Published in final edited form as:
J Proteome Res. 2008 April ; 7(4): 1490–1499. doi:10.1021/pr700804c.

Application of Protein Microarrays for Multiplexed Detection of Antibodies to Tumor Antigens in Breast Cancer

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Abstract

There is strong preclinical evidence that cancer, including breast cancer, undergoes immune surveillance. This continual monitoring, by both the innate and the adaptive immune systems, recognizes changes in protein expression, mutation, folding, glycosylation, and degradation. Local immune responses to tumor antigens are amplified in draining lymph nodes, and then enter the systemic circulation. The antibody response to tumor antigens, such as p53 protein, are robust, stable, and easily detected in serum, may exist in greater concentrations than their cognate antigens, and are potential highly specific biomarkers for cancer. However, antibodies have limited sensitivities as single analytes, and differences in protein purification and assay characteristics have limited their clinical application. For example, p53 autoantibodies in the sera are highly specific for cancer patients, but are only detected in the sera of 10-20% of patients with breast cancer. Detection of p53 autoantibodies is dependent on tumor burden, p53 mutation, rapidly decreases with effective therapy, but is relatively independent of breast cancer subtype. Although antibodies to hundreds of other tumor antigens have been identified in the sera of breast cancer patients, very little is known about the specificity and clinical impact of the antibody immune repertoire to breast cancer. Recent advances in proteomic technologies have the potential for rapid identification of immune response signatures for breast cancer diagnosis and monitoring. We have adapted programmable protein microarrays for the specific detection of autoantibodies in breast cancer. Here, we present the first demonstration of the application of programmable protein microarray ELISAs for the rapid identification of breast cancer autoantibodies.

Keywords

Breast Cancer; Autoantibodies; Tumor Antigen; Biomarker; Proteomics

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Introduction

The early diagnosis of breast cancer is associated with a significant improvement in morbidity and overall survival[1]. While mammography is a highly successful screening modality, there remains a need for serologic biomarkers for the early diagnosis, monitoring, and classification of breast cancer. Despite this, very few serologic biomarkers have been developed for the diagnosis and management of breast cancer. CA27.29, CA15-3, and CEA are currently used in clinical practice, but by the 2007 biomarker guidelines for the American Society of Clinical Oncology, their use is limited to management of late stage disease[2]. Despite advances in genomics and proteomics, few, if any, biomarkers for early detection are in late stage clinical development for breast cancer. This may reflect the molecular heterogeneity of breast cancer[3], a physical isolation of early breast cancer from the systemic circulation, or technologic limits of detection of early molecular changes.

The molecular changes in breast carcinogenesis are reflected in antigen upregulation, mutation, altered glycosylation, and altered degradation. These changes in protein structure can be specifically detected by the immune system, which carries altered antigens to draining lymph nodes for amplification of the immune response, to then enter the systemic circulation. This in vivo amplification system is highly specific and adaptive for changes in protein structure. Many tumor-specific autoantibodies have been identified in the sera of patients with breast cancer[4, 5]. Of these, several have been analyzed in detail with hundreds of serum samples, including p53 antigen [6], her2/neu [7], MUC1[8],c-myc [9], ECPKA [10], and NY-ESO-1[11, 12]. While the specificity of these antigens for cancer is high (>90%), the sensitivities of individual antigens are low (10-20%). Multiplexed analysis using several antigens can increase sensitivity of detection up to 70% [5, 9]. The majority of the published assays, while promising, depend on individual recombinant protein production and small, single-institution serum sets. The structure, quantity, and purity of recombinant protein used in ELISAs can dramatically affect the detection of autoantibodies, demonstrating that contaminating or misfolded proteins can lead to false-positive antibody detection[13]. Many of these assays must be cautiously interpreted, and they remain to be validated in standard biomarker multi-institutional phase 3 blinded retrsopective longitudinal repository studies or phase 4 prospective screening studies[14].

Of the known tumor antigens that induce detectable antibodies in early cancer, mutated p53 protein was the first identified [15] and the most studied [6]. The development of autoantibodies to p53 protein is thought to be due to accumulation of mutant p53 antigen in the cell nucleus. P53-specific autoantibodies correlate with cancer ($p<10^{-4}$), and are associated with p53 point mutations, but epitope mapping using overlapping peptides has suggested that immunogenic regions are in the N- and C-termini of the molecule and not the mutation hotspots in the core region[16-18]. P53 autoantibodies have been detected in high-risk individuals months prior to the diagnosis of lung cancer and angiosarcoma[19], and prior to relapse of early-stage breast cancer[20].

A number of techniques have been developed to detect novel autoantibodies in patient sera. Traditionally, this has been done with serologic expression cloning (SEREX) of phage expression libraries derived from tumor cells. Over 2000 autoantigens recognized by patient sera have been identified with SEREX[11, 21, 22], though relative few have gone on to validation studies. A modification of SEREX screening is combinatoryal phage display, which expresses tumor antigens as fusions with phage proteins. Phage display relies on biopanning of phage libraries using patient serum to identify candidate antigens, then spotting proteins on phage-protein microarrays. This approach has been used to identify tumor antigens in cancer, many of which are out-of-frame translation products or expressed from the 3′ UTR23-26. Autoantigens can also be detected by fractionation of tumor cell

lysates and then immunoblotting with patient or control sera. This has been used to demonstrate antibodies in the sera of lung and prostate cancer[27-30].

