Protein Microarrays for Cancer Diagnostics and Therapy

Jimmy Cheng-Ho Lin

Johns Hopkins Medical Institutions, Baltimore, Md., USA

Key Words

Cancer • Proteomics • Mass spectrometry • Protein array

Abstract

In this postgenomic age, cancer will be understood in intricate detail beyond the genomic level. New technologies are emerging that allow global proteomic level characterization, profiling and understanding. One of the most exciting emerging technologies of cancer proteomics is protein microarray. In this review, the different types of protein microarrays are discussed, including the methods, challenges and techniques of each type. Subsequently, the application of these specific methods to cancer diagnosis, prognosis and therapy will be overviewed, providing a general review of current methods, and proposing how protein arrays will help shape the future of oncoproteomics.

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Introduction

With the completion of the human genome [1, 2] and the development of new generations of sequencing technologies, the genetics of cancer is now being understood in unprecedented detail. Large-scale sequencing projects have been completed for all the annotated genes for breast

[3], colorectal [4], glioblastoma [5, 6], pancreatic [7] and lung cancers [8, 9]. Even though cancer is in essence a genomic disease, through different processes such as mutation, copy number variation, insertion, deletion and methylation, the resulting functional elements of cancers are proteins [10]. Therefore, the study of the cancer proteome is just as central as understanding the cancer genome.

The ultimate goal for researchers is to be able to diagnose cancers early, make specific prognostic predictions and cure the patient of the disease. In each of these steps, proteins have played a large role. For example, many of the methods for early detection of cancers rely upon protein markers, such as prostate-specific antigen for prostate cancer and cancer antigen 125 for ovarian cancer. Conversely, many of the small-molecule drugs or antibodies are specific inhibitors of proteins, such as tyrosine kinase inhibitors. Whether it is the finding of new biomarkers or developing new therapeutic drugs, it is important to understand, profile and target proteins, and cancer proteomics allows the large-scale understanding of the whole complement of proteins in different cancer cells or in patients with cancer. Therefore, the role of cancer proteomics cannot be underestimated.

In this paper, the focus is on one of the most exciting technologies for cancer proteomics – protein microarray. This technology has allowed for critical understanding

of proteomes and provides organizing principles as researchers pursue cancer proteomics. This review will describe the technology and basic scientific principles, as well as the applications of this technology within cancer research. Global knowledge of the field, as well as some specific applications, would provide researchers and clinicians with an overview of cancer proteomics and how it can be translated into cancer diagnostics, prognostics and therapeutics.

Protein Microarray: Technology Overview

Protein microarray is the simultaneous analysis of a large number of different proteins in one single experiment, allowing for a parallel assessment of a large number of proteins. While the term 'protein microarray' has been used by different authors to encompass different meanings, here, protein microarray is used as the general term for the high-throughput parallel chip-based investigation of protein identity, quantity, interaction and function. There are two general types of protein arrays: (1) forward-phase protein arrays are arrays to detect proteins, composed of different capture molecules, and (2) reverse-phase protein arrays are arrays composed of proteins on which subsequent analyses can be performed [11]. Unlike DNA, where nucleotide arrays can take advantage of hybridization of complementary base pairs, protein microarray detection takes advantage of the alternate interactions to detect and recognize proteins, such as using antibodies, peptides, nucleic acids or aptamers. The technologies for both types of arrays will be discussed separately below and have been compared for clinical use [12].

Forward-Phase Protein Microarray

For forward-phase protein microarrays, the elements on the array are capture molecules. As previously mentioned, these can include antibodies, proteins, nucleotides or aptamers. Of the different types, the most common is the antibody array. Antibody arrays are so common that some researchers refer to them simply as protein microarrays or protein biochips [13]. Antibody arrays, as the name suggests, take advantage of the specific interaction between antibodies and proteins, and use antibodies as the capture or bait probes. They are printed and immobilized on a solid surface, nitrocellulose, hydrogel or membrane (e.g. polystyrene or polyvinylidene fluoride) in a highly multiplex configuration. Some of the surfaces used include modified glass, such as ones coated with amine-reactive reagent, poly-L-lysine or streptavidin.

With the array of capture probes, the analyte, such as a cellular lysate or a serum sample, is applied and incubated on this surface to allow for interactions between the capture probes and the analyte.

After hybridization-like capturing, the next step is to detect what the specific captured elements are. There are two general categories of detection formats – label-based and sandwich. Label-based methods require pretreatment of the samples prior to incubation in the microarray (fig. 1a). The analyte can be either directly labeled with fluorophores or quantum dots [14] or indirectly tagged after capture with a secondary labeled antibody. An example image of an array is shown in figure 1e. For the sandwich method, while no prelabeling is needed, two different antibodies are required for each target (fig. 1b). The first antibody is placed as the capture probe and a second antibody that binds to another epitope of the target is used to detect the binding. This increases both the specificity and sensitivity. However, it is often not easy to develop two specific antibodies to two different nonoverlapping epitopes on the same protein.

