed using RNA from tumor specimens packaged into  $\lambda$ -phage vectors and expressed recombinantly in *Escherichia coli*. Recombinant proteins are transferred onto nitrocellulose membranes. The membranes are incubated with sera from cancer patients or control donors. The clones that are only reactive to serum from cancer patients are subcloned

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to monoclonality, and the nucleotide sequence of the inserted cDNA is determined.<sup>11,12</sup> Multiple breast cancer antigens have been identified using SEREX, including NY-BR-1 through NY-BR-7,<sup>3,13</sup> cancer-testis antigens NY-ESO-1 and SSX2,<sup>14</sup> ING1-a candidate breast cancer suppressor gene,<sup>14</sup> fibulin,<sup>15</sup> hMena,<sup>16</sup> lactate dehydrogenase-A (LDH-A),<sup>15</sup> thyroid hormone-binding protein (THBP),<sup>15</sup> and replication-protein A,<sup>17</sup> to name a few. The diagnostic value of these SEREX-identified antigens remains to be tested in large-scale studies. Furthermore, it is unknown how the use of *E. coli* as the protein expression system affects the identified antigenic repertoire. Most likely a significant number of antigens cannot be adequately identified using this technique.

Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) can be used to separate thousands of individual cellular proteins from tumor tissue or cell lines. The separated proteins are transferred onto membranes. The membranes are probed with sera from cancer patients or normal donors. The proteins that only react with sera from cancer patients will be identified by mass spectrometric analysis and/or amino acid sequencing. With this method, antibodies to RS/DJ-1, an oncogenic protein that regulates RNA-protein interaction, were identified in the sera from breast cancer patients.<sup>18</sup> A limitation of this strategy is its relatively low throughput.<sup>19</sup>

A high-throughput approach to autoantibody discovery is protein array as robotic microarray spotters allow the grouping of thousands of proteins, in replicate, onto a single glass slide and make it possible to evaluate the presence of serum antibody to hundreds of proteins simultaneously. Several protein microarray platforms have been developed for high-throughput analysis. The recombinant protein arrays use clones from cDNA expression libraries or peptide phage display libraries;<sup>20–22</sup> the native protein arrays use proteins derived from tumor tissue or cell lines.<sup>23,24</sup> Recent studies on prostate,<sup>20</sup> lung,<sup>21</sup> ovarian,<sup>25</sup> and breast cancer<sup>26</sup> have used the phage display technology. This approach involves the construction of a T7 cDNA phage display library from tumor tissue or a cell line. The candidate antigen peptides are expressed and displayed on the surface of a phage. The advantage of this approach is that the libraries can be enriched with peptides specifically recognized by patient serum using a process called biopanning before they are spotted on the array. Biopanning entails successive rounds of immunoprecipitation of phage libraries using patient serum to select the peptides recognized by antibodies in patient serum and using normal donor serum to remove the peptides recognized by antibodies in normal serum.<sup>20,27,28</sup> The limitation is that the peptide sequence is short and the immunogenicity of the noncoding sequence as detected in some of the studies may be difficult to interpret.<sup>20</sup> Using bacteria or virus-expressed full-length recombinant proteins allows the study the immunogenicity of candidate antigens at a whole protein level, but still misses post-translational modifications such as phosphorylations and glycosylations, which may be essential to the immunogenicity of the proteins. In that respect, arraying proteins isolated from tumors or tumor cell lines may be better suited for uncovering immunogenic proteins. Fractionated proteins from a tumor cell lysate can be used to spot the array.<sup>23,24</sup> In the study by Qiu et al., protein lysates from the A549 human lung adenocarcinoma cell line were separated into 1840 fractions that were spotted in duplicate, along with various controls, on nitrocellulose-coated slides. Sera from lung cancer patients and healthy controls were each hybridized to an individual microarray. The