The advent of proteomic technologies, in particular protein microarrays, allows for rapid, flexible, high-throughput screening of thousands of tumor antigens for the detection of autoantibodies in the sera of cancer patients. This approach has been successfully used to screen autoimmune patient sera[31] and to identify antigens expressed in ovarian cancer[32]. Because the identity of each feature on the array is known, this is a powerful technique for the detection of novel antibodies, but production of protein microarrays is challenging. Protein arrays produced by printing isolated proteins, which are now commercially available, require production and purification of recombinant antigen, raising issues of batch-to-batch variation, purity, and limited shelf-life. Glycan arrays are also being developed to detect alterations in glycosylation, which can also be highly immunogenic[33].

While established and highly quantitative analytical methods are available for validation and large-scale serum screening, there is a clear need for discovery tools for the rapid detection of antibodies to thousands of tumor antigens in patient sera. Here, we present evidence of the adaptation of programmable protein microarrays[34] for the detection of autoantibodies in breast cancer sera. Just-in-time protein expression avoids the potentially denaturing steps of protein isolation, storage, printing, and storage that occur with printed protein microarrays. To demonstrate that programmable protein microarrays can be used for serologic screening, we provide a direct comparison with a recombinat protein p53 autoantibody ELISA. We show that of 1,705 non redundant expressed antigens, dominant antibodies in melanoma and cancers of the breast and ovaries are detected. The application of these assays for biomarker detection will be discussed.

Materials and Methods

Patient Sera

Sera used in these analyses were obtained from the Lurie Breast Cancer Tissue and Blood Repository and the Specialized Research Program in Breast Cancer at the Dana-Farber Cancer Institute. Sera derived from breast cancer patients were obtained at the time of presentation with invasive breast cancer. Sera from patients with early stage ovarian cancer and matched healthy women were obtained from the Brigham and Women's Hospital. Control sera were from healthy women undergoing blood donation. Written consent was obtained from all subjects under institutional review board approval.

Generation of recombinant Survivin-GST and GST protein

The pGEX-4T-1 plasmid (Amersham, Piscataway, NJ) encoding full-length glutathione S-transferase (GST) protein and GST fused to the full-length survivin gene (Survivin-GST) were expressed in BL21 DE3 cells (Stratagene, La Jolla, CA) with 0.1M IPTG induction for 4 hours and purified according to manufacturer's recommendations. Bacterial cells were sonicated in cold PBS with protease inhibitors and lysozyme, then 1% Triton X-100 was added. Cleared supernatants were applied to glutathione CL-4B Sepharaose columns (Pharmacia, Piscataway, NJ) and bound protein was eluted in 50 mM Tris pH8.0 with 10 mM reduced glutathione. Protein purity was confirmed with SDS-PAGE and concentration determined at OD 280nM.Recombinant EBNA-1 protein was obtained by ABI (Columbia, MD)

P53 antibody ELISA

The p53 protein ELISA assay was performed per manufacturer's recommendations (p53 ELISA^{PLUS} Autoantibody Kit, Calbiochem, Gibbstown, NJ). 100 μl each of calibrators,

controls, and serum samples were added in duplicate. The calibrators were used neat, 1:1.5, 1:2, 1:3, 1:4, and 1:6 to obtain a calibration curve. Serum samples were diluted 1:100 and bound for 1 hour at room temperature (rt). The samples were removed and the wells washed 5 times with wash buffer. The wells were incubated with 100 μl of detector antibody conjugate for 1 hour at room temperature and washed 5 times with wash buffer. To develop the wells, 100 μl of substrate solution was added and incubated in the dark at room temperature. After 30 minutes, 50 μl of stop solution was added. The absorbance was determined at 450 nm using a Wallac plate reader. A calibration curve was created using the absorbances of the diluted calibrators. Dilutions of 1:1.5, 1:2, 1:3, 1:4, and 1:6 correspond to titers of 1, 0.67, 0.5, 0.33, 0.25, and 0.16 Units [U]. A p53 autoimmune index was calculated for each sample based on the formula ($Absorbance_{sample}$-$Absorbance_{cut\text{-}off}$)/($Absorbance_{undiluted\ calibrator}$-$Absorbance_{cut\text{-}off}$). The cut-off value is defined as 0.15 U. Any sample below this value is designated p53 negative. Samples within 20% of the cut-off were designated critical, and samples above 20% of the cut-off were designated p53 positive.

Survivin and EBNA-1 antibody ELISA

Recombinant GST, survivin-GST, or EBNA-1 protein was applied at 5 μg/ml to Nunc C96 Maxisorp plates (Fisher, Pittsburgh, PA) in carbonate buffer (pH 9.6) overnight at 4°C. Plates were washed in PBS-0.05% Tween (PBST) and blocked with PBS-Tween with 2% milk, overnight at 4°C. Serum was added in duplicate at 1:500 dilution in blocking buffer, overnight at 4°C. After washing, 1:1000 goat anti-human IgG-HRP secondary antibody was added (Zymax, Invitrogen, Carlsbad, CA) for one hour at rt. After washing, TMB-Plus (Dako, Carpinteria, CA) was added and the reaction stopped with 1N H_2SO_4. Absorbance was read at 450 nM, and GST signal was subtracted from Survivin-GST signal.

