Proteomic Analysis of Tumor Biomarkers in Human Clinical Specimens Using the BioOdyssey™ Calligrapher™ MiniArrayer

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Introduction

Reverse-phase protein microarray (RPPA) technology is a powerful emerging analytic strategy for interrogating the proteomes of tissues and cells. As a high-throughput screening platform, RPPA permits rapid quantitative identification of novel cancer biomarkers associated with oncogenesis and disease progression. RPPA should be useful in accelerating the understanding of cellular differentiation, transformation, angiogenesis, tumorigenesis, and metastasis. This new technology has the ability to quickly elucidate alterations in protein expression levels, detect posttranslational modification and mRNA processing events, and dissect molecular networks associated with drug administration or exposure to environmental factors (for example, toxins, infectious agents, or radiation). With this new ability, cancer researchers, clinicians, and, more importantly, patients should realize a significant benefit in biomarker discovery, molecular diagnostics, drug development, and personalized medicine.

Innovations that increase the production, quality, and performance of protein microarrays are critical to realizing the full potential of this screening platform. Laboratories engaged in high-throughput protein biomarker identification and discovery require protein microarray products and methods that are easy to analyze, are easy to automate, and ensure consistent results. The amount of information obtained from current protein microarray experiments is limited to what one probe and slide can generate, and thus these experiments can consume an excessive amount of expensive resources, reagents, and clinical tissue samples. Realizing the full scientific and healthcare benefits of RPPA will require that sample throughput and sensitivity be maximized. Significant cost reductions can be achieved by using innovative multiplex detection systems to probe multiple samples with multiple analytes in a single assay.

In our laboratory, we have developed a line of ready-to-use protein microarrays for cancer research. Arrays are fabricated using lysates prepared from an extensive collection of human tumor and normal clinical specimens representing a variety of organs, tissues, and cancer subtypes. Our approach is similar to that used to evaluate protein expression in the NCI-60 cancer cell lines (Nishizuka et al. 2003) and biopsy samples in clinical trial research (Gulmann et al. 2005). We are also developing sensitive detection systems that would permit automated multiplex analysis of multiple targets in a single assay. The development and manufacturing of our products require a robust and consistent arraying platform with exceptional spot-tracking features for quality control and run validation.

Detection sensitivity is an inherent challenge in the use of protein microarrays, especially for the detection of specific proteins in complex biological samples such as cell or tissue lysates. Spot sizes are often small (~100–300 µm), and the amount of target protein available for detection is correspondingly reduced. The choice of detection methods (chromogen, chemiluminescence, fluorescence, etc.) also influences the detection limits of the assay. Finally, data capture and image analysis hardware and software vary greatly in sensitivity, flexibility, and of course cost. Therefore, to maximize the data obtained from an experiment, it is important to maximize sensitivity.

We wished to evaluate parameters affecting the use of protein microarrays and the quality of the data obtained from experiments using them. To determine specificity and sensitivity criteria in our system, we prepared a special batch of microarray slides using lysates from lung, breast, and colon tissue. Slides were interrogated with individual antibodies specific for known biomarkers involved in oncogenesis, vascularization, the extracellular matrix, and cell maintenance. Target visualization was performed using a chromogen detection system, and microarray images were acquired and processed.

Methods

Six pairs of patient-matched normal and tumor tissue protein extracts from colon, breast, and lung were spotted in triplicate on specially prepared nitrocellulose-coated glass slides (Grace Bio-Labs, Inc.). Arrays were printed using a BioOdyssey Calligrapher miniarrayer with TeleChem Stealth SMP6 micro spotting pins, producing 160 µm spots, delivering ~2.0 nl/spot of a 1.0 mg/ml protein solution. Lysates were spotted in

