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RESEARCH ARTICLE

Antibody array-based technologies for cancer protein profiling and functional proteomic analyses using serum and tissue specimens

Marta Sanchez-Carbayo

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Abstract In the context of other proteomic technologies, targeted antibody arrays are strongly contributing for protein profiling and functional proteomics analyses in serum specimens. Protein-protein interactions, post-translational modifications, and interaction between protein and DNA or RNA can all shift the activity of a protein from what would have been predicted by its level of transcription. Functional proteomics studies the interaction of proteins within their cellular environment to determine how a given protein accomplishes its specific cellular task. Accordingly, the promise of protein profiling and functional proteomics is that by chronicling the function of aberrant or over-expressed proteins, it will be possible to characterize the mechanism of the disease-sustaining proteins. The further understanding of the disease networks will eventually lead to targeted cancer therapy and specific biomarkers for diagnosis, prognosis or therapeutic response prediction based on disease specific proteins. This review describes how such strategies reported to date in serum specimens may assist in characterizing tumor biology, and for the diagnosis, surveillance, prognosis, and potentially for predictive and therapeutic purposes for patients affected with solid and hematological neoplasias.

Keywords Antibody arrays · Functional proteomics · Serum

Abbreviations

DNA	desoxirribonucleic acid
RNA	ribonucleic acid
MS	mass spectrometry
2D	two-dimensional electrophoresis
TAA	tumor-associated antigen
Cy3	cyanine 3
Cy5	cyanine 5
IL	interleukin
PSA	prostate-specific antigen
SA	streptavidin
SPRI	surface plasmon resonance imaging
VEGF	vascular endothelial growth factor
PrEST	protein epitope signature tags
OCT	optimal cutting temperature
RCA	rolling circle amplification
RLS	Resonance light Scattering
ECL	Enhanced ChemiLuminescence
TSA	tyramide signal amplification
TIF	tumor interstitial fluid
CSF	cerebrospinal fluid
HUPO	Human Proteome Organization

M. Sanchez-Carbayo (✉)
Tumor Markers Group, Spanish National Cancer Research Center,
310A, Melchor Fernandez Almagro 3,
28029 Madrid, Spain
e-mail: mscarbayo@cnio.es

Proteomics in oncology: concepts

Cancer can be described as a genetic disease, driven by the multistep accumulation of genetic and epigenetic factors. These molecular alterations result in uncontrolled cellular proliferation, cell cycle deregulation, decrease in cell death or apoptosis, blockage of differentiation, invasion, and metastatic spread. The particular genetic and protein expression alterations that occur as part of the crosstalk between these pathways, will in great part determine the biological behavior of the tumor including its ability to grow, recur, progress, and metastasize. The advent of high-throughput methods of molecular analysis can comprehen-

sively survey the genetic and protein profiles characteristic of distinct tumor types and identify targets and pathways that may underlie a particular clinical behavior. The driving force behind oncoproteomics is the belief that certain protein signatures or patterns are associated with a particular malignancy and clinical behavior. If so, the correlation of clinical parameters with defined protein expression patterns that reflect the mutated genetic program that caused or was involved in cancer progression, would allow tumor stratification, predict disease progression, and even define improved tailored therapeutic modalities. The technological challenges to achieve these goals are significant since the human proteome is not defined. One potential approach to finding cancer-associated protein signatures is proteomic antibody array-based techniques using non-invasive body fluids such as serum specimens.

While the amino acid sequence of a protein is uniquely determined by a nucleotide sequence, the genetic code of a protein is not a complete predictor of the function of a protein. Many in vivo factors can alter the activity level or function of a protein as cells are influenced by a complex system of communication with other cells and factors in their microenvironment. Protein-protein interactions, post-translational modifications, and interaction between protein and DNA or RNA can all shift the activity of a protein from what would have been predicted by its level of transcription. Functional proteomics studies the interaction of proteins within their cellular environment to determine how a given protein accomplishes its specific cellular task. Accordingly, the promise of functional proteomics is that by chronicling the function of aberrant or over-expressed proteins, it will be possible to characterize the mechanism of the disease-sustaining proteins. The further understanding of the disease networks will eventually lead to targeted cancer therapy and specific biomarkers for diagnosis, prognosis, or therapeutic response prediction based on disease-specific proteins. In addition, the response of proteins to molecular-targeted therapy could be monitored to determine the efficacy of the targeted therapy and potential viable future therapies involving the same protein pathway [1].