Generation of NAPPA protein microarrays

The arrays were prepared by printing purified plasmid DNA. Expression plasmid DNA in DH5alpha cells was grown overnight at 37°C in 1.5 mL liquid culture (Terrific Broth) and purified using Nucleobond anion exchange resin. The DNA (1 μg/μL) was printed along with the capture antibody (anti-GST antibody, 50 ng/μL, Amersham), protein crosslinker (BS3, 2mM, Pierce) and bovine serum albumin (3 mg/mL). Printing was done using an Affymetrix 427 or Genetix arrayer with 300um solid pins. The slides were expressed using previously published protocols[34]. For testing serum, the arrays were incubated with 300 uL of 1:300 diluted sera for 1hr at room temperature. Serum antibody binding was detected by probing the array with a HRP conjugated anti human IgG (1:500, Jackson lab) for 1hr at room temperature. The slides were developed for fluorescence detection by treating the slides with Tyramide signal amplification (Perkin Elmer) as per manufacturer's instructions. Data analysis was performed using Microvigene software (Vigenetech 2.9.9.2).

Statistical Analysis

Protein ELISA analyses were performed in duplicate or triplicate and positive autoantibodies were confirmed on repeat experiments. Individual antigens on microarrays were spotted as duplicates or quadruplicates. We defined an autoantibody signal as positive if it had at least a 3fold change compared to control sera (Figure 2), to the median of the sera tested (Figure 4), or to the median of all antigens on the array (Figure 5). To compare levels of antibody signal between two groups (as in Figures 1A and 1C) we used the Wilcoxon Rank Sum non-parametric test. To measure agreement in NAPPA and standard ELISA's detection of positive signals in sera, we used KAPPA analysis. When examining levels of p53-specific antibody in two patients who had positive signals prior to neoadjuvant chemotherapy (Figure 1B), we fitted a linear regression to log-transformed levels of antibody signal. To determine if the levels declined over time after chemotherapy was administered we tested if the regression slope was different from 0.

Results

Autoantibody Detection in Breast Cancer: Clinical Parameters

The clinical application of tumor antigen-specific antibodies as biomarkers require that they be specifically detected in cancer patients, be detectable in early stages, and/or be associated with tumor burden or disease outcome. The most well-studied autoantigen in breast cancer is p53. As with other p53-associated cancers, p53-specific antibodies are detected in the sera of 10-15% of breast cancer patients, and are strongly associated with missense mutations in the core domain of the molecule[35-37]. There have been multiple, but sometimes conflicting, studies correlating p53 antibodies to clinical stage, subtype, or outcome. These conflicting results may reflect underlying tumor heterogeneity, sample collection, or variation across different patient populations, but most likely is directly related to differences in antigen preparation and structure used in the assays[38]. For example, screening for antibodies with protein was more specific for cancer patients than screening with overlapping peptides [39]. This is not surprising, since many B cell epitopes are conformational[40, 41]. P53 antibodies have been shown to correlate with poor prognosis in breast cancer [42, 43], but improved prognosis in ovarian cancer[44], although the specificity of the p53 point mutations in the two cancers are quite similar.

The greatest potential impact of a biomarker in breast cancer would be to detect early-stage disease. Autoantibodies have been detected as early as DCIS[45], but, again, the association of p53-specific antibodies and tumor stage has shown mixed results. A higher frequency of autoantibodies was observed in late stage breast cancer[43, 46]. However, studies in the U.K. and in Sweden found that p53 antibodies did not correlate with disease status[47-49]. In lung cancer, p53 autoantibodies decreased in most patients during therapy[50, 51], arguing that the presence of these antibodies is associated with tumor burden, and they could be used as biomarkers to monitor response to therapy.

To demonstrate the detection of p53-specific antibodies in breast cancer, we used a set of well-annotated sera from 46 healthy female donors and 186 breast cancer patients, obtained at the time of diagnosis. The detection assay used was a commercially-available ELISA assay that uses recombinant, baculoviral-generated p53 antigen coated onto 96-well plates. The left panel of Figure 1 shows the levels of p53-specific antibodies in normal individuals and in cancer patients stratified by stage. p53-specific antibody levels were significantly lower in normal controls than in cancer patients using Wilcoxon Rank Sum test (p-value<0.0001). In comparison, levels of antibody to the EBV viral antigen EBNA-1 did not differ significantly between normal controls and cancer patients (p-value=0.1764, right panel of Figure 1A). Responses to p53 were detected as early as stage II.

To confirm that p53 antibodies are associated with tumor burden, figure 1B demonstrates p53 antibodies that were detected in two patients at time of diagnosis. Linear regression was fitted to log-transformed levels of p53-specific antibodies versus times. After neoadjuvant chemotherapy, the antibody concentration in the sera markedly declined over time (p-value=0.0002). The loss of these antibodies persisted long after chemotherapy completion. In contrast, we do not find any change in antibody concentration to the viral antigen, EBNA-1 (Figure 1B, right). This suggests that the rapid decline in anti-p53 antibody titer is antigen-specific, but we cannot rule out a more global effect of chemotherapy on antibody-specific immunity.

The three major subtypes of breast cancer, hormone receptor positive, her2/neu positive, and ‘triple-negative’ breast cancer, have markedly different RNA microarray expression profiles protein expression, and biologic behavior[52]. As shown in Figure 1C, the ER-negative breast cancer subtype had higher levels of p53-specific antibodies than the ER-positive subtype (p-

value=0.0373). In comparison, there was no significant difference between the levels of p53-specific antibodies between the Her2-positive and Her2-negative subtypes of breast cancer (p-value=0.6698).