**Table 1.** Serum Antibody Responses to TAAs Detected in Breast Cancer Patients

tumor antigen	serum antibody positivity in breast cancer patients	references
HER2	11%	Disis et al. <sup>2</sup>
	55%	Disis et al. <sup>51</sup>
	7%	Disis et al. <sup>52</sup>
P53	48%	Willsher et al. <sup>6</sup>
	46%	Regele et al. <sup>53</sup>
	26%	Green et al. <sup>54</sup>
	26%	Mudenda et al. <sup>55</sup>
	21%	Gao et al. <sup>56</sup>
	5%	Angelopoulou et al. <sup>57</sup>
	15%	Regidor et al. <sup>58</sup>
	12%	Peyrat et al. <sup>59</sup>
	8%	Dalifard et al. <sup>60</sup>
	9%	Crawford et al. <sup>5</sup>
MUC1	10%	Goodell, unpublished
	8%	Kotera et al. <sup>61</sup>
	26%	von Mensdorff-Pouilly et al. <sup>46</sup>
	20–23%	Chapman et al. <sup>50</sup>
	20%	Goodell, unpublished
Endostatin	42–66%	Bachelot et al. <sup>62</sup>
Lipophilin B	27%	Carter et al. <sup>63</sup>
HSP90	37%	Conroy et al. <sup>64</sup>
Cyclin B1	43%	Suzuki et al. <sup>65</sup>
Fibulin	75%	Pupa et al. <sup>66</sup>
Cyclin D1	8%	Goodell, unpublished
Cathepsin D	5%	Goodell, unpublished
TOPO2 $\alpha$	7%	Goodell, unpublished

intensity measures of duplicate spots (within-slide) and duplicate slides (between-slides) were highly reproducible, exhibiting correlation values  $>0.9$ .<sup>23</sup> The disadvantage of this method is that each spot on the array may have multiple proteins, and subsequent identification of the individual immunogenic protein can be challenging. The inability to control protein orientation during immobilization also remains a limitation.<sup>29</sup> Similar to SEREX, the candidate markers that emerged from protein array screening remain to be validated in large populations.

High-throughput technologies have now allowed the identification of hundreds of candidate autoantibodies for use as biomarkers. While array-based approaches are being developed as diagnostic assays, we hypothesize that only a limited number of autoantibodies may be needed for adequate sensitivity and specificity. The identification of a limited panel of antigens that may provide broad population coverage within a specific malignancy will allow the development of clinical grade ELISA assays, greatly facilitating clinical application. The ability to develop a successful diagnostic assay, however, is dependent on several factors such as the ability to detect the autoantibody in the premalignant state, the prevalence of the autoantibody in a specific population, or even the specificity of the autoantibody for a specific tissue type. Population-based studies of individual autoantibodies can give some indication of whether the detection of humoral immunity may aid in discriminating cancer patients from noncancer bearing individuals.

### Autoantibodies in Breast Cancer

Table 1 shows the frequency of autoantibodies associated with known breast cancer antigens. Serum antibodies to a few of most well-studied breast cancer antigens, p53, HER-2/neu,

and MUC1, will be discussed below, and from these descriptions, some general conclusions can be drawn as to characteristics which may prioritize a candidate autoantibody for diagnostic development.

p53 is one of the most extensively studied tumor antigens. It is an approximately 53 kDa nuclear phosphoprotein which normally plays the role of tumor suppressor as an intermediary of natural cell death. Wild-type p53 acts in a dominant fashion to suppress uncontrolled cell growth, serving as a mediator of cell cycle arrest or apoptosis. In normal cells, p53 is present at a very low level and exclusively in the nuclei. p53 mutations can occur in up to 50% of all cancers.<sup>30</sup> Mutation inactivates normal function, resulting in 'immortalized' cells. Mutant p53 accumulates in the cancer cell cytosol and nucleus and, thus, is specific to cancer cells. Many studies have shown that p53 mutations may occur early in the transformation of some cancers, an essential characteristic for use as an early cancer detection tool.<sup>26,27</sup> Increased p53 protein in tumor cells is indicative of a mutated p53 gene, and the increased level of p53 may elicit an immune response resulting in anti-p53 autoantibody in serum.<sup>31,32</sup> Antibodies against the p53 protein have been detected in the serum of patients with many cancers such as breast cancer, Burkitt's lymphoma, lung cancer, and pancreatic cancer.<sup>33</sup> There is a strong correlation between accumulation of p53 in primary tumor cells and presence of serum p53-specific antibodies in patients with different tumor types.<sup>34</sup>

