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Antibody Profiling with Protein Antigen Microarrays in Early Stage Cancer

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Abstract

Introduction—Proteins not present in normal cells, i.e., cancer cells, may elicit a host immune response that leads to the generation of antibodies that might react with these tumor-associated proteins. In recent years, a growing number of reports have showed that autoantibody profiling may provide an alternative approach for the detection of cancer. However, most studies of antigen-autoantibody reactivity have relied on recombinant proteins. Recombinant proteins lack the proper post-translational modifications present in native proteins. Because of this limitation, native or natural protein antigen microarrays are gaining popularity for profiling antibody responses.

Areas covered—1) to illustrate some examples of autoantibodies as signatures for early stage cancer; 2) to briefly outline the various protein antigen microarray platforms; 3) to illustrate the use of native or natural protein microarrays in the discovery of potential biomarkers; and, 4) to discuss the advantages of native protein antigen microarrays over other approaches.

Expert opinion—The nature of protein microarray platforms is conducive to multiplexing, which amplifies the potential for uncovering effective biomarkers for many significant diseases. However, the major challenge will be in integrating microarray platforms into multiplexed clinical diagnostic tools, as the main drawback is the reproducibility and coefficient of variation of the results from array to array, and the transportability of the array platform to a more automatable platform.

Keywords

Autoantibody profiling; Antigen microarray; Biomarker; Cancer diagnostic; Humoral response; Native antigen microarray; Natural antigen microarray; Protein microarray; Reverse capture microarray; Serologic detection

1.0. Introduction

Diagnosing diseases based on a simple blood test is a particularly interesting concept. The human serum or plasma proteome is likely to contain most human proteins. At the same time, it is the most generally informative proteome from a medical viewpoint. Almost all

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Declaration of interest:

Brian Liu serves on the Scientific Advisory Board of Inanovate, Inc., and is one of the inventors and has licensing rights to the "reverse capture" native protein antigen microarray platform described in the article.

cells in the body communicate with the blood directly or through extracellular fluids, and many release at least part of their contents into the blood stream upon damage or death. Some medical conditions, such as myocardial infarction, are diagnosed based on the increase of specific serum or plasma proteins. And for cancer, it is difficult to argue convincingly that there is any cancer that does not produce some specific pattern of protein change in the body's working fluid.

The technical challenge to analysis of the serum proteome, however, arises from both the dynamic range of protein amounts as well as from the structural complexity of these proteins. Serum and plasma proteins are present at unequal concentrations. Many of the high-abundance proteins are derived from major organs such as the liver. It is highly unlikely that changes within a smaller organ, such as a tumor in the prostate or ovary, would readily produce and secrete vast amounts of tumor related proteins into circulation. In fact, many of the classical tumor markers such as alpha-fetoprotein, prostate specific antigen, carcino-embryonic antigen, and human chorionic gonadotropin, are found in the ng/ml range in serum or plasma. In addition, secreted proteins from tissues or cells such as cytokines are also found in the ng/ml range in serum or plasma.

There are additional intrinsic limitations to identifying proteins in biological fluids. One such limitation is that some proteins in biofluids are in a constant state of flux. Proteins may be rapidly cleared from the body, transiently expressed and/or subjected to proteolysis. The challenges involved with mining the biological proteome must be overcome in order to detect circulating cancer biomarkers.

One potential strategy is to take advantage of the body's own immune system. With cancer, the immune system plays a vital role. For example, cancer sera contain antibodies that react with a unique group of autologous cellular antigens called tumor-associated antigens (TAAs). Proteins not present in normal cells may elicit a host immune response. It has been postulated that the production of antibodies to TAAs constitutes an integral component of the anti-tumor immune response in cancer patients. And since the immune response is generated locally, even small amounts of antigens, so few that they might be undetectable by any other means, may be identified and amplified by the immune response [1]. Furthermore, unlike the antigens found in serum or plasma, which may be in low abundance, transiently expressed by the cells or tissue of origin or rapidly cleared, the antibodies that react with these antigens are stable. In fact, the half-life of an IgG in a body's serum is approximately 21 days.