Several high-throughput techniques are available today for functional proteomics. These techniques can be applied not only to in vitro and in vivo models but also to clinical samples to further characterize protein functions in a multiplexed manner. Immunocapture through immunoblotting, precipitation, histochemistry and protein, and tissue microarrays are tools usually applied to clinical samples (tissue and body fluids). Immunoprecipitation can identify interactions between proteins and can be applied if the clinical sample proteins are of adequate size and stability. Unknown partner proteins in a multiprotein complex can be identified using sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by mass spectrometry (MS) analysis and peptide mass fingerprinting as is done routinely for non-clinical samples. MS cannot only provide sequence from which to identify the protein, it is precise enough to detect co- and post-translational changes such as phosphorylation, glycosylation, acetylation, and alternate cleavage sites. In this review, antibody array-based technologies will be described for protein profiling and functional proteomic analyses to be applied using non-invasive serum specimens.

Antibody array-based techniques in the context of other proteomic approaches

Several antibody array-based techniques are available today for protein profiling and functional proteomics. It is important to correctly classify antibody array-based targeted approaches in the context of other proteomic strategies that may be undertaken to investigate cancer proteomes [2–8]. The terminology of untargeted and targeted proteomics refers to whether the proteins to be measured are known and considered in the experimental design (targeted) and the number of proteins that can be detected and characterized (decided at front in targeted approaches). Untargeted platforms such as two-dimensional electrophoresis and mass spectrometry are best suited for first pass comparisons of proteomes unknown at front in the experimental design to identify relatively few, novel, and known proteins that may exhibit the greatest differences in abundance. These techniques in their low- and high-resolution versions were initially considered the mainstay or standard of proteomic technologies [6–8]. Targeted platforms measure and quantify known proteins of interest identified previously, and are suited for analyses of quantitative differences in abundance among known protein families and pathways. Tissue arrays and multiplexed western blots are considered targeted proteomic approaches [8]. However, antibody and protein microarrays are considered the main targeted techniques used for large-scale analysis of many samples and known proteins. These two latter represent the most versatile among the proteomics techniques available to date, since antigens, peptide, complex protein solutions, or antibodies can be immobilized to capture and quantify the presence of specific either proteins or antibodies, respectively [6–8]. Immobilization of proteins either as purified or phage-displayed protein versions or in format of complex protein solutions have led to tumor-associated antigen (TAAs) or reverse-phase arrays [9–12]. TAAs arrays utilized on serum specimens enhance the detection of autoantibodies against TTAs, which can be utilized for cancer diagnosis and patient outcome stratification and the characterization of protein-antibody interactions. The rationale of TAAs arrays in clinical practice is

related to the presence in the cancer sera of antibodies which react with a unique group of autologous cellular antigens or TAAs [9, 10]. Complex protein extracts can also be spotted onto membranes and probed with antibodies targeting specific proteins and pathways on the so-called reverse-phase arrays [11, 12]. Overall, the versatility of targeted platforms allows controlling and estimating the reproducibility, scalability, and precise antibody and protein quantification, leading to high sensitivity and coverage. One of the major advantages of the antibody arrays approach is that it allows experimental designs to address specific hypothesis, and biological interpretation of the results obtained, making them critical for protein profiling and functional proteomic analyses in oncology. However, the number of proteins amenable for these analyses depends on the availability of antibodies with high affinity and specificity to bind a target protein [6–8]. Because of the little overlap between studies conducted with targeted and untargeted approaches using the same specimens, confirmation of the advantages and pitfalls of these types of high-throughput proteomic technologies remains an elusive goal. Overall, any of these proteomic strategies are impacting on protein profiling and functional proteomic studies and the discovery of cancer-specific candidates (Table 1). In this review, these proteomic technologies have only been summarized to set up the main differences among them.