Multiple studies have focused on the evaluation of autoantibodies to p53 as a diagnostic tool due to reports suggesting that the antibody responses to p53 can occur early in the course of a cancer and predict undetected malignancy or premalignancy. One of the earliest reports described the evolution of the p53 antibody response in patients at high risk of developing lung cancer, heavy smokers.<sup>35</sup> Although study subjects were free of cancer at the time antibody assessment started, rising titers of p53 antibodies preceded the development of early stage lung cancers bearing p53 mutations in two patients. Additional studies have shown that p53 specific antibodies can be detected prior to clinical diagnosis of cancer.<sup>36,37</sup> Serum p53 antibodies have been detected in 11.6% (5/43) of early stage breast cancer patients with ductal carcinoma in situ of the breast, a preinvasive lesion. Three of the 5 seropositive patients had lesions no larger than 5 mm.<sup>38</sup>

The detection of autoantibodies may be used as an adjunct to more standard serologic tests being evaluated to aid in cancer diagnosis. Muller et al. showed that the addition of p53 specific antibody detection to conventional tumor markers (CEA for colon cancer, AFP for hepatocellular carcinoma, CEA and CA15-3 for breast cancer, CA72-4 for gastric cancer) led to an increase in diagnostic sensitivity of 8% without decreasing specificity.<sup>39</sup> The methods to measure anti-p53 antibody in serum are straightforward. The recombinant protein is available through a commercial source. Furthermore, a cell lysate-based assay that utilizes BT-20, a cell line that overexpresses p53, to measure anti-p53 has also been developed to be Clinical Laboratory Improvement Act (CLIA)-compliant.<sup>40</sup> The disadvantage of the use of p53 humoral immunity as a single biomarker is the lack of specificity for any particular cancer.

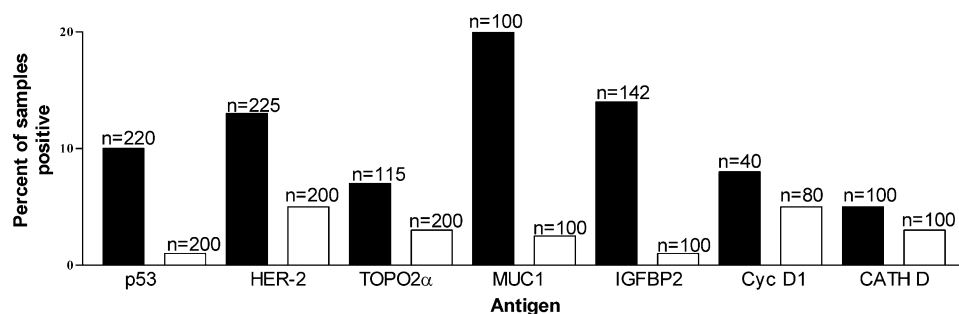
Another well-known TAA for breast cancer is HER-2/neu. HER-2/neu is an approximately 185 kDa protein and is a member of the epidermal growth factor receptor group, a transmembrane phosphoglycoprotein receptor presumed to act as a growth factor receptor. The nonmutated protein is

expressed at low levels in normal cells but constitutively overexpressed at high levels by malignant cells. The gene for HER-2/neu is present in many normal tissues as a single copy. Amplification of the gene (rather than mutation) and/or overexpression of the protein on the cell surface has been identified in multiple cancers. Protein overexpression occurs in approximately 30% of breast cancers, particularly premenopausal breast cancer, and is associated with more aggressive disease and a poor prognosis in patients with positive lymph nodes.<sup>41</sup>