Antibodies other than p53 antigen have been detected in early-stage breast cancer patients, with varying frequencies. Survivin-specific antibodies have been detected in 11 of 46 patients breast cancer in Japan[53] but only 5 of 64 patients in the U.S. [9]. We generated recombinant survivin protein as a GST-fusion in bacteria. Using the same sera as in Figure 1C(i), we examined survivin-specific antibody levels (Figure 1C(ii)). We found no significant difference in survivin-specific antibody levels between ER-positive and ER-negative subtypes of breast cancer (p-value=0.9720). However, survivin-specific antibodies had higher levels of expression in the Her2-negative subgroup of breast cancer than in the Her2-positive subgroup (p-value=0.0076).

Development of NAPPA Tumor Antigen Protein Microarrays

Standard ELISAs offer a simple, rapid method to identify immune responses to a single antigen. However, this approach has low to moderate throughput and requires the expression and purification of every antigen to be tested. Therefore, ELISA has limited utility for proteome-scale applications, where the intent is to assess the global immune responses to thousands of proteins. High density antigen microarrays offer a significant advantage in throughput but can be difficult to produce given the expense and difficulty in producing large numbers of purified antigens.

We have developed programmable protein microarrays that comprise anchored cDNAs that are transcribed and translated in situ to produce corresponding epitope-tagged proteins[34]. Upon protein synthesis, the proteins are rapidly captured at the site of synthesis through a high affinity capture molecule, usually an antibody, that recognizes the epitope tag. This allows for a large number of antigens to be freshly synthesized and captured for profiling the immune response against entire proteomes.

Adaptation of NAPPA protein microarrays for Serologic Screening

To validate the assay, we obtained commercially available p53 antibody positive and negative sera (Calbiochem). The responses of the sera were confirmed by ELISA analysis using recombinant p53 antigen adhered to 96-well plates (Figure 2A). p53 autoantibodies were also detected by NAPPA protein microarray, and three control antigens (S100A7, p21, and ML-IAP) were not detected (Figure 2B,C). To confirm expression of all four antigens, the arrays were spotted in quadruplicate and probed with anti-GST antibody (Figure 2B, left), p53 negative sera (middle) and p53 positive sera (right). The p53 positive and negative sera were provided as internal standard with the commercially-available ELISA assay. Postive signal on NAPPA was defined as 10% above signal from non antigen containing negative control. The sensitivity of p53 detection by NAPPA and standard ELISA were tested by diluting the p53 positive sera, figure 2C. Comparable detection levels were observed with diluting the serum six fold.

Accessibility of p53 antigenic epitopes on NAPPA protein microarrays

Nearly all protein microarrays and many ELISAs are produced with proteins that bear epitope tags on either or both of their termini. In the context of detecting immune responses to these proteins, one potential concern is that these polypeptide tags might block access to antigenic epitopes via steric hindrance. As accessibility of antigenic epitopes to applied sera is essential for the detection of autoantibodies, we wished to confirm that multiple regions of known epitopes of the p53 autoantigen were accessible to antibodies directed at them. In figure 3, 7 different monoclonal antibodies directed at epitopes spread throughout the p53

protein (D01, D02, D07,D013,D014,240, and 243) were used to detect determinants on the p53 molecule expressed by NAPPA. The antigenic epitopes of these monoclonal antibodies have been well-characterized[54] and are depicted in Figure 3. As shown with the anti-GST control which detects the c-terminal GST fusion protein, all p53 antigenic determinants are specifically detected by NAPPA fluorescence, 96-well ELISA, and by western blotting of expressed recombinant protein. The negative control (no antigen) and the control antigen p21-GST are not detected. This demonstrates that multiple regions of the molecule are accessible to antibody detection on the array. Moreover, the trends of detection are similar for these antibodies for both NAPPA and ELISA. Data on the quantitation of NAPPA and ELISA signals are provided in Supplemental Table 1, but the sensitivity of detection of epitopes must be interpreted with caution, as each monoclonal antibody has unique binding characteristics. Furthermore, one advantage of NAPPA is that, if needed, tumor antigens on the array can also be readily expressed with N-terminal tags or with non-GST tagged antigens to allow for flexibility of epitope expression (data not shown).

NAPPA protein microarrays specifically detect p53 autoantibodies in breast cancer patient sera

To confirm the selectivity of autoantibody detection by tumor antigen expressed on NAPPA protein microarrays, sera from 15 patients with metastatic breast cancer were screened for p53 autoantibodies using a commercially-available protein ELISA kit (Calbiochem). Based on published reports and our own data (Figure 1A), we assumed that majority of sera did not have detectable antibodies to p53 antigen. We defined sera to have a detectable level of antibodies to p53 antigen on NAPPA and standard ELISA as those that have fold change of 3 or more when compared to the median. Fold changes were computed as ratios of the serum signal to the median signal of all 15 sera assayed. We found a significant concordance between NAPPA and standard ELISA (Kappa=1.0, p-value=0.0095) with the same 2 of the 15 patients having detectable autoantibodies to p53 antigen in both NAPPA and ELISA (Figure 4)

Application of Proteomic Technologies for Antibody Detection

Although specific for cancer patient sera, p53-specific autoantibodies occur with limited frequency in patients and alone have limited utility as cancer biomarkers. For new autoantigen discovery, we selected over 1,700 antigen clones from our PlasmID repository to build a custom high density antigen array, which was printed onto a derivatized glass surface [55]. The gene collection represented 1,116 cancer related genes of which 539 were linked to breast cancer, as determined by the literature mining tool, MedGene [56]. The protein types included signalling molecules like kinases (84), transcription factors (206), and transmembrane proteins (535). The success of protein expression and capture on the array was measured by probing the array with an anti GST antibody that recognizes a C terminal GST epitope on every antigen, figure 5A. We determined that 90% of the proteins had readily detected protein signal over background (10% above the average of non antigen containing spots).