Antibody array formats

Current formats Depending on whether the antibodies are immobilized on a planar or spherical surface, antibody arrays have been classified into planar and suspension/bead formats, respectively (Fig. 1). Innovation in the immobilization surfaces and detection strategies are leading to an increasing number of planar arrays and bead-based antibody array technologies. Planar antibody arrays represent the most versatile type, as shown along the clinical applications presented for the discovery of targets, functional networks, and biomarker candidates below. The main planar label-based formats comprise one antibody and sandwich assays. One antibody and sandwich assays present advantages and pitfalls over each other. In both formats, the target protein is always captured by one (or more) immobilized "capture" antibody in the array. In one antibody label-based assays, the targeted proteins are detected through labeling with a tag. In sandwich assays, a second not immobilized "detection" antibody interacts with a different epitope for a given monomeric protein enabling detection by forming a 'sandwich' (Fig. 1). In the direct labeling, proteins are labeled with a fluorophore (including cyanines such as Cy3 or Cy5). In the indirect labeling, proteins are labeled with a tag that is later detected by a labeled antibody [7]. By multiplexing with different fluorescent labels for each sample, one antibody label-based assays may allow the incubation of more than one sample simultaneously. These assays can be designed to be competitive if the analytes belonging to the co-incubated test and reference solutions compete for binding at the antibodies. The competition in one antibody (two color) assays is ratiometric and does not imply that the analytes are saturating the antibodies. This competition has been suggested to lead to improvements in linearity of response and dynamic range as compared to non-competitive assays

Table 1 Main characteristics of array based proteomic techniques

Technique	Printed molecule	Pitfalls	Most frequent application
Antibody (forward phase) arrays	Highly specific antibodies	Availability of antibodies	Protein profiling Biomarker discovery
		Cross-reactivity	Signaling Post-translational modifications
Bead-based multiplexed arrays	Antibodies coating differentially identifiable beads	Degree of multiplexing limited by number of differentially identifiable beads	Protein Profiling Cytokine Signaling Biomarker discovery
Reverse-phase arrays	Lysate protein extracts	Limited number of analytes analyzed even with multisectored slides Crossreactivity	Protein profiling Biomarker discovery Signaling Post-translational modifications
Antigen arrays	Purified proteins and peptides	Significance of Autoabs in progression is controversial	Antibody profiling Immune response evaluation Biomarker discovery

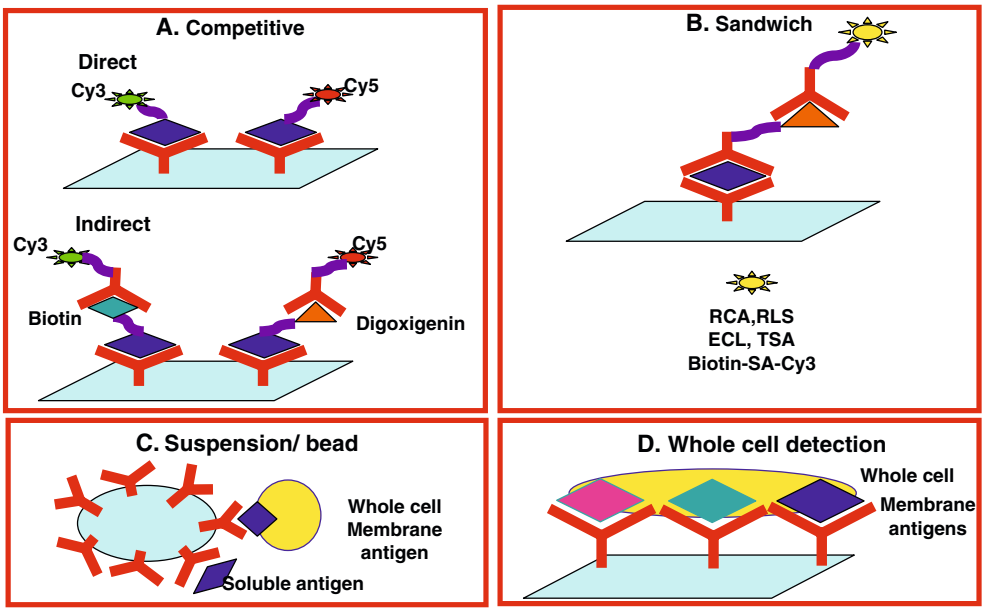

Fig. 1 Main formats of planar and suspension antibody arrays. *RCA* rolling circle amplification, *RLS* resonance light scattering, *ECL* enhanced chemiLuminescence, *TSA* tyramide signal amplification, *SA* streptavidin

[5–8]. The main disadvantage is related to the potential disruption of the analyte-antigen interaction by the label, which may also limit the detection, as well as the sensitivity and specificity.