Similar to investigations of antibody response to p53, endogenous humoral immunity to HER-2/neu directly correlates to overexpression of the protein by the patient's tumor, and HER-2/neu-specific autoantibodies can be detected in patients with early stage disease, indicating that the presence of antibodies are not simply a reflection of tumor burden. HER-2/neu antibodies at titers of >1:100 were detected in 12 of 107 (11%) breast cancer patients versus 0 of 200 (0%) controls ( $p < 0.01$ ).<sup>2</sup> Detection of antibodies to HER-2/neu also correlated to overexpression of HER-2/neu protein in the patient's primary tumor. Nine of 44 (20%) patients with HER-2/neu positive tumors had HER-2/neu-specific antibodies, whereas 3 of 63 (5%) patients with HER-2/neu-negative tumors had detectable antibodies ( $p = 0.03$ ). Furthermore, we have recently shown that antibodies are associated with the extent of protein overexpression in primary tumor.<sup>42</sup> The presence of HER-2/neu-specific antibodies in breast cancer patients and the correlation with HER-2/neu-positive tumors implies that immunity to HER-2/neu develops as a result of exposure of patients to HER-2/neu protein expressed by their own cancer.

HER-2/neu specific autoantibodies have been found in the sera of patients with colon cancer, and, again, their presence correlates with overexpression of protein in the primary tumor ( $p < 0.01$ ).<sup>43</sup> HER-2/neu has also been demonstrated to be a shared tumor antigen in patients with prostate cancer. Antibody immunity to HER-2/neu was significantly higher in patients with prostate cancer (15.5%, 31/200) compared with controls (2%, 2/100,  $p = 0.0004$ ), and titers greater than 1:100 were most prevalent in the subgroup of patients with androgen-independent disease (16%, 9/56).<sup>44</sup> Studies such as those described here provide the basis for evaluating antibodies to HER-2/neu as a potential tool for cancer diagnostics, but also underscore the questionable utility of single antibody evaluation for diagnosis. Although the specificity of the approach may be significant, that is, few responses are found in noncancer bearing individuals, the sensitivity of antibodies to identify all patients with HER-2/neu overexpressing tumors is low.

Similar to HER2, MUC1 is also expressed on the cell surface. Mucins are a family of glycoproteins with high molecular weight that have a large number of tandem repeat domains that vary in length. MUC-1 has been found to be expressed abundantly in many epithelial tumors including the majority of breast cancers,<sup>45</sup> and circulating immune complex containing polymorphic epithelial mucin has been detected in breast cancer patients and patients with benign breast tumors.<sup>46,47</sup> An evaluation of the immunogenicity of MUC1, however, identifies a significant potential obstacle in the use of autoantibodies for cancer diagnosis. A large study, involving 101 patients with breast cancer, 40 women with benign breast tumors and 96 healthy controls, suggested that MUC1-specific antibody immunity was found more often among women with benign disease than in women with breast cancer. Indeed, a negative correlation was found between presence of MUC1



**Figure 1.** Antibodies to tumor associated antigens are more frequently detected in sera from breast cancer patients than from normal donors. Shown are the percentages of individuals positive for serum antibody to 7 tumor antigens. Gray columns show the response in patients; white columns show the response in control normal donors. The number of patients or controls tested for each antigen were indicated at the top of the column. The antibody responses to TOPO2 $\alpha$ , IGFBP2, cathepsin D (CATH D), MUC1, and cyclin D1 were measured using recombinant ELISA. The antibody responses to p53 and HER2 were measured using capture ELISA as previously described.<sup>40</sup> A sample was defined as positive if the antibody concentration was greater than the mean  $\pm$  3SD of the reference population.

antibodies and extent of disease, such that the rate of positive response dropped from 38% in women with nonmalignant lesions to 26% in women with newly diagnosed breast cancer. A further drop to 18% was found in women with recurrent or progressive breast cancer. Earlier this year, the same investigators went on to demonstrate that within a population of 127 women with BRCA1 or BRCA2 mutations, MUC1 antibody levels were significantly lower than those found in 370 age-matched controls.<sup>48</sup>