To detect an immune response, we probed the high density cancer antigen arrays with serum from a healthy control and sera from patients with melanoma, ovarian and breast cancer. As a positive control, we chose to express the Epstein Barr nuclear antigen (EBNA), which derives from the highly prevalent Epstein Barr Virus (EBV). More than 90% of the population responds to this antigen, which acts as a good internal control indicating that the antigen detection assay is working)[57].

In figure 5B, we detected responses to the EBNA antigen from the control, breast, and ovarian cancer sera. The serum from the melanoma patient did not have detectable

antibodies to EBNA, although a strong response to a well-documented melanoma antigen called ML-IAP (Melanoma inhibitor of apoptosis protein) was detected, figure 5B (ii)[58]. For breast and ovarian sera, we detected responses to the tumour antigen p53 as well as other potential cancer antigens, figure 5B (iii,iv). Responses to p53 and ML-IAP were confirmed by standard ELISA (data not shown).

Discussion

Host factors that drive immune recognition of cancer

The evidence for active immunosurveillance of breast cancer is mounting. Polymorphisms in MHC molecules have long been associated with the development of clinical autoimmune disorders, and epidemiologic studies have shown mild assocation with protective HLA-DQ and HLA-DR class II alleles in breast cancer[59]. Lymphocytic infiltrates, as in ovarian cancer, colon cancer, and melanoma, have been shown to be associated with improved overall survival in breast cancer[60, 61]. However, less than half of patients with mutated p53 antigen develop a detectable antibody response. In longitudinal studies, patients who fail to make antibody responses at presentation, do not have detectable antibodies at disease relapse[6], arguing that host immune memory and genetic variability drive the development of autoantibodies to tumor antigens.

Antibody binding to cognate antigen results in uptake of antigen/antibody complexes by Fc gamma receptors (FcGR) on antigen presenting cells. This enhances MHC class II antigen presentation and amplification of memory B cells bearing the specific immunoglobulin receptor. Polymorphisms in subsets of FcGR (IIa and IIIa) alter the affinity of FcGR binding to immunoglobulin and are associated with the development of clinical autoimmune disorders[62]. These polymorphisms are associated with altered response to antibody-mediated therapy in lymphoma[63]. In particular, we have found that structural polymorphisms in complement factor H are associated with clinical response to trastuzumab [64]. Furthermore, dying and apoptotic tumor cells elicit danger signals via toll-like receptors (TLR) for dendritic cell activation and antigen presentation[65]. Not surprisingly, polymorphisms in TLR4, which impact innate immunity, affect response to chemotherapy in breast cancer[66]. Thus, host factors that impact antibody binding (FCG receptors), and activation of innate and adaptive immunity (complement and toll-like receptors) could profoundly influence the ability of patients to develop autoantibodies to tumor-derived antigens.

Protein structure, immunogenicity, and lessons from p53

The structural features that enhance immunogenicity of proteins are not well known, but charged surfaces, coiled-coil motifs, and nucleic acid binding are associated with autoantibody development in autoimmunity[67]. Alterations in post-translational modifications, such as phosphorylation and aberrant glycosylation, can also be strongly immunogenic[68]. Cancer-associated changes in apoptosis and cleavage by granzymes also render antigens more immunogenic[69].

Given these factors, it is perhaps surprising that immunity to p53 antigen is detected across multiple cancer types and across multiple ethnic backgrounds in Europe, the U.S. and Asia. In our multiplexed protein microarray analysis of over 1,700 unique antigens, p53 is an immunodominant antigen which induces striking immunoreactivity compared with other tumor antigens. We have detected these antibodies in multiple stages and subtypes of breast and ovarian cancer. The structural cause of this immunodominance is not known, especially since epitope mapping studies have suggested that antibody binding to p53 is localized outside of the mutational hotspots. It has been hypothesized that one reason for p53 immunogenicity is due to protein binding to hsp70, causing immune activation[70].

Understanding the structural factors that drive antibody-mediated immunity will be critical for biomarker detection as well as immunotherapies.

Proteomic approaches to discovery of autoantibody signatures

The advent of proteomics allows for a global assessment of the breadth of the immune response to cancer. For biomarker development in particular, multiplexing autoantibody assays is critical to enhance sensitivities, while maintaining specificities[5, 9, 23]. Since the protein structure, content, and post-translational modifications are different for different proteomic techniques, it is not surprising that different antigens are identified. SEREX relies on phage expression and immunoblotting, and results in the detection of denatured epitopes that can be expressed in phage. Antibody detection by phage display has led to out-of-frame and 3′UTR epitopes, perhaps reflecting the cDNA libraries used, or defective ribosomal products present in cancer cells[71]. Fractionation of tumor cell lysates can detect cancer-specific post-translational modifications.

The programmable protein microarrays presented here may have distinct advantages for autoantibody detection in cancer. First, antigen selection is only limited by the availability of full-length sequence-verified open reading frames. Second, the cDNA's that are spotted on the arrays can be readily manipulated for epitope identification and mutational analysis. For serological assays, anitgens produced in situ by NAPPA behaved similarly to standard ELISA methods. Sensitivity of detection was comparable between NAPPA and commercial ELISA tests in titration experiments with the same antibodies. Accessbility to the epitopes of the antigen was unaffected by the differences in the methods of display. Moreover, good concordance of signal was observed between the two methods. The difference is that NAPPA can easily be multiplexed without sacrificing the performance features of a standard ELISA. Large number of antigens can be readily expressed with high levels of success for large scale studies.