Although these ordered arrays are standardized and the identity is not in question, allowing two possible samples to undergo the same treatment for comparison is still useful in this context. Similar to 2-dimensional differential in gel electrophoresis, the samples can be labeled with different fluorophores (e.g. Cy3 and Cy5) without significant differences in size and charge. Subsequently, the mixture of the 2 samples can be analyzed with 1 protein microarray and imaged with the 2 colors independently. The intensity ratios can be used to detect perturbations of a sample from a control.

The largest difficulty with methods that rely upon antibodies is specificity and affinity matching [15]. First, it is not always easy to obtain antibodies that bind specifically to the protein of interest and which do not cross-react with other similar proteins. Second, even with specific antibodies, for the different proteins on the same microarray, they will often not have the same affinity to the targets, meaning that the concentrations of the analytes will be outside the linear detection range. This makes quantification much more difficult and reduces the reproducibility and accuracy of the measurements.

Besides antibody, other molecules are also being explored for capture probes. One of the types of molecules being investigated is aptamers [16–18]. Aptamers are short single-stranded DNA or RNA oligonucleotides that can sensitively and specifically detect and capture proteins. Nucleotide sequences are less dependent on physiological conditions, such as buffer composition, pH

Fig. 1. Protein microarray types. **a** Label-based antibody array – where the analyte is pretreated to allow detection. **b** Sandwich antibody array – where 2 antibodies are used to detect the analyte. **c** Reverse-phase array – where the analyte is immobilized on the surface. **d** Single-protein array – where only 1 protein of interest is used and immobilized on the surface. **e** Sample picture of a readout from a protein array (see text for more discussion).

and temperature, than antibodies and proteins. Furthermore, nucleotide sequences can be easily generated, and large libraries with high diversity and complexity can be generated, amplified and selected, using competitive screens such as SELEX (systematic evolution of ligands by exponential enrichment) [19]. However, these methods are still being perfected and have yet to be in widespread use.

Reverse-Phase Protein Microarray

A complementary approach to forward-phase protein microarrays is the reverse-phase protein microarray, also called protein lysate array. Instead of the analyte being captured from the solution phase, for reverse-phase protein microarrays, the analytes themselves are immobilized and arrayed on the surface or membrane such that an array is comprised of hundreds of different patient samples or cellular lysates. Subsequently, the antibodies can then be applied to the microarray to detect specific

epitopes, protein sequences or structures (fig. 1c). For example, for clinical samples such as serum, the reverse-phase protein array could be composed of a multiplex array of serum proteins from different patients sometimes at different dilutions. This allows the investigation of a large number of samples in one single experiment. When an array is incubated with one detection protein and a single analyte, the endpoint can be measured and compared across multiple samples.

Instead of mixtures of protein lysates, individual proteins have also been placed in protein arrays (fig. 1d). Instead of understanding different samples, different proteins are queried for their function and interactions. This method can be used to investigate protein-protein interactions, to identify the substrates of kinases or to determine the protein targets of small molecules [20]. Protein arrays with individual proteins have been used more for basic science research, such as querying function and binding of specific proteins. Examples include identify-

ing small-molecule modulators of epidermal growth factor (EGF) signaling [21] or determining the protein interaction network for ErbB receptors [22]. While this is a powerful technology, it is still currently used more for experimental research than for clinical diagnostics, prognostics and therapeutics.

As shown, the whole spectrum of protein array technology is multifaceted and diverse in its current applications and potential uses. Whether forward or reverse in phase, or with different labeling or label-free detection methods, the highly multiplex parallel analysis of complex mixtures and samples is a powerful tool for a diversity of potential applications. With the development of better antibodies, aptamers or more specific capture devices, along with miniaturization and more sensitive detection, the possibilities for protein microarrays could be the first steps in the large-scale simultaneous detection of the cancer proteome in detail that has not been appreciated before.

Protein Microarray: Applications

As one of the fastest emerging technologies, protein microarrays have started to be applied extensively to cancer research. There has been an increasing amount of applications of different protein array methods for different types of cancer, from understanding to diagnostics to therapeutics. Below are some highlights of different applications for different tumor types with specific technologies and methods.

Forward-Phase Microarrays

Because forward-phase protein microarrays allow for the identification, quantification and measurement of large numbers of proteins within a cell, it has been applied to a large number of different tumor types. In the following, some of the applications of forward-phase arrays on breast, lung, pancreatic and prostate cancer will be discussed.