These clinical reports of autoantibodies against TAA detected in cancer patients with much higher frequency than control donors demonstrate that autoantibodies can be raised against both intra- and extracellular proteins, that circulating autoantibodies can be found in both early stage as well as preinvasive tumors, and that TAA autoantibodies can be identified in high risk patients who are not yet tumor bearing. All these characteristics would be a benefit for a diagnostic assay. However, autoantibodies can also be associated with benign disease or even be detected at lower levels in tumor bearing individuals than controls. Moreover, measurement of a single autoantibody will not provide the adequate sensitivity needed for a diagnostic test. These observations underscore the need to fully characterize an autoantibody response across multiple populations prior to clinical development.

### Detecting Serum Antibody Response to a Panel of TAAs

The use of a single antibody as predictor of disease exposure has obvious limitations because the test is only valid to patients whose tumors harbor the antigens. For example, anti-p53 antibodies have been studied in over 9489 patients with a wide variety of tumors.<sup>36</sup> Despite the strong specificity of the response, only 20–40% of patients with cancers harboring p53 missense mutations will have p53 antibodies in their sera. Since no single serum antibody marker exists in all the patients, we seek to identify a combination of markers that may increase patient coverage. This statement is based on the assumption that multiple serum antibodies specific to these TAAs can be detected simultaneously from the same patient. Our laboratory has developed multiple CLIA-compliant ELISA assays to measure TAA-specific antibodies in serum.<sup>40,49</sup> We recently investigated the serum antibody response to 7 well-characterized TAAs (p53, HER2, MUC1, topoisomerase II alpha (TOPO2 $\alpha$ ), insulin-like growth factor binding protein 2 (IGFBP2), Cyclin D1, and cathepsin D) in a heterogeneous breast cancer popula-

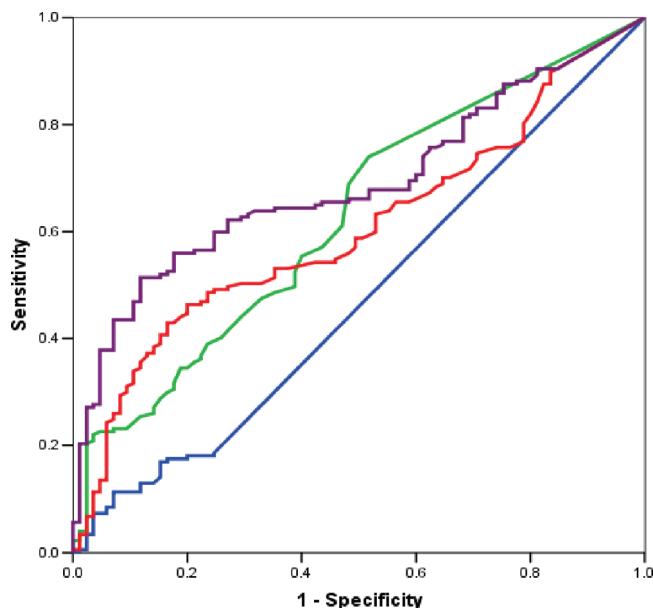
tion and control age- and gender-matched donors using validated ELISA assays (Figure 1). Approximately 18% of the breast cancer patients had early stage disease and approximately 82% had late stage disease. Patients were between the ages of 18–75. The controls met all requirements for donation to the regional blood center and were between the ages of 18 and 75. The antibodies to TOPO2 $\alpha$ , IGFBP2, and cathepsin D were measured by indirect ELISA using commercially available recombinant proteins as previously described.<sup>49</sup> Briefly, alternate columns on 96-well plates were coated overnight with purified human TOPO2 $\alpha$  (Topogen, Columbus, OH), IGFBP2 (Sigma Chemicals, Inc., St. Louis, MO), cathepsin D (U.S. Biological, Swampscott, MA), MUC1 (Abnova, Corp., Taipei), cyclin D1 (Research Diagnostics, Inc., Concord, MA), or carbonate buffer alone, blocked for 1 h with PBS/BSA, and washed with PBS/Tween. After washing, 50  $\mu$ L/well of control or experimental sera was added in duplicate titration sets. After overnight incubation at 4  $^{\circ}$ C, plates were washed again and anti-human/HRP conjugate was added at 50  $\mu$ L/well. Plates were washed again after a 45 min incubation at 4  $^{\circ}$ C and developed using TMB reagents (KPL, Gaithersburg, MD). The antibody responses to HER-2/neu and p53 were performed by capture ELISA as previously described.<sup>40</sup> Briefly, 96-well plates were coated with 520-C9 (monoclonal antibody to HER2) or TIB-116 (monoclonal antibody to p53) before the addition of SKBR3 cell lysate (HER2+) or BT-20 cell lysate (p53+) which serve as antigen sources. Serially diluted, purified human IgG provided a standard curve. A sample was defined as positive if the value was greater than mean  $\pm$  2SD (for HER2) or mean  $\pm$  3SD (for p53) of the previously analyzed reference population which were shown to be negative by Western blot analysis. Positive results for each assay were confirmed by Western blot analysis. With the use of Western blot as gold standard, the sensitivity of the assays is 77% (HER2) and 100% (p53), and the specificity of the assay is 89% (HER2) and 93% (p53).