The application of autoantibody biomarkers in breast cancer

There are a number of clinical settings where the development of biomarkers for breast cancer would have a significant clinical impact. Biomarkers for early detection of primary breast cancer would be particularly useful for patients at increased risk of cancer development or as a complement to mammography or magnetic resonance imaging (MRI) to identify occult cancers. Monitoring for early relapse will have a greater impact if therapies that target minimal residual disease are proven to be beneficial. The antibodies that are associated with improved outcome (tumor rejection) may also have therapeutic potential.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Yanhui Hu, Andreas Rolf, Janice Williamson, and Mauricio Fernandez from the Harvard Institute of Proteomics, Harvard Medical School for their support. We thank Dr. David Lane for providing the p53 monoclonal antibodies. This study was supported by a research grant from the Early Detection Research Network 5U01CA117374-02 and the Friends for an Earlier Breast Cancer Test.

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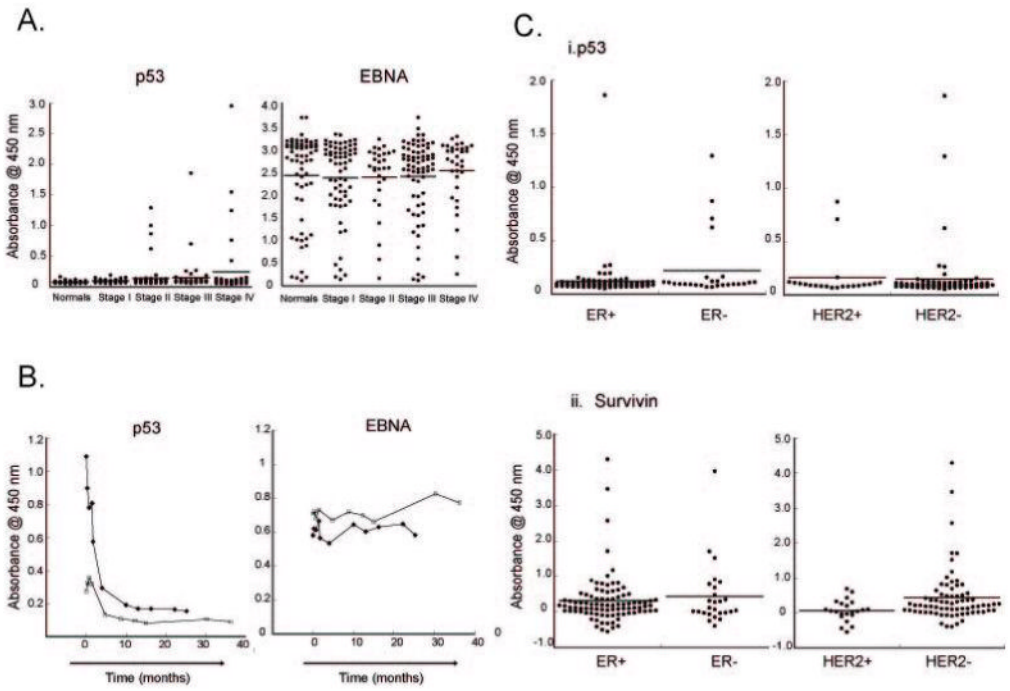

Figure 1.

Detection of p53-specific antibodies in early-stage breast cancer. **A.** Sera derived from breast cancer patients and healthy female donors were tested for p53-specific antibodies (left) and EBNA-1 specific antibodies (right) by protein ELISA. The sera used consisted of normals (n=46); Stage I (n=29); Stage II (n=70); Stage III (n=47) and Stage IV breast cancer (n=40). The median level is shown with a bar. P53 antibody detection correlates with presence of breast cancer ($p<0.0001$). In contrast, EBNA-1 antibodies are detected at comparable intensity in normals (n=64) and patients (n=132), independent of stage. **B.** Rapid and durable loss of p53 antibodies with neoadjuvant chemotherapy. Two patients with early-stage Her2+ breast cancer who had detectable antibodies to p53, underwent neoadjuvant chemotherapy treatment with trastuzumab and chemotherapy over a 14-week period. P53 antibodies were detected in serial serum samples by ELISA, and show a rapid loss with the onset of treatment. **C**. Autoantibodies in breast cancer can be subtype-specific. i) Detection of p53-specific antibodies in breast cancer subtypes. Sera from Figure 1A were divided by tumor subtype. Subgroup analysis showed borderline significant preferential detection of anti-p53 antibodies in patients with ER- (n=26) compared with ER+(n=93) cancer, but no preference between HER2+ (n=22) vs. HER2- (n=73) breast cancer. ii) Selective detection of survivin-specific antibodies in HER2- breast cancer. Sera from the breast cancer patients in (A) were tested for anti-survivin specific antibodies using recombinant GST-tagged survivin. Subgroup analysis showed selective detection of anti-survivin antibodies in patients with HER2- breast cancer, independent of estrogen receptor (ER) status.