The factors leading to a humoral response in cancer patients are not clear. These antibodies to TAAs, called autoantibodies, may arise through several mechanisms and may be a result of the over-expression of various proteins, release of proteins during apoptosis (including intracellular proteins), reduced degradation of proteins, and/or modifications of proteins associated with a disease state such as cancer. In recent years, there is a growing group of investigators that have exploited the serum autoantibody repertoire from cancer patients to identify TAAs. The growing list of TAAs include 5 α -reductase [2], p53 [3], prostasomes [4], glucose-regulated protein-78 kDa (GRP78) [5], MUC1 [6], survivin, NY-ESO-1, and others [7]. Since many of the TAAs include intracellular proteins that are involved in the altered regulation, structure, or function of a cell, cancer autoantibodies or humoral responses have been suggested to be reporters for identifying aberrant cellular mechanisms underlying malignancy [8]. Interestingly, Zhang et al. [9,10] demonstrated that cancers of the prostate, breast, and lung showed distinct profiles of autoantibody reactivity in which specific TAAs were preferentially targeted. These results strongly suggest that autoantibody profiling might provide an alternative approach for discriminating between cancer patients and control individuals [9,10].

An “Achilles heel” of humoral responses to TAAs is their sensitivity. Zhang and colleagues highlighted this limitation and demonstrated that antibody reactivity to individual tumor antigens rarely exceeded 20% in the cancer patient populations [9,10]. However, successive addition of tumor proteins to a mini-array of seven antigens resulted in a dramatic increase in the number of positive antibody reactions in cancer patients, up to >60% in some patients [9,10]. Thus, the ability to detect the reactivity of multiple antigens, such as using a multiplex assay system, would improve the sensitivity and perhaps specificity for an assay. Therefore, the purpose of this review is to briefly outline the various protein antigen microarray platforms that would allow the detection of multiple autoantibody-antigen reactivities.

2.0. Protein microarray platforms

2.1. Recombinant protein approaches

Currently, one of the most commonly used methods to identify TAAs is by serological identification of antigens by recombinant expression cloning (SEREX). SEREX is an immunoproteomic strategy developed by Michael Pfreundschuh and colleagues [11,12]. Briefly, SEREX uses fresh tumor specimens to construct a cDNA library, which is then cloned into λ -phage expression vectors [11,12]. These recombinant phages are used to infect *Escherichia coli* bacteria, with the recombinant proteins expressed during the lytic phase of phage infection [11,12]. These proteins from the lysed *E. coli* are transferred to nitrocellulose membranes, and subsequently probed with patient's serum to identify and select the clones with reactivity to the patient's IgG antibodies [11,12]. Corresponding cDNA inserts from the reactive clones can then be isolated, and the tumor antigens determined by their DNA sequences [11,12] (see Figure 1). SEREX has demonstrated to be a useful method to identify serological targets and TAAs in a variety of tumors, and over 2000 tumor antigens have been identified [13]. An online database housing the cDNA sequences identified through SEREX, as well as information on the libraries from which they were derived, is the Cancer Immunome Database [14].

Despite the many positive utilities of SEREX, this technique has several drawbacks. Most notably, this platform utilizes recombinant proteins that are expressed in *E. coli* bacteria. The recombinant proteins generated from the cDNA library may not represent the native form of the proteins associated with the tissue or cells of origin. Post-translational modification (PTM) of proteins, such as glycosylation, is often found in cancer. Glycan structures are important determinations of many different biological processes, including protein-protein interaction, cell adhesion and migration, and inter-cellular signaling. Alterations to glycan structures can contribute to the development and progression of cancer and other diseases, and examples of glycan variations associated with cancer have been found on major serum proteins such as α -fetoprotein [15], haptoglobin [16], α -1-acid glycoprotein [17], and α -1-antitrypsin [18]. In addition, glycan alterations on MUC-1 in cancer have been observed previously, including truncations in the O-glycosylation that lead to the exposure of core structures [19,20]. Such modifications in the glycoproteins will not be represented by the cDNA inserts in SEREX. Without the relevant PTMs, the patient serum antibodies may fail to identify the antigen targets. In addition, the SEREX approach is biased toward high-titer IgG antibodies within a patient serum, which means that low abundance autoantibody-antigen reactivity may be overlooked [21].