In the sandwich label-based format, immobilized "capture" antibodies capture unlabeled proteins, which are detected by another "detection" antibody using several methods to generate the signal for detection (Fig. 1b). The use of these two "capture" and "detection" antibodies against different epitopes of a given analyte increases the specificity for the target protein to be measured as compared to label-based assays. The reduced background of these assays increases also the sensitivity. The sandwich format allows only non-competitive assays, since only one sample can be incubated on each array [2–8]. This format requires standard curves of known concentrations of analytes to achieve accurate calibration of concentrations. As compared to label-based assays, sandwich arrays are more difficult to develop in a multiplexed manner since matched pairs of antibodies and purified antigens may not be available for each target, and the potential cross-reactivity among detection antibodies increasing with additional analytes. The practical size of multiplexed sandwich assays limits to 30-50 different targets [6–8]. This contrasts with one antibody assays where only availability of antibodies and space on the substrate limit the number of targets analyzed.

Proteins in suspension can also be detected using bead/suspension arrays (Fig. 1c) [13–15]. These arrays use different fluorescent beads, each coated with a different antibody and spectrally resolvable from each other [13–15]. The beads are incubated with a sample to allow protein binding to the capture antibodies, and the mixture is incubated with a cocktail of detection antibodies, each corresponding to one of the capture antibodies. The detection antibodies are tagged to allow fluorescent detection. The beads are passed through a flow cytometer system, and each bead is probed by two lasers, one to read to the color or identity of the beam, and another to read the amount of detection antibody on the bead [13–15]. Multiplexed bead-based flow cytometry assays represent an active area of development. Differentially identifiable beads coated with either proteins, autoantigens, or antibodies can identify a variety of bound antibodies or proteins using a flow cytometer system [13–15]. Other antibody array approaches have been developed as modifications of the one antibody and sandwich label-based arrays. These alternate strategies allow detection of proteins on whole cells without protein isolation (Fig. 1c,d) [3, 4]. Advances in instrumentation and bead chemistries are making this approach very valuable for the detection of circulating cancer cells. As another version of this concept, suspensions of cells can be incubated on antibody arrays, and the amount of cells that bound each antibody can be quantified by dark field microscopy. These arrays have the potential of characterizing multiple membrane proteins in specific cell populations or changes in cell surfaces induced by drug therapies.

Emerging formats Several examples can be provided to delineate recent remarkable innovations achieved to monitor specific post-translational modifications as well as to increase the limits of detection or enable the technology to profile protein extracts obtained from very few individual

cells. In a first example, antibody arrays are adapted to detect differences in the content of glycans (sugars or carbohydrates) of proteins. These carbohydrate post-translational modifications on proteins are known to be important determinants of protein function in both normal and disease biology. Antibody array designs have been developed to allow efficient, multiplexed study of glycans on individual proteins from complex mixtures [16, 17]. Once multiple proteins are captured using antibody microarrays, these post-translational modifications can be detected using lectins or glycan-binding antibodies [17]. In pancreatic cancer, profiling of both protein and glycan variation in multiple serum samples using parallel sandwich and glycan-detection assays, has identified the cancer-associated glycan alteration on proteins in the serum of pancreatic cancer patients [17]. These antibody arrays for glycan detection are opening a novel field of glycobiology research in the context of neoplastic diseases for functional proteomics and the discovery of potential targets and cancer biomarker candidates.

High sensitivity, in the femtomolar range, allowing protein quantification from limited sample quantities (only six cells) can be achieved by the so-called antibody "ultramicroarrays" [18]. These arrays were initially tested for the detection of interleukin (IL)-6 and prostate-specific antigen, finding detection levels using purified proteins in the attomolar range [18]. Remarkably, this strategy should enable proteomic analysis of clinical specimens available in very limited quantities such as those collected by laser capture microdissection.

Another critical technical development that is being applied to antibody arrays increasing the limits of detection is quantum dot technology. By offering remarkable photostability and brightness and low photobleaching, quantum dots allow detection of proteins in biological specimens (serum, plasma, body fluids) at picogram/milliliter concentration, as has been shown to detect several cytokines [19]. Models of quantum dot probes include conjugation of nanocrystals to antibody specific to selected markers and the use of streptavidin-coated quantum dots and biotinylated detector antibody [19]. By allowing monitoring of changes in protein concentration in physiological range in body fluids, the methodology can potentially be applied to other types of planar and suspension arrays.