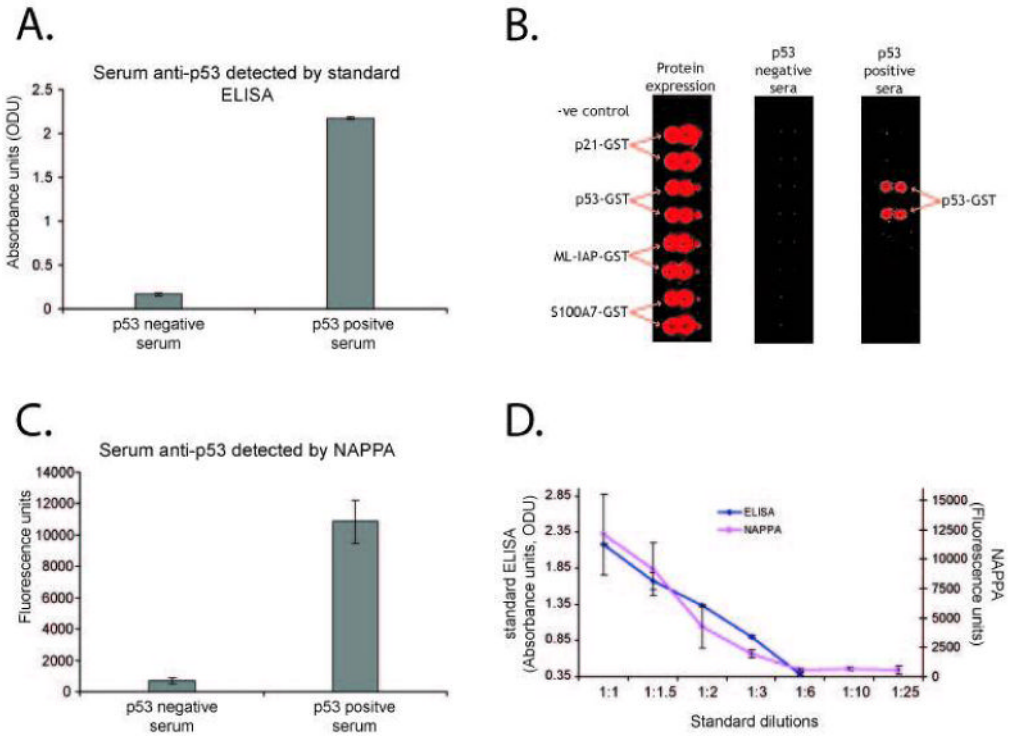

Figure 2.

Development of protein microarrays for detection of autoantibodies. A. Detection of p53 antigen with standard ELISA, using reference p53-antibody positive sera and negative sera. B and C. 4 antigens (S100A7, p21, ML-IAP, and p53) are expressed in microarray format, and p53 antigen is specifically detected with the reference sera. All four proteins are strongly expressed, as measured by detection of GST tag using anti-GST antibody. D. Sensitivity of detection. P53 positive serum was serially diluted and tested by standard ELISA and NAPPA. Standard deviation for ELISA was based on two samples and for NAPPA was based on four features on the array.

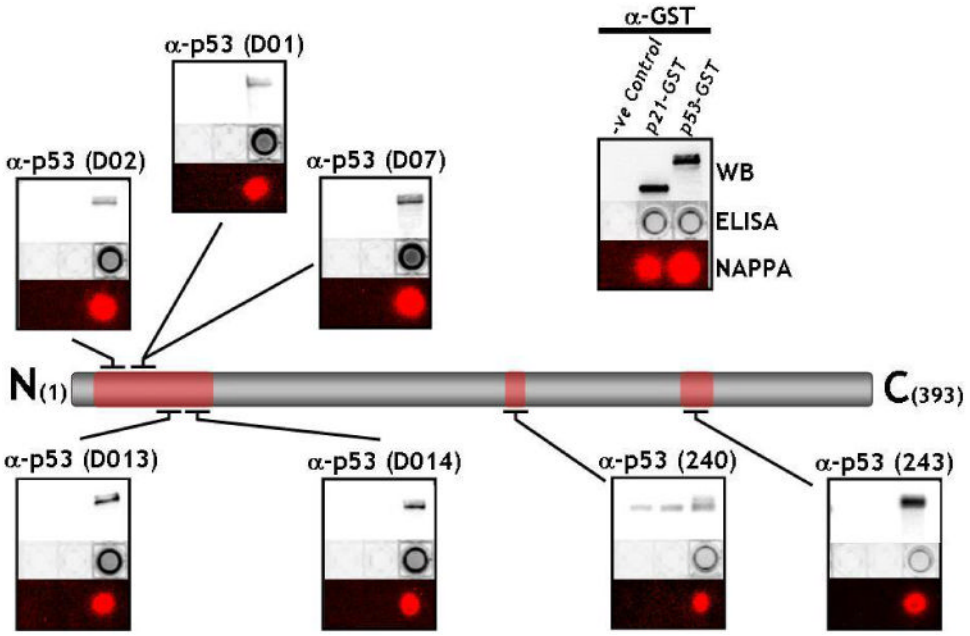

Figure 3.

Detection of multiple distinct epitopes of p53 antigen expressed by NAPPA. 7 different monoclonal antibodies to p53 antigen (D01, D02, D07,D013,D014,240, and 243) were used to detect determinants on the p53 molecule expressed by NAPPA. As shown with the anti-GST control which detects the c-terminal GST fusion protein, all p53 antigenic determinants are specifically detected by NAPPA fluorescence, 96-well ELISA, and by western blotting of expressed recombinant protein. The negative control (no antigen) and the control antigen p21-GST are not detected.