2.2. Microarrays for humoral response profiling

Microarray formats, pioneered for DNA testing, are an attractive choice in cancer humoral response biomarker discovery. With the capacity to immobilize hundreds or even thousands of proteins on a single surface, the microarray format enables measurement of a

comprehensive panel of antibodies to specific antigens, with incorporated redundancies and controls. Such microarrays are created by spotting antigens directly onto an array surface. When incubated with patient samples, the spotted antigens serve to capture autoantibodies whose reactivity can be determined through either the use of a secondary antibody detector, such as fluorescently labeled rabbit anti-human IgGs, or through the use of a direct label, i.e., fluorescently labeled autoantibodies directly from a serum specimen. Thus, antigen or protein microarrays enable high-throughput and scalable analyses and are powerful tools for screening the immune response in cancer patients to elucidate autoantibodies and TAAs.

2.3. Combinatorial phage-protein microarray

One form of recombinant antigen microarray relies on the use of combinatorial phage display for the creation of phage-protein microarrays. Wang et al. developed a phage-protein microarray for the identification of serum immunoreactivity to antigens derived from prostate cancer tissues [22]. Similar to SEREX, a library of cDNA was cloned into a bacteriophage system, whereby the prostate cancer cDNAs could be expressed. However, unlike the SEREX approach, the cDNAs were expressed as fusion proteins on the surface of bacteriophages. The bacteriophages containing fusion proteins are collected, and several rounds of biopanning with IgGs from normal sera are completed to remove irrelevant phages and to enrich the library for those antigens binding autoantibodies from the sera of prostate cancer patients. Following biopanning, the phage clones are collected and spotted onto an array surface to create the phage-protein microarray, with each spot on the array representing one phage-protein [22]. Using this approach, Wang et al. identified a set of 22 antigens that could differentiate the serum of prostate cancer patients from that of controls [22]. It was further determined that this set of antigens performed better than serum prostate specific antigen (PSA), the classic prostate cancer screening marker [22].

Using the same phage-protein microarray approach, other investigators have shown that autoantibody profiling could be useful in the identification of other cancers. Zhong et al. reported on a similar process for the development of a comprehensive screening strategy for non-small cell lung cancer (NSCLC) [23]. Using these diagnostic arrays, plasma samples (stage I NSCLC and risk-matched controls) were assayed with the goal of selecting a set of markers for early-stage NSCLC. Using a predictive model, the investigators were able to accurately classify a high percentage of non-cancer and cancer samples, corresponding to 87.5% specificity and 82.6% sensitivity [23]. In addition, the predictive model was able to correctly classify a plasma sample as cancer even when the sample was taken up to 5 years before the diagnosis of lung cancer by CT scan [23]. Similar studies were demonstrated for ovarian cancer [24] and breast cancer [25].

Although these studies demonstrate the power of autoantibody profiling using phage-protein microarrays, there are some limitations to this approach. The most significant of which is the short peptide sequence that is expressed by the phage. Many of these peptide sequences mimic the target epitopes, but the actual antigen detected by the autoantibody may be difficult to identify. In fact, many of the phage clones used to classify a plasma or serum sample were found to contain non-coding sequences [22], making the identification of the actual antigen target somewhat difficult. And as with SEREX, antigens with post-translational modifications, e.g., glycosylated cancer antigens, cannot be detected with this platform.

2.4. ProtoArray™ high-density protein microarray

To circumvent the above limitation, a high-density recombinant full-length protein antigen microarray was developed. This platform, the ProtoArray platform, is commercially available from Invitrogen [26] and is licensed from Michael Synder of Yale University [27].

This platform utilizes full-length human proteins expressed as N-terminal glutathione S-transferase (GST) fusion proteins purified from insect cells (baculovirus expression system) to maximize native folding and post-translational modifications. GST-tagged human proteins are purified and arrayed onto nitrocellulose-coated slides. To control for the quality of the printing process, random samples of microarrays can be probed with an anti-GST antibody. Since the proteins contain a GST fusion tag, probing the microarrays with an anti-GST antibody identifies irregular spot morphology or missing spots. The advantage for the ProtoArray is its ability to array high-density full-length proteins. Currently, over 8200 proteins translated from genes throughout the human genome are arrayed onto a single slide. Several investigators have utilized these arrays for autoantibody profiling for various cancers [27–30]. In particular, Gnjjatic et al. recently used the ProtoArray platform to profile immune responses to ovarian and pancreatic cancer [29]. Using sera from 60 patients with pancreatic cancer, 51 patients with ovarian cancer, and 53 age-matched healthy controls, the binding of IgG antibodies against >8000 GST-tagged human proteins was compared. They identified that 202 proteins were preferentially immunogenic in ovarian cancer sera compared to 29 in pancreatic cancer, with few overlaps [29]. Similarly, Babel et al. demonstrated that they could identify 43 tumor-associated antigens for the diagnosis of colorectal cancer [30]. Three of these candidate antigens were found to be significantly expressed on colorectal cancer cell lines and colonic mucosa by immunoblotting and immunohistochemistry [30]. Thus, these studies demonstrate that the ProtoArray microarray platform is suitable for autoantibody discovery in cancer.

