Use of Reverse Phase Protein Microarrays and Reference Standard Development for Molecular Network Analysis of Metastatic Ovarian Carcinoma*

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Cancer can be defined as a deregulation or hyperactivity in the ongoing network of intracellular and extracellular signaling events. Reverse phase protein microarray technology may offer a new opportunity to measure and profile these signaling pathways, providing data on post-translational phosphorylation events not obtainable by gene microarray analysis. Treatment of ovarian epithelial carcinoma almost always takes place in a metastatic setting since unfortunately the disease is often not detected until later stages. Thus, in addition to elucidation of the molecular network within a tumor specimen, critical questions are to what extent do signaling changes occur upon metastasis and are there common pathway elements that arise in the metastatic microenvironment. For individualized combinatorial therapy, ideal therapeutic selection based on proteomic mapping of phosphorylation end points may require evaluation of the patient's metastatic tissue. Extending these findings to the bedside will require the development of optimized protocols and reference standards. We have developed a reference standard based on a mixture of phosphorylated peptides to begin to address this challenge. ***Molecular & Cellular Proteomics 4:346–355, 2005.***

Major discovery efforts brought about by advances in genomic and proteomic technologies have resulted in many new potential drug targets. Most of these new targets are proteins involved in cellular signaling, and a number of array-based technologies are being developed to assess and validate these candidates (1–6). These emergent technologies provide the opportunity to measure the varying activities of enzymatic networks in cell lines before and after perturbation *in vitro*. However, these models may not accurately recapitulate the activity of a protein-signaling network within a cell *in situ* because these networks exist within the context of the inter- and intracellular microenvironment. Therefore, it is critical that protein array technologies be developed to measure the status of kinase-driven molecular networks as they exist within the context of the cellular milieu in both normal and diseased tissues. Protein microarrays can be used to profile the working state of cellular signal pathways in a manner not possible with gene microarrays since post-translational modifications cannot be accurately portrayed by global gene expression patterns alone (3, 7–20).

Unique and potentially revolutionary opportunities exist for protein microarray technology. Because most new drug targets for cancer and many other diseases are signaling-related, a proteomic approach that can elucidate ongoing post-translational phosphorylation events now makes it possible to generate a diagnostic portrait, based on the activity of the drug targets themselves, of who will respond to a particular therapy and who will not. Thus, providing clinicians with knowledge of which pathways are active in a patient's tumor will enable them to specifically apply targeted therapy. The technology may also be used to monitor total and phosphorylated proteins over time, before and after treatment, or between disease and non-disease states, allowing us to infer the activity levels of the proteins in a particular pathway in real time (21–25). Proteomic approaches to molecular network analysis may provide more valuable clinical information than just "response" analysis and is an enabling tool for true patient-tailored therapy.

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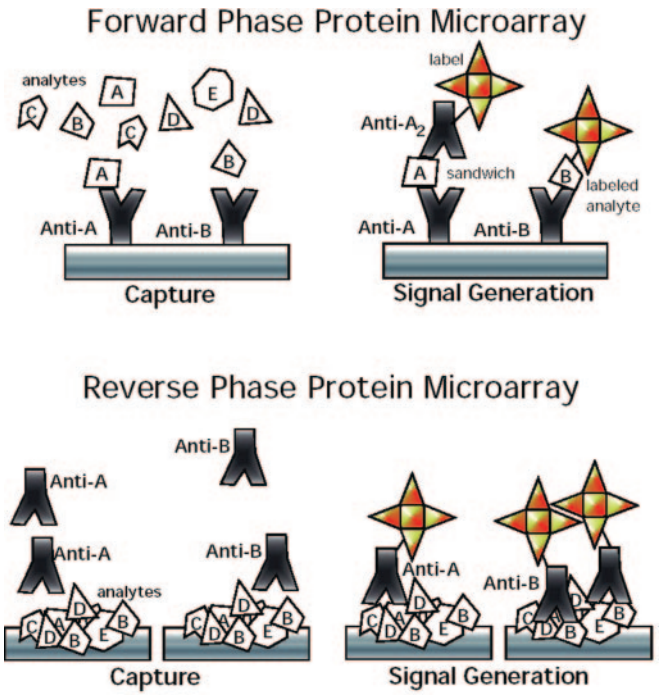

FIG. 1. **Classes of protein microarray technology.** Forward phase arrays (*top*) immobilize a bait molecule such as an antibody designed to capture specific analytes with a mixture of test sample proteins. The bound analytes are detected by a second sandwich antibody or by labeling the analyte directly (*upper right*). Reverse phase arrays immobilize the test sample analytes (*e.g.* lysate from laser capture microdissected cells) on the solid phase. An analyte-specific ligand (*e.g.* antibody; *lower left*) is applied in solution phase. Bound antibodies are detected by secondary tagging and signal amplification (*lower right*).

PROTEIN MICROARRAY FORMATS

Protein microarray formats can be divided into two major classes: forward phase arrays and reverse phase arrays (RPAs).[1] In the forward phase array format, the analyte(s) of interest is captured from the solution phase by a capture molecule, usually an antibody, that is immobilized on a substratum and acts as bait molecule (1, 2) (Fig. 1). In a forward phase array, each spot contains one type of immobilized antibody or bait protein. Each array is incubated with one test sample such as a cellular lysate or serum sample representing a specific treatment condition, and multiple analytes from that sample are measured simultaneously. In contrast, the RPA format immobilizes an individual complex test sample in each array spot such that an array is comprised of hundreds of different patient samples or cellular lysates. In the RPA format, each array is incubated with one detection protein (*i.e.* antibody), and a single analyte end point is measured and directly compared across multiple samples (17, 24, 26–29) (Fig. 1). Probing multiple arrays spotted with the same lysate concomitantly with different phosphospecific antibodies provides the effect of generating a multiplex readout. Efforts are ongoing in our laboratory to multiplex the arrays even further through the use of dual color infrared dye-labeled antibodies as well as quantum dots. Using these technologies, it is hoped that multiple analytes can be measured on the same spot on the same array (30, 31). The utility of reverse phase protein microarrays lies in their ability to provide a map of known cell signaling proteins. Identification of critical nodes, or interactions, within the network is a potential starting point for drug development and/or the design of individual therapy regimens (21, 22). The array format is also amenable to extremely sensitive analyte detection (Fig. 2) with detection levels approaching attogram amounts of a given protein and variances of less than 10% (1, 32). Detection ranges could be substantially lower in a complex mixture such as a cellular lysate; however, the sensitivity of the RPAs is such that low abundance phosphorylated isoforms can still be measured from a spotted lysate amount of less than 