Another technical innovation allowing detection of proteins at picomolar concentrations utilizes surface plasmon resonance imaging (SPRI) measurements of RNA aptamer microarrays. The adsorption of proteins onto the RNA microarray is detected by the formation of a surface aptamer-protein-antibody complex. The SPRI response signal is then amplified using a localized precipitation reaction catalyzed by the enzyme horseradish peroxidase that is conjugated to the antibody. This enzymatically amplified SPRI methodology has initially been characterized for the detection of human thrombin at the fM concentration range. The appropriate thrombin aptamer for the sandwich assay can be identified from a microarray using several potential thrombin aptamer candidates. The SPRI method has also been optimized to detect the protein vascular endothelial growth factor (VEGF) at a biologically relevant pM concentration. This incipient technology shows a potential for increasing this sensitivity for detecting proteins in body fluids [20]. The sensitivity achieved for VEGF allows its measurement in the serum for selecting or monitoring antiangiogenic therapies for breast, lung, or colorectal cancer. In the same line of research, an independent study using a 17-multiplexed photoaptamer-based array has exhibited limits of detection below 10 fM for several analytes including the VEGF and endostatin, among others, in serum samples. Since photoaptamers covalently bind to their target analytes before fluorescent signal detection, the arrays can be vigorously washed to remove background proteins, providing the potential for superior signal-to-noise ratios and lower limits of quantification in biological matrices. Interestingly, the affinity of the capture reagent can be directly correlated to the limit of detection for the analyte on the array [21].

Strategies and applications of antibody arrays for serum proteomics

The increasing number of strategies of antibody arrays is improving, emerging, and challenging in their applications in protein profiling and functional proteomic cancer research. Significant contributions of proteomics research using antibody arrays reported to date have derived from a wide spectrum of experimental designs using different specimens varying from in vitro and in vivo models (not covered in this review), bodily fluids, and tissues. Representative examples of these strategies in clinical specimens comprising from single experiments to comparison of relatively low or medium size datasets obtained under different conditions (e.g., normal, preneoplastic, inflammation, and cancer) are described in this review.

The initial report applying antibody microarrays in serum cancer for the discovery of biomarker candidates was performed using direct labeling methods for prostate cancer, comparing several substrates for antibody printing [22]. As part of optimization analyses, data from "reverse-labeled" experiment sets accurately predicted the agreement between antibody microarrays and enzyme-linked immunosorbent assay measurements [22]. Comparison of protein profiles of patients with prostate cancer and control serum samples identified five proteins (von Willebrand factor,

immunoglobulin M, α1-antichymotrypsin, villin, and immunoglobulin G) that had significantly different levels between the prostate cancer samples and the controls. This initial study using direct labeling protocols is one of the critical analyses that led to multiple developments enabling the immediate use of high-density antibody and protein microarrays [22]. The use of amplification protocols, such as the two-color rolling circle amplification method served to improve the detection of low abundant proteins. This method has also been shown to provide adequate reproducibility and accuracy for protein profiling on serum specimens and clinical applications [23–25]. Sandwich assays can also measure protein abundances in body fluids such as the serum using amplification detection methods such as resonance light scattering [26], enhanced chemiluminescence [27], or the tyramide signal amplification method [28] (Fig. 1, reviewed in 7). A report designed antibody arrays for bladder cancer by selecting antibodies against targets differentially expressed in bladder tumors versus their respective normal urothelium identified by gene profiling [29]. Serum protein profiles obtained by two independent sets of antibody arrays served to segregate bladder cancer patients from controls. Protein profiles also provided prognostic information by stratifying patients with bladder tumors based on their overall survival in an independent printing set. In addition, serum proteins, such as c-met, that were top ranked at identifying bladder cancer patients were associated with pathological stage, tumor grade, and survival when validated by immunohistochemistry of tissue microarrays containing bladder tumors [29]. Such strategy provides experimental evidence for the use of several integrated technologies strengthening the discovery process of cancer-specific biomarker candidates and functional proteomic analyses of disease progression.