2.5. Native/natural protein antigen microarrays

Even with the use of the ProtoArray™ microarray platform, which maximizes protein folding and post-translational modifications (PTMs), such PTMs remain dependent on the expression systems that are used and may not reflect the unique modifications that are associated with a human disease state such as cancer. Thus, there is a need to develop native or natural protein antigen microarray platforms.

2.6. Reverse capture native protein antigen microarray

Recently, we developed a “reverse capture” autoantibody microarray platform that facilitates the detection of autoantibodies to antigens derived from diseased tissues or cells [31,32]. The reverse capture platform utilizes a high-density antibody microarray that has been spotted with well-characterized, highly specific monoclonal antibodies. The arrayed monoclonal antibodies serve to capture their corresponding antigens in their native state when incubated with proteins isolated from either cell lines or tissues. These captured antigens then serve as bait for fluorescently labeled IgGs purified from patient sera or plasma samples (Figure 2). Using IgGs from individual preoperative prostate cancer patients and IgGs from control patients with benign prostatic hyperplasia (BPH), we demonstrated that there were differential autoantibody reactivity profiles between prostate cancer and BPH patients [31,32]. We have extended our initial proof-of-concept study. Using a nano-particle slide, which increased the signal-to-noise ratio, and a set of 27 defined monoclonal antibodies to capture specific antigens, we compared 41 prostate cancer patients with 39 benign prostate hyperplasia controls. Using the reverse capture approach, we identified a subset of antigens that might be able to differentiate prostate cancer from BPH in patients with increased serum PSA [33]. Although still preliminary, our top five autoantibody signatures combined in a multiplex assay achieved a sensitivity of over 95% at 80% specificity for discriminating between prostate cancers and BPH whereas the “gold standard” serum PSA test achieved a sensitivity of only 12.2% at 80% specificity [33]. If validated, a panel of these autoantibody signatures may complement the current PSA assay, perhaps as a secondary test, and may prevent unnecessary biopsies in patients with BPH.

Using our reverse capture approach, Tang et al. recently demonstrated that they could identify 35 autoantibody signatures that were significantly elevated in the plasma samples from patients with mucinous epithelial ovarian cancer when compared with healthy controls [34]. In addition, functional annotation of the autoantibody targets identified nine target antigens involved in integrin and Wnt signaling pathways [34]. Furthermore, immunohistochemistry of archived ovarian specimens showed significant over-expression of 8 of the 9 target antigens in mucinous ovarian cancer tissues, suggesting that autoantibodies from mucinous ovarian cancer patients may arise as a result of over-expression of these proteins [34].

One additional advantage of our reverse capture platform is its ability to capture and detect circulating immune complexes for a specific target. Circulating immune complexes (CIC) are formed when antibodies are bound to the free antigen in the blood. For example, approximately 20% of women with ovarian cancer have low concentrations of serum cancer antigen 125 (CA125), and this important tumor marker cannot be used to monitor their disease. One possibility for the low concentrations is the formation of circulating immune complexes to CA125. Using our reverse capture platform where we arrayed mouse monoclonal antibodies to CA125, we tested for the presence of CIC to CA125 in patients with ovarian cancer that have low serum CA125 [35]. The arrayed monoclonal antibodies will capture the free serum CA125 antigen as well as any CIC. We can detect the free serum CA125 antigen using a detection antibody specific to CA125, e.g., rabbit anti-CA125 antibody. Since CIC is defined as complexes with antibodies to the antigen in a patient, using the same array in parallel, we can detect the presence of CIC to CA125 with a polyclonal anti-human IgG as the detection antibody [35]. Using this approach, we showed that CIC to CA125 is present in some patients with ovarian cancer with low or normal CA125 concentration and hypothesized that the presence of CIC may interfere with the ability to detect free CA125 in these patients [35].