Breast cancer is one disease where forward-phase arrays have been greatly taken advantage of, for understanding the cell lines and tissues themselves as well as the serum and plasma of patients. For example, Woodbury et al. [23] used a multiplex microarray to detect biomarkers in the serum of breast cancer patients using tyramide signal amplification. In addition to biomarker detection in serum, Celis et al. [24] analyzed yet another type of patient sample – interstitial fluid. Using a large number of different methods, including cytokine anti-

body arrays, they analyzed the microenvironment of breast cancer cells in search of potential biomarkers and therapeutic targets. In a subsequent similar study, Celis et al. [25] studied the adipocytes that also contribute to the tumor environment using multiple methods including antibody arrays and provided a picture of the mammary fat proteome related to high-risk breast cancer. Furthermore, cytokine arrays have also been used by themselves for breast cancer research. For example, Lin et al. [26] used a forward-phase array of antibodies for cytokines to detect protein expression levels of different cytokines in breast cancer, identifying the important role of interleukin 8 in breast cancer oncogenesis.

Comparisons of the biology between breast cancer and normal cells have also been explored. For example, Hudelist et al. [27] used forward-phase antibody-based protein arrays to understand the global protein expression within breast cancer cells compared to normal cells. With a commercial panel of 378 monoclonal antibodies, proteins that were increased in expression in breast cancer included casein kinase I ϵ , p53, annexin XI, CDC25C, eIF-4E and MAP kinase 7. The samples were detected using fluorescence and were verified using immunohistochemistry. They further discussed the general use of protein microarrays for comparison of cancer tissues [28]. In another study, Smith et al. [29] also used forward-phase protein arrays with antibodies, but instead of understanding the biological mechanism, the researchers were profiling the patterns for drug resistance to doxorubicin in different breast cancer cell lines. Using an array of 224 antibodies including diverse pathways, the protein expression changes related to doxorubicin resistance were better understood. This shows the effectiveness of this tool to understand proteomic effects of different therapeutic regimens, which not only allows for better understanding of the drug and disease, but also has potential for personalized medicine.

With the increase in commercially available arrays, it is now easier to investigate different signatures of cancer cells. For example, Vazquez-Martin et al. [30] used Human Cytokine Array III (Raybiotech Inc.) to investigate 42 cytokines and growth factors in specific cell lines as well as sera. Not only was there high correspondence between these methods, but interleukin 8 was linked to metastasis and endocrine resistance of HER2-overexpressing cancers and was hypothesized to be a biomarker for therapy monitoring. In addition, metastatic breast cancer has also been studied with recombinant single-chain variable-fragment antibody arrays. For example, Carlsson et al. [31] compared sera from metastatic breast can-

cer patients to normal controls and identified a possible 11-analyte biomarker signature to distinguish cancer versus healthy serum proteomes with a 95% sensitivity and specificity. These methods, when combined with diagnostic approaches from mass spectrometry, may prove to be complementary evidence and tools for cancer diagnosis.

In addition to ovarian cancer, pancreatic cancer has also been studied using forward-phase protein arrays especially for detecting biomarkers in sera. For example, Orchekowski et al. [32] used antibody arrays to measure protein content in serum from a total of 141 patients with pancreatic cancer, benign pancreatic disease and normal patients. Using 92 proteins, detection by 2-color, rolling-circle amplification, they identified signatures specific to cancer samples that had a sensitivity and specificity of over 90% for distinguishing patients with cancer from normal controls. In another study, Ingvarsson et al. [33] used recombinant single-chain variable-fragment antibody microarrays to obtain protein signatures in sera from patients with pancreatic adenocarcinoma along with healthy controls. They were able to derive a 21-analyte signature that can be correlated to a shorter life expectancy in cancer patients.

For lung cancer, both the sera and the tissues have been profiled with forward-phase protein arrays. For example, in serum, Gao et al. [34] used a protein microarray of 84 antibodies on nitrocellulose-coated microscope slides to query the serum from 80 total serum samples from patients with lung cancer, chronic obstructive pulmonary disease and healthy controls. They experimented with 2-color rolling-circle amplification and found that 7 proteins showed significant differences between lung cancer and normal tissues including C-reactive protein and serum amyloid A, mucin 1 and α_1 -antitrypsin. These were suggested as possible biomarkers for the detection of lung cancers. Lung cancer tissues have also been studied. For example, using arrays of over 300 antibodies, Bartling et al. [35] studied the tumor samples from 12 patients with squamous cell lung carcinoma and with matching controls. They found 29 proteins with differential protein expression, suggesting possible targets of pathways important in these cancers.