Cytokine profiling on serum and plasma specimens represents one of the most described applications of antibody arrays technology, especially for autoimmune diseases. In neoplastic diseases, they have been evaluated to a lower extent, although the implementation of cytokine antibody arrays is increasing in many aspects of cancer research, such as the discovery of biomarker candidates, molecular mechanisms of cancer development, preclinical studies, and the effects of cancer compounds. Studies linking clinical material (serum) and in vitro systems have revealed the potential of cytokine profiling using antibody arrays for characterizing hematological neoplasias [8–10], or in serum of patients with breast cancer [30, 31]. Cytokine profiles can support differentiation between cancer patients from control subjects and also stratify patients with leukemia based on clinical outcome. Several reports have also compared the reproducibility and differences among the several technologies available for multiplexing cytokine measurements, including not only planar antibody arrays but also bead-based technologies [13–15]. Cytokine profiles of cell lysates have also been analyzed by means of cytokine arrays and compared to those obtained on body fluids and tissue extracts [30]. Commercially available cytokine arrays were applied to conditioned media of cancer cells to dissect functional cytokine-secreted signatures associated to the overexpression of critical breast cancer target genes in breast cancer cells. This strategy revealed that the enhanced synthesis and secretion of members of the IL-8 chemokine family may represent a new pathway involved in the metastatic progression and endocrine resistance of HER2-overexpressing breast carcinomas [31]. Not only this, in vitro strategy served to identify a potentially relevant signalling pathway but also identified a cancer protein-specific signature with clinical applications in blood specimens.

Other body fluids such as the tumor interstitial fluid (TIF) which perfuses the tumor environment has also been utilized for protein functional proteomic profiling using antibody arrays. Analysis of the TIF could identify factors present in the tumor microenvironment that may be associated with tumor growth and progression. TIFs collected from small pieces of freshly dissected invasive breast carcinomas have been analyzed by cytokine-specific antibody arrays. The approach provided a snapshot of more than 1,000 proteins (either secreted, shed by membrane vesicles, or externalized due to cell death) produced by the complex network of cell types that make up the tumor microenvironment. Considering that the protein composition of the TIF reflects the physiological and pathological state of the tissue, it should provide a new and potentially rich resource for the discovery of diagnostic biomarker candidates and for identifying more selective targets for therapeutic intervention [32, 33]. Interestingly, labeling and hybridization methods have been optimized for multiple protein detection on cerebrospinal fluid specimens, characterized by low protein concentrations [34]. Non-invasive body fluids such the saliva, sputum, or urine specimens represent potential samples for clinical application of antibody arrays. It is required to optimize labeling and hybridization protocols to the sensitivities required for such specimens.

Strategies and applications in tissue specimens

It is also feasible to characterize proteomic profiles of protein extracts of tissue specimens using antibody arrays. By comparing malignant and normal counterparts, it is possible to identify differentially expressed proteins associated with disease progression. This strategy has been performed in lung cancer comparing tumor samples from patients with squamous cell lung carcinoma and normal

lung tissue controls with a high number of antibodies printed on antibody arrays [35]. Among the differentially expressed proteins, up-regulated proteins were shown to correlate with a high messenger RNA expression obtained from paired gene microarray data. Thus, using a tumor profiling strategy, antibody microarrays served to identify functional networks and biomarker candidates in lung cancer [35]. In line with this strategy, it is possible to characterize protein profiles of neoplastic subpopulations obtained from frozen resected tumor specimens using laser capture microdissection [36]. This procedure is especially critical for data interpretation in heterogeneous tumors such as breast or prostatic cancer. For example, profiling of protein extracts of breast tumor versus the adjacent normal breast tissue identified a number of proteins with increased expression levels in malignant specimens such as casein kinase Ie, p53, or annexin XI. Decreased expressed proteins in the malignant tissue included the multifunctional regulator 14-3-3. Immunohistochemistry in paraffin-embedded normal and malignant sections derived from the same patient using antibodies against these proteins served to validate the data obtained using the antibody microarrays [36]. In this exercise, protein profiling of a single neoplastic patient using a commercially available microarray served to identify molecular functional determinants of cancer progression in breast cancer. It seems reasonable to insist on that the clinical validation with high number of specimens on independent sets of clinical material is critical to verify the clinical significance of cancer-specific discovery analyses.