As this platform utilizes antibodies as capture reagents, it has the potential of testing not only for the autoantibodies directed against the immobilized target antigens, but also for the targeted antigens themselves. However, unlike the typical intrinsic limitations of detecting circulating tumor antigens in the serum or plasma (i.e., rapidly cleared from the body, transiently expressed and/or subjected to proteolysis), the autoantibodies directed against the antigens are stable. And as illustrated above, this platform is able to detect CIC in sera of ovarian cancer patients where their serum CA125 concentration is within the normal range [35].

Although the reverse capture platform allows the capture of native protein antigens, the platform is limited by the selection, quality, and specificity of the monoclonal antibodies that are spotted onto the array, i.e., tested by Western blotting to demonstrate binding only to their specific antigens. Thus, the platform favors a targeted approach. As a result, the platform may fail to detect relevant antigenic targets because the necessary capture antibodies are not immobilized on the array.

2.7. Whole proteome lysate fractionation microarray

To overcome the limitations of the reverse capture platform, a whole proteome lysate fractionation microarray has been developed [36,37,38]. Whole lysates (from tumors or cells) can be separated into distinct fractions containing native antigens by a two-dimensional liquid chromatography strategy. The 1st dimension separation is chromatofocusing. Chromatofocusing is essentially an ion-exchange chromatography, where the elution conditions are obtained by dropping the pH so that the proteins elute in the order of their isoelectric points. This method utilizes two buffers, starting at a high pH of 8.5 and then switches to a low pH of 4.0, and a positively charged column. As the run proceeds, pH

gradient forms in the column and proteins elute from the column according to the charge they assume from the gradient, which is based on the isoelectric point (pI) of the proteins. When the pH of the column is greater than the pI of the protein, the net charge on the protein is negative, and the protein is bound to the positively charged column. When the pH of the column is less than the pI of a protein, the net charge on the protein is positive, and the protein is repelled from the column. When the pH of the column is equal to the pI of a protein, the net charge on the protein is zero, and the protein elutes from the column with the buffer flow.

The 2nd dimension is reverse-phase high pressure liquid chromatography. In the reverse phase, the stationary phase is non-polar and the mobile phase is polar. The mobile phase for protein separations consists of an ion suppressing agent, trifluoroacetic acid (TFA) in water, and then elution of proteins with TFA in increasing amounts of an organic solvent, such as acetonitrile. The degree of hydrophobicity of a protein is the basis of migration. The extent to which a protein is hydrophobic is determined by the percent content on non-polar amino acids, which include valine, leucine, isoleucine, phenylalanine, tryptophan and methionine. At a low concentration of organic solvent, a protein will absorb onto the stationary phase. As the organic solvent concentration increases, the protein desorbs from the column at the critical concentration of organic solvent. The greater degree of hydrophobicity a protein possesses, the higher the concentration of organic solvent is required for desorption from the column. Following the 2nd dimension, the fractions are then arrayed onto a slide which can then be probed with antibodies derived from sera or plasma (see Figure 3).

Although the use of a slightly different protein fractionation approach to define the humoral immune response in an array format has been reported in leprosy [39], one of the first studies using this whole proteome array with 2-D liquid chromatography fractionation in cancer was reported by Bouwman and colleagues [36,37]. These investigators separated prostate cancer LNCaP cell lysate into 1760 protein fractions and spotted these fractions onto a nitrocellulose coated microscope slide [37]. The slides were probed with serum from either prostate cancer patients or controls, and the resultant autoantibody reactivity was measured. The investigators identified 40 fractions that exhibited higher autoantibody binding from the prostate cancer sera than from control sera [37]. Additionally, using statistical modeling and cross-validation techniques, the researchers demonstrated that the autoantibody reactivity profiles could be used to classify samples with an accuracy of 80% or greater [37].