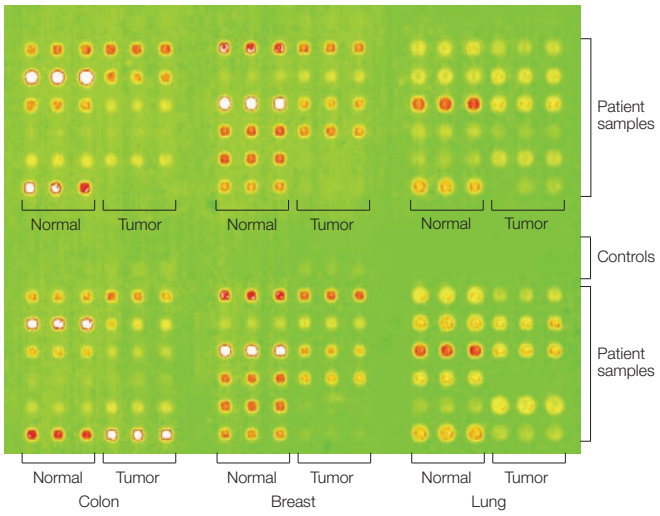

Fig. 1. False-color image of protein levels in a microarray of human clinical specimens. Normal and tumor lysates from six patients were arrayed and probed with an antibody to a circulating protein thought to correlate with differences in vascularization. Each row in a grid represents samples from a single patient. The first three spots in a row are from normal cells and the last three are from tumor cells. Upper and lower grids are duplicates of the same patient samples. The top two rows of the bottom grid consist of negative control spots to assess background noise of the assay.

modified RIPA protein extraction buffer according to the manufacturer's recommended protocols. Positive (purified IgGs) and negative (buffer only and BSA) controls were also included in the arrays. Sample tracking and spot placement were validated using the BackTracker™ file system, which is built into the miniarrayer software.

Slides were treated using protocols similar to those for western blotting of proteins transferred to nitrocellulose membranes (Krajewski et al. 1996). Monoclonal and polyclonal primary antibodies raised against β-actin, tubulin (Imgenex Corp.), glyceraldehyde-3-phosphate dehydrogenase and laminin-1 (Proteus BioSciences, Inc.), human IgG, and rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) were used according to suppliers' recommendations and detected using HRP-conjugated secondary antibodies (Protein Biotechnologies, Inc.). Color development was achieved by incubating the slides in tetramethylbenzidine (TMB) peroxidase substrate (Sigma-Aldrich Co.), and the slides were subsequently scanned using an ArrayIt SpotWare colorimetric microarray scanning system (TeleChem International, Inc.).

Results and Discussion

Protein arrays were used to evaluate several criteria associated with the manufacture and use of this platform as a tool for cancer research. After slides were spotted, labeled, and imaged, false-color images were generated to reveal differential protein target content within each matched pair of samples. Figure 1 shows the differential abundance of a circulating protein marker in normal and tumor samples derived from multiple tissues and multiple patients. For example, in breast tumors, all normal samples displayed higher levels of the protein than the tumor samples. Spot size and deposition were within acceptable limits (10% CV) as was signal-to-noise ratio for colorimetric detection of protein biomarkers using immunological detection methods. Sensitivity using TMB was in the low picogram range.

In our laboratory, the BackTracker software feature that comes with the BioOdyssey Calligrapher miniarrayer adds a unique advantage over those offered in other array systems we have tried. Because we have to create our own source plates for the different protein array products we manufacture, the ability to easily predefine the robot printing program for each unique array layout requirement, which the BackTracker file system provides, is especially valuable.

This study demonstrates the successful use of the BioOdyssey Calligrapher miniarrayer for accurately and reproducibly printing protein arrays of complex biological samples derived from human tumors and normal tissues. The BackTracker software feature enhances our ability to rapidly redefine protein array templates and has significantly improved our product manufacturing capabilities. Rapid screening of multiple clinical specimens for protein biomarkers in a single assay in an easy-to-use platform not only is possible but also is a valuable first step in determining further analytical strategies.

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