As shown in Figure 1, we have found that breast cancer patients have increased antibody response to p53, HER2, MUC1, topoisomerase II alpha (TOPO2 $\alpha$ ), insulin-like growth factor binding protein 2 (IGFBP2), Cyclin D1, and Cathepsin D. The most frequently found antibody response was directed against MUC1, which was detected in 20% of the patients, compared to a responses rate in controls of approximately 3%. Thirteen percent and 10% of breast cancer patients had antibodies to HER-2/neu and p53, respectively, while only 5%

of controls had antibodies to HER-2/neu and only 1% of controls had p53-specific antibody responses. Cyclin D1 antibodies were found in 8% of patients, TOPO2 $\alpha$  antibodies were found in 7% of patients, and 5% of patients had cathepsin D autoantibodies. Cathepsin D antibodies and TOPO2 $\alpha$  antibodies were found in 3% of controls, and 5% of controls had cyclin D1 antibodies. Antibodies specific for tumor-associated antigens were found in patients with both early and late stage disease. This data demonstrates that breast cancer patients can generate immune responses to multiple antigens simultaneously. Although the serum antibody response rate to the best performing single antigen, MUC1, is no more than 20%, addition of HER-2/neu to the panel increased the percent of positive samples to 25%, and addition of p53 and IGFBP2 increased the rate of positivity to 31%. Thus, 31% of the breast cancer patients analyzed have serum antibodies to at least 1 of 4 antigens tested, suggesting that diagnostic sensitivity may be improved by using a panel of serum antibodies for detection of malignancy.

Recent publications support the idea of using a combination of autoantibody markers for cancer diagnosis. Using a phage display library constructed from prostate cancer tissue, Wang et al. analyzed serum samples from 119 prostate cancer patients and 138 control using protein array. Serum antibody responses to the 22 phage-displayed peptide detector, as built from the training set of samples, could discriminate subjects with prostate cancer and a control group with 88% specificity and 82% sensitivity.<sup>20</sup> Moreover, assessment of serum antibody immunity performed better in distinguishing prostate cancer from controls than assessment of serum prostate-specific antigen levels, the currently used screening test for prostate cancer.<sup>20</sup> However, only 4 out of the 22 peptides were derived from in-frame, named coding sequences. The remaining phage peptides were generated from untranslated sequences. A caveat to the phage display and protein array data is that the peptide antigens identified from each study may be dependent on the tumor specimen used for the construction of library and the serum used for biopanning. The biologic meaning of the noncoding sequences identified in some of the studies is also difficult to interpret.