The results of protein profiling of tumor protein extracts using antibody arrays can be validated in several manners in order to confirm that potential identified functional networks and biomarker candidates are cancer specific. On one hand, gene profiling of matched tumors can prove that the increased protein expression is associated with increased transcript profiles [35]. At the protein level, it can also be tested that the differential expression of proteins can be detected using an independent method such as immunoblotting [35], or enzyme immunoassays [22, 29]. Clinical validation of differential protein expression patterns can be confirmed by immunohistochemistry using the same antibodies that were printed on the antibody arrays on paraffin-embedded normal and malignant tissues providing high reliability on the results found by protein profiling. If tissue arrays with well-characterized independent set of tumors are available, it is possible to evaluate clinico-pathological correlations of novel cancer-specific proteins identified with antibody arrays in the serum, cancer cells, or in tissue specimens with tumor stratification, disease progression and clinical outcome [29].

The use of comprehensive gene profiling analyses using tissue material can identify tumor targets relevant of specific neoplasias for antibody arrays design. Such approach can be applied in antibody-based proteomics to generate protein-specific affinity antibodies to functionally explore the human proteome. Specific protein epitope signature tags (PrEST) can be identified and used to raise mono-specific, polyclonal antibodies, and be subsequently analyzed on paraffin-embedded sections of malignant and normal tissue. Genome-based, affinity proteomics, using PrEST-induced antibodies, is an efficient way to rapidly identify a number of disease-associated protein candidates of previously both known and unknown identity [37]. A descriptive and comprehensive protein atlas for tissue distribution and subcellular localization of human proteins in both normal and cancer tissues is being created [38]. The subsequent antibodies generated can be used for analysis of corresponding proteins in a wide range of assay platforms, including (1) immunohistochemistry for detailed tissue profiling, (2) specific affinity reagents for various functional protein assays, and (3) capture ("pull-down") reagents for purification of specific proteins and their associated complexes for structural and biochemical analyses [38].

A critical part in proteomics research deals with optimization of sample preparation for comprehensive protein measurements. Protease inhibitors can be added in order to overcome accelerated protein degradation due to the presence of secreted proteases. Novel tissue sample handling approaches to enrich (>95% purity) epithelial cells from fresh human tissue samples include the use of an epithelial cell surface antibody. This purification method showed several advantages for proteomic analyses on tissue specimens since a large quantity of cells available for downstream analysis were available and it showed high reproducibility [39]. Flow cytometry, sorting analyses, pull-downs of protein extracts, or spectrometry techniques represent alternative approaches to enrich cell populations of interest before protein profiling using antibody arrays.

Thus, quality control is a critical consideration as proteins and modifications such as phosphorylation may be unstable in improperly handled clinical samples. Optimal outcome can be found when clinical samples are flash frozen immediately upon removal from the patient. Small samples are recommended to be directly embedded in an optimal cutting temperature-like medium and frozen in situ so that thawing is retarded when samples are removed from freezer storage. Furthermore, inclusion of protease inhibitor cocktails that may include phosphatase and other inhibitors in any fixative or lysis background may further protect frozen samples.

As commented above, tissue microarrays using core specimens of tissue paraffin archived blocks which are recasted to create whole-cell microarrays of tumor specimens are considered targeted proteomic approaches that may complement antibody array proteomic analyses. Once tissue specimens are

placed on the tissue array, they can be analyzed concurrently with immunohistochemistry, fluorescence in situ hybridization, and RNA-RNA in situ hybridization. It is necessary to mention that for RNA analyses, prior formalin fixation, and paraffin embedding may limit these later techniques. Newer protocols are under development to improve protein and RNA resolution from these fixed archival samples.

Conclusions

The parallel analysis of multiple proteins in small sample volumes is being applied to measure multiple protein abundances and for functional proteomic analyses using antibody arrays. Application on biological specimens is serving to address disease progression, clinical subtypes, and outcomes in exploratory analyses. Modifications to antibody arrays are leading to protein profiling strategies that may also result into novel cancer targets and biomarker candidates such as: (a) detecting specific protein post-translational modifications, (b) measurement of enzyme activities, (c) quantification of protein cell-surface expression, (d) characterizing signalling pathways, and (e) the development and characterization of antibodies including identification of binding partners to proteins derived from studies for drug discovery or novel epitope mapping for determining regions of proteins than bind specific antibodies.