In prostate cancer, there have been some examples of applications of forward arrays on sera. In one study, Miller et al. [36] used microarrays of 184 antibodies to detect protein abundances in sera from a total of 53 patients with prostate cancer and controls. Polyacrylamide-based hydrogels on glass showed better signals than poly-L-lysine-coated glass with a photoreactive cross-linking

layer. Five proteins showed significant differences including von Willebrand factor, immunoglobulin M, α_1 -antichymotrypsin, villin and immunoglobulin G. In another study, Shafer et al. [37] also studied the serum but used 2-color, rolling-circle amplification. They identified thrombospondin 1 as a possible biomarker with 79% sensitivity and 81% specificity.

Many other cancers have been studied including bladder [38], colorectal [39, 40], leukemia [41, 42], gastric [43], glioblastoma [44], hepatocellular [45, 46], intestinal [47], squamous cell [48], mantle-cell lymphoma [49] and ovarian cancer [50].

Reverse-Phase Microarrays

While forward-phase arrays allow the understanding of many different attributes for one sample, reverse-phase arrays allow researchers to understand one specific process in a large number of different cell types or patient samples. Specific protein arrays with single proteins in each location allow for the characterization and functional annotation of specific proteins, networks or pathways. While there have been various antibody arrays performed on breast cancers, reverse-phase methods have also been applied. For example, Rapkiewicz et al. [51] used reverse-phase protein arrays to analyze both archival cytology aspirate smears and frozen fine-needle aspiration samples. They showed that even at low abundances, whether phosphorylated or not, proteins can still be reliably and quantitatively measured.

Global panels of cancers have been studied with reverse-phase arrays. Nishizuka et al. [52] used reverse-phase protein microarrays to extensively characterize the 60 human cancer cell lines in the NCI-60 panel. For each, up to 10 twofold serial dilutions were used with quantification by mouse monoclonal antibodies. Other large-scale characterizations based on panels of different cell types have also been performed. For example, Mendes et al. [53] were able to use a reverse-phase array to analyze 90 different cell lines from 12 different cell types for the signal transduction in different pathways such as PI3-K, EGFR and VEGF angiogenesis. In another study, Boyd et al. [54] studied signal pathways by examining the phosphorylation of 100 proteins in 30 different breast cancer cell lines; the surprising finding was that the signatures from proteomic methods were different from cancer subtypes based on transcriptomic studies.

Many other specific cancers have been studied using reverse protein arrays, and potential markers that separate different disease tissues and cancer types have been

discovered. Ovarian cancer, for example, has been studied by reverse-phase protein microarray [55]. Specifically, Wulfskuhle et al. [55] profiled the levels of activated extracellular-regulated kinase (ERK1/2), Akt and glycogen synthase kinase 3 β in different tumors. Using the different levels of the proteins, different subtypes of ovarian cancers were differentiated. Prostate cancer has also been studied based on reverse-phase protein arrays. For example, Grubb et al. [56] obtained pure cell populations by laser capture microdissection and arrayed these samples to understand the changes in cell signaling focusing on the phospho-specific endpoints. In addition, esophageal cancers have also been studied with reverse-phase protein arrays for disease progression and pathway activation [57, 58].

Besides specific protein biomarkers, there are increasingly different uses with reverse arrays. For example, autoantibodies have also been studied. In their study, Qiu et al. [59] arrayed protein lysates from a human lung adenocarcinoma cell line into 1,840 fractions and hybridized sera from 33 totally healthy individuals and lung cancer patients to detect the tumor antigens that react with the antibodies. With the growth of the libraries and patient samples available for research use, there will be increases in the use of reverse protein arrays. As patient care develops that takes into consideration individual variation, panels of lysate arrays would be the optimal tool to survey the effect of different drugs or protein biomarkers in a large population of diverse samples.

Conclusion and Future

With the great advances in cancer genetics and the promise of personalized medicine, cancer proteomics will become one of the dominant driving forces to develop new diagnostics and therapies. Protein array technologies show great promise. Initial studies with small numbers of samples or antibodies already show the potential of identifying possible cancer biomarkers or therapeutic targets. As these methods continue to mature and develop, no doubt new diagnostics and drugs will result. As protein microarrays grow in sophistication and popular use, forward array use will increase due to more sensitive and specific antibodies or aptamers, while reverse array use will increase with more patient samples collected. As these libraries grow and the technology resolution continues to increase, the combination of the growth of biological sample and technological advance will transform the use of protein microarrays in cancer detection, prognosis and therapeutics. However, hurdles such as amplification or large-scale sequencing methods still exist and will require revolutionary technologies to propel this field. As nucleotide sequencing enters the next generation or possibly the third, perhaps sooner rather than later, proteomics will start its own revolution into the next generation. The ultimate promise is that one day, researchers will be able to detect and identify protein populations in vivo in different tissues as patients enter the hospital, and these signatures will guide not only diagnostics, but also monitoring of physiology or determination of therapy.

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