We have applied the whole proteome lysate fractionation microarray to identify specific antibody-antigen reactions in an experimental late stage breast cancer vaccine that was developed by Dr. Karen S. Anderson at the Dana Farber/Harvard Cancer Center. Under approval with our Institution Review Board (IRB), patients with late stage breast cancer underwent tumor vaccination with autologous tumor cells engineered to express granulocyte macrophage-colony stimulating factor. The tumor cells were lethally irradiated and subcutaneously injected back into the donor patient. Patients were inoculated on days 1, 8, 15, 29, and then every two weeks until the vaccine supply ran out. Using differentially dye-labeled IgGs from pre-vaccination sera and post-vaccination sera and applying them to a whole proteome breast cancer lysate fractionation microarray, we were able to determine the ratio of the differential dye intensities at each fraction on the microarray. Our results showed several antigen fractions have increased antibody-antigen activity in the post-vaccine sera (Figure 4). Some of the reactive antigens in the fractions identified by mass spectrometry include MUC5B, HSP70, S100 proteins, and mammaglobin-B. Future work will test the hypothesis that specific vaccines targeting these antigens may allow us to increase the effectiveness of the vaccines, as well as to determine which antigen response might indicate a positive anti-tumor outcome. Thus, the whole proteome lysate native protein antigen

microarray platform allows us to monitor the humoral response generated by a vaccine, and at the same time, to identify potential targets that might increase the efficacy of the vaccines.

3.0. Expert opinion

Currently, antigen microarrays are still in an early stage of development. However, the data generated by these platforms has strengthened the argument that antigen microarrays and autoantibody profiling are a valuable and viable platform for biomarker discovery in early stage cancer. The main technical challenge that protein microarray technologies and cancer autoantibody profiling serve to overcome is the difficulty of detecting significant low-abundance proteins within complex biological fluids, as well as the intrinsic limitation that some proteins in biofluids are in a constant state of flux. Additionally, the nature of microarray platforms is conducive to multiplexing, which amplifies the potential for uncovering effective biomarkers for many significant diseases.

As post-translational modifications of proteins are highly common in cancer, native protein microarrays, by their ability to eliminate the dependence on recombinant antigens, will facilitate the identification of antibody signatures to disease related post-translational modifications. For example, the abundant O-glycosylation found on mucins is a well documented case where studies have shown that the O-glycopeptide epitopes are candidates for natural autoantibodies induced in cancer patients [40]. Such studies illustrate the need to continue the development and use of native or natural protein antigen microarrays.

As protein antigen microarrays are multiplexed platforms, they are highly compatible with standard ELISA approaches for biomarker validation. For example, our reverse capture protein antigen microarray platform is primarily antibody based. Thus, it can easily have the potential to be adapted to the ELISA platform. However, to attain clinical relevance, the reproducibility of these antigen microarrays must be improved. Currently, the main drawback to experimental reproducibility is the coefficient of variation of the results from slide-to-slide. The linearity range of the assay depends on the antibody-antigen affinity. Linearity can be achieved only when the concentration of the analyte and antibody matches the affinity constant [41]. In addition, some of this variation is due to the technical variability during the arraying or spotting of the capture antibody or protein antigen on the slide surface, the consistency of binding to the array surface, and variations in background noise from slide-to-slide due to the slide chemistry or coating surface. Conditions such as humidity, temperature, dust, and pin washing will affect the variations in the printing step. As for slide surface, presently, there are a number of possible surfaces available for protein arrays. In general, chip surfaces can be divided into two major categories: flat (planar), and 3D (mostly gel-like) surfaces [42,43]. The proteins are bound to the surface of these chips by either covalent bonds or simple electrical charge. However, unlike DNA, the surface charge of proteins is highly variable and the use of simple uniform electrostatic interaction for the immobilization of different proteins results in large variation in the amount of protein bound. Secondly, the structural conformation of proteins deposited onto a planar surface does not mimic “native” proteins. Third, there is no control over the orientation of the proteins on the surface [44]. Therefore, protocols should be developed to promote consistency and include standards or calibrators so that one can normalize the results from slide-to-slide, as this will allow for more valid comparisons within and between experimental results. Finally, as the level of throughput continues to increase, bioinformatics takes on an ever greater importance in the organization and use of experimental data. As standards do not yet exist that can address all of the variabilities of a protein microarray, clinical validation of results will require an iterative process using independent sets of human clinical material and to integrate data from other technologies, e.g., ELISA.