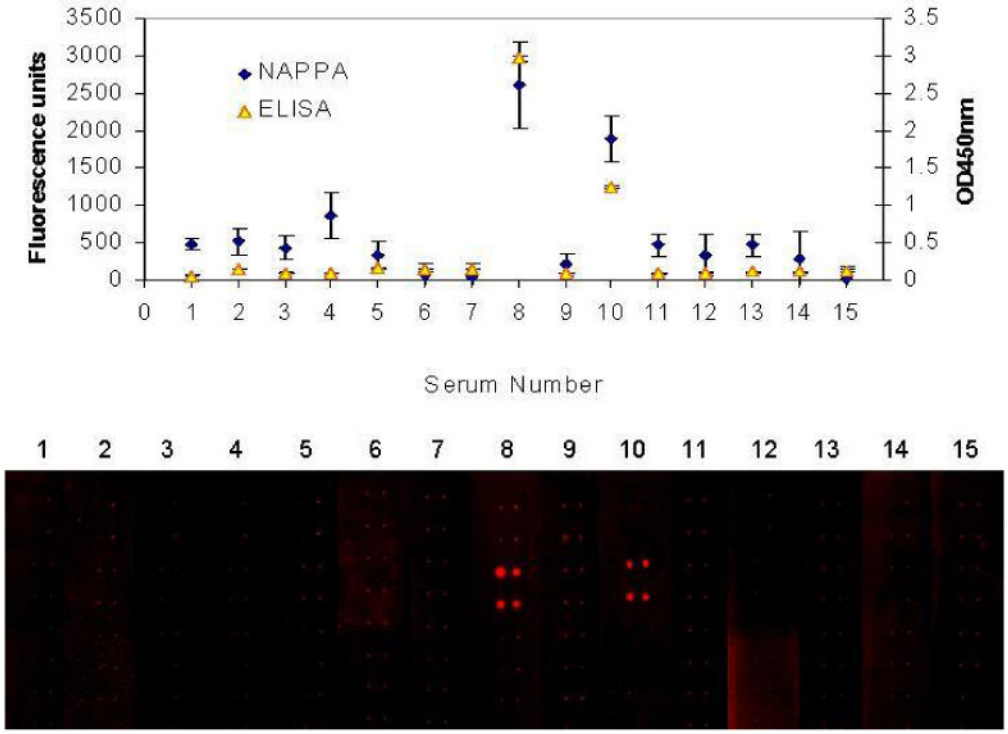

Figure 4.

Comparable specificities of NAPPA microarray and protein ELISA for the detection of p53 antibodies in metastatic breast cancer patient sera. 15 sera from patients with metastatic breast cancer were diluted 1:100 and tested for p53-specific antibodies by protein ELISA and by NAPPA microarray. Two sera were positive by protein ELISA and were also positive by NAPPA microarray. The images of the quadruplicate-spotted antigens detected are shown in red on the bottom.

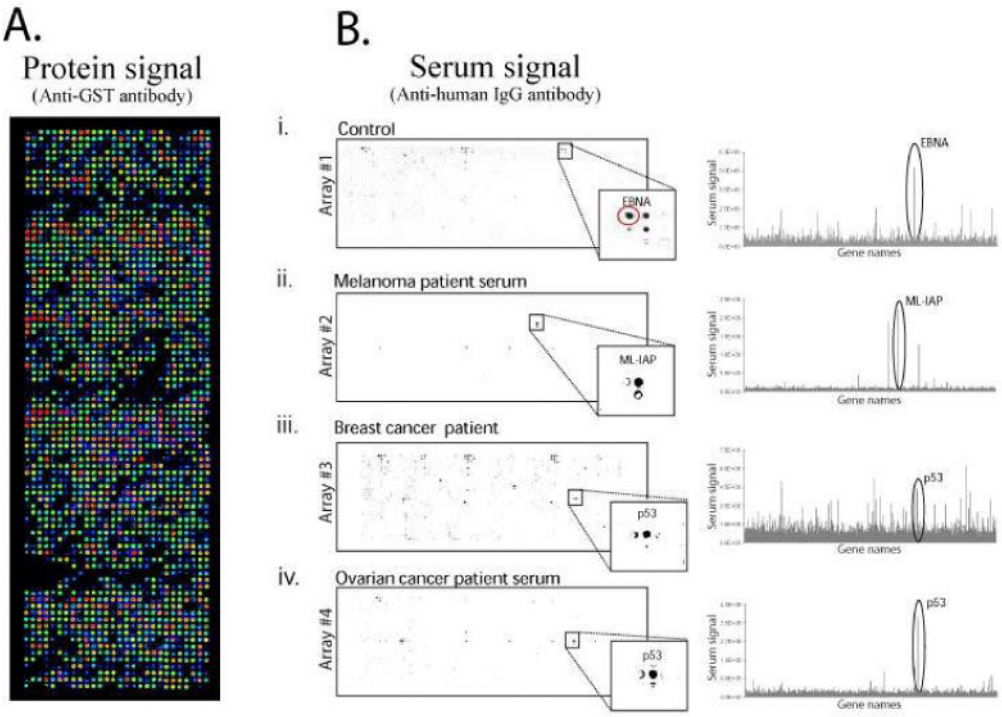

Figure 5.

Development of custom tumor antigen high-density programmable protein microarrays for the detection of autoantibodies in patient sera. A) Over 1,700 candidate tumor antigens were expressed and captured in a microarray format, and protein expression was detected using anti-GST antibody. B. The arrays were probed with sera from a healthy individual, and patients with melanoma, breast and ovarian cancer. All arrays were incubated with 1:300 diluted serum and developed using a HRP conjugated anti-human IgG and TSA. (i) Shows EBNA response from healthy serum (red circle, CV=40% across 4 slides) (ii) Response to ML-IAP from melanoma serum (CV not tested) (iii) Response to p53 from breast cancer sera (CV=11% across 4 slides) (iv) Response to p53 from ovarian cancer sera (CV=49% across 2 slides).