We questioned whether a combination of well-defined antibody markers for breast cancer may have improved sensitivity and specificity over using a single marker to discriminate cancer versus controls. As shown in Figure 2, samples from 184 breast cancer patients with late stage disease and 134 controls without malignancy were tested for responses to p53, HER-2/neu, IGFBP-2, and TOPO2 $\alpha$ , and responses were used to construct receiver operating characteristic (ROC) curves. Data presented here indicates that response to p53 alone was not a significant predictor of breast cancer (AUC = 0.48,  $p = 0.538$ ), but combining responses to 2 antigens (p53 and HER-2/neu) resulted in an AUC of 0.61 ( $p = 0.006$ ), and combining responses to all of the 4 antigens increased the area under the curve to 0.63 ( $p = 0.001$ ). Using an algorithm weighted on logistic regression coefficients of independent antibody markers resulted in an AUC of 70% ( $p < 0.001$ , Figure 2). This data suggests that a panel of autoantibodies is more efficient at discriminating cancer from controls than the use of a single antibody measurement. It has to be emphasized that most of the serum samples were obtained from patients with late stage disease. Whether the findings apply to early stage patients remains to be investigated. It is also noted that most of the patients in our study have received previous treatment for their



**Figure 2.** Antibody responses to a panel of tumor-associated antigens can distinguish between breast cancer patients and healthy controls. Serum samples from 184 breast cancer patients and 134 healthy controls were tested for responses to p53, HER2, IGFBP-2, and TOPO2 $\alpha$ , and responses were used to construct ROC curves. Response to p53 alone was not a significant predictor of breast cancer (AUC = 0.48,  $p = 0.538$ , blue line), but combining responses to 2 antigens (p53 and HER-2) resulted in an AUC of 0.61 ( $p = 0.006$ , green), and combining responses to all 4 antigens increased the area under the curve to 0.63 ( $p = 0.001$ , red). Using an algorithm weighted on logistic regression coefficients of independent antibody markers resulted in an AUC of 70% ( $p < 0.001$ , purple).

disease. It is important to validate the markers in the future using samples from newly diagnosed breast cancer patients. A recent publication by Chapman et al. also tested 137 patients for antibodies to a panel of 6 antigens (p53, MUC1, c-myc, NY-ESO-1, BRCA2, and HER-2/neu) and found that a total of 64% and 45% of patients with primary breast cancer and DCIS, respectively, were positive for at least 1 of the 6 antigens. Response rates to single antigens in this population ranged from 3% to 34%, but response rates for healthy controls were not reported.<sup>50</sup> Although preliminary, these data support further investigation to develop a multiplexed serum antibody based assays for breast cancer diagnosis.

### Conclusion and Future Directions

It is well-recognized that the immune surveillance against cancer can lead to the generation of serum antibodies recognizing TAAs, even at early stage of the disease. As summarized in this review, multiple TAA-specific serum antibodies have been reported in breast cancer patients. Preliminary data presented here as well as published data on other types of cancer support the idea of developing a serum assay evaluating the antibody response to a panel of tumor antigens for breast cancer diagnosis. The ideal screening assay will be easy to perform and compliant with CLIA standards. With the availability of novel high-throughput technologies such as phage display and protein array, multiple candidate markers have emerged. These candidates will need to be validated in larger populations with a comparison to known autoantibody markers to determine which biomarkers have the highest diagnostic

value. Furthermore, candidate biomarkers must be characterized as to whether the autoantibody is detected in early invasive or even preinvasive disease. The availability of well-characterized serum samples from newly diagnosed patients and samples collected before the clinical onset of disease will be fundamental to the validation of some of the candidate markers as described in the paper. Despite the challenges that lie ahead, the assessment of a panel of autoantibodies specific for TAA holds great potential as a new diagnostic tool in the fight against cancer.

### Abbreviations:

CLIA, Clinical Laboratory Improvement Act; DCIS, ductal carcinoma in situ; HER-2/neu, protein product of the erb-b2 gene, human epidermal growth factor receptor 2; MUC1, mucin 1; PAGE, polyacrylamide gel electrophoresis; ROC, receiver operating characteristic; SEREX, serological analysis of recombinant cDNA expression libraries; TAA, tumor associated antigen.

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