The major challenge will be in integrating microarray platforms into multiplexed clinical diagnostic tools. As noted above, there are concerns about the quality and reproducibility of the microarray assays. These range from the activity and reproducibility of spotting of reagents on the array to the image processing procedure, and even the data analysis methods used. A good review of the validation and quality control for protein microarrays is a review by Kricka and Master [45]. Rather than being employed as stand-alone diagnostic tools, protein microarrays will more likely be used in tandem with other techniques such as ELISA or other automatable systems, as these systems can achieve coefficient of variation of less than 10% [45]. For example, several comparisons of microarray and conventional immunoassays have been published [45,46]. Although some comparison studies demonstrated good correlation between microarrays with ELISAs [45], others, such as a comparison of human cytokine assays performed with a commercially available membrane protein microarray and ELISA, did not demonstrate good concordance [46]. In the comparison of human cytokine, diluted human whole blood was stimulated with live bacteria or LPS [46]. Plasma cytokine levels were then measured by ELISA and membrane protein microarray. Of the cytokines measured, there was not a 100% concordance with the ELISA results. Specifically, although TNF- α was demonstrated to be elevated in the plasma following stimulation by live bacteria or LPS by ELISA, the membrane protein microarray was not able to detect the increase [46]. Thus, only when the appropriate QC, QA, and validation steps are in place for the microarray platforms, perhaps with the use of microfluidics and automated systems, will these platforms have the ability to emerge from the confines of the research laboratory to a clinical laboratory. Only then, will protein microarrays gain traction and enable the field to evolve to have a clinical impact in cancer diagnostics and theranostics.

Article Highlights

- Early stage cancers could be detected by using a simple blood test.
- Autoantibody profiling provides a means to detect cancer biomarkers in the blood.
- Microarrays provide an attractive platform for the discovery of cancer biomarkers.
- Various protein microarray platforms are reviewed.
- Native protein microarrays allow for detection of proteins in their natural form including post-translational modifications which may be important to many types of cancers.
- Whole proteome fractionation microarrays allow for the screening of biomarkers across the entire proteome of a cell line or tissue.

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Figure 1.

Serological identification of antigens by recombinant expression cloning (SEREX): A cDNA library is generated from a tumor and cloned into a bacteriophage expression vector. The recombinant phages are then used to infect *Escherichia coli* bacteria, and the recombinant proteins are expressed during the lytic phase of the infection. The proteins are transferred to a membrane, and incubated with patient sera to identify clones expressing immunoreactive proteins. The reactive proteins can then be identified by sequencing their corresponding cDNAs.

Figure 2.

A schematic of the native protein antigen “reverse capture” microarray platform. Well-characterized monoclonal antibodies are printed onto a glass slide. Native protein antigens derived from cell lines or cancer tissues are immobilized onto the array by the printed monoclonal antibodies, with the corresponding antigen captured by a specific monoclonal antibody. Using the immobilized antigens as bait, labeled autoantibodies from a patient and control are then allowed to react with the immobilized antigen. The amount of fluorescent dye-labeled autoantibody between test and control will determine reactivity with the immobilized antigen. We termed this array a “reverse capture” microarray since the standard nomenclature for “capture” refers to the analyte/antigen and not the antibody.

Whole Proteome Lysate Fractionation Microarray

Figure 3.

Whole proteome lysate fractionation microarray. Tumor or cell lysates are separated into distinct fractions using a 2-D liquid chromatography fractionation strategy where the 1st dimension is separation by isoelectric points and the 2nd dimension is separation by hydrophobicity. Following the 2nd dimension, the fractions are arrayed onto nitrocellulose coated microscope slides. The slides are then incubated with fluorescent dye-labeled serum antibody isolated from cases and controls. The informative spots on the arrays and their corresponding fractions containing the associated antigens are then validated and the protein antigens subsequently identified by mass spectrometry.

Figure 4.

This figure is an image of a whole proteome breast cancer lysate microarray slide scanned for fluorescence after incubating with antibodies isolated from serum from a patient with late stage breast cancer that have underwent autologous tumor vaccination. Post-vaccination antibodies were labeled with Cy3 (green) fluorescent dye, and pre-vaccination antibodies from the same patient were labeled with Cy5 (red) fluorescent dye. Note that there was little reactivity from the pre-vaccination antibodies, but numerous post-vaccination reactive “spots” are seen.