The use of antibody array methods not only results in added benefit for cancer diagnostics and patient stratification but also provides complementary information for the characterization of the biology underlining tumorigenesis and tumor progression. Protein profiling using antibody arrays is contributing to reveal the importance of monitoring multiple cell-signalling endpoints and thus, mapping specific cellular networks not only in protein extracts from in vitro and in vivo models (not covered in this review) but also form tissue or body fluid specimens [40, 41]. Changes in glycan contents, phosphorylation status, or cleaved states of key signalling proteins can easily be evaluated using antibody arrays as well [42]. It is possible to test whether one pathway might become blocked with chemotherapeutic agents. Analyses of these pathways might reveal relevant information for designing individual targeted therapies and/or combinatorial strategies directed at multiple nodes in a cell-signalling cascade. This strategy might be tested to predict response to novel drug therapies using the protein extracts of the tumors or in body fluids specimens.

Antibody-based microarrays represents a rapidly emerging technology for proteomic analyses that is advancing from the first proof-of-concept studies to increasing protein profiling applications in cancer biomarker development. The increasing number, scope and effectiveness of the formats, methods and applications of antibody arrays are likely to markedly accelerate the characterization of cancer-specific pathways, networks, and post-translational modifications. Identifying cancer-associated protein changes may lead to the discovery of cancer-associated targets and biomarker candidates that may assist in disease predisposition, diagnosis, prognosis, patient monitoring, and possibly for therapeutic purposes on various sample types, such as serum, plasma, and other bodily fluids; cell culture supernatants; tissue culture lysates; and resected tumor specimens. As standards do not yet exist that bridge all of these applications, the current recommended best practice for clinical validation of results is to approach study design in an iterative process using independent sets of human clinical material and to integrate data from several measurement technologies. The main problems described in poorly delineated experimental designs include lack of uniform patient inclusion and exclusion criteria, low patient numbers, poorly supporting clinical data, absence of standardized sample preparation, and limited analytical verification providing estimations of the intra and inter-assay reproducibility.

Several challenges and limitations remain to be improved in the design and application of antibody arrays: (1) the mechanisms by which proteins or antibodies are immobilized in substrates such as nitrocellulose are poorly understood for certain applications; (2) the limited dynamic ranges of 2 or 3 orders of magnitude for certain labeling protocols can be increased; (3) achieving accuracy and reproducibility similar to clinical immunoassays at the very low pico/femtomolar detection level; (4) the immunoreactivity might be affected by the molecular protein complexity and potential protein denaturation; (5) lack of standards and calibrators for all the antibody and reagents utilized; and (6) development of high-affinity and highly-specific antibodies is not possible for all the potential target antigens under study.

The large increase in technical modalities of antibody arrays are requiring standardized processes for storing and retrieving data obtained from different technologies by different research groups. In this regard, it is necessary to acknowledge the multi-institutional effort of the Human Proteome Organization towards the standardization of protocols for critical parameters in serum or plasma proteomic analyses, including protein profiling using antibody arrays. Initial studies provided guidance on pre-analytical variables that can alter the analysis of blood-derived samples, including choice of sample type, stability during storage, use of protease inhibitors, and clinical standardization. It is also critical to standardize statistical strategies for high-confidence protein identification and data analysis. These efforts and strategies towards integrating proteomic datasets would lead towards accurate and comprehensive representation of human proteomes.

Thus, the most significant contribution of proteomic research using antibody arrays for the discovery of molecular networks, targets, and cancer biomarker candi-

dates is expected to derive not from single experiments, but from the synthesis and comparison of large datasets obtained under different conditions (e.g., normal, inflammation and cancer) and in different in vitro and clinical samples from serum and various tissues and organs. The technology will continue providing unique opportunities in cancer diagnostics, patient stratification, predicting clinical outcome, and therapeutic response using serum specimens and other clinical samples. Continued progress in the technology will surely lead to extensions of these applications and the development of new ways of using the methods. Further innovations in the technology and in the experimental strategies will further broaden the scope of the applications and the type of information that can be gathered. In the near future, the detailed characterization of the specific protein expression profiles or protein atlases of each tumor will also serve to better detect, monitor, and stratify the clinical outcome risk of each specific cancer patient so that they may benefit of tailored interventions based on the aggressiveness of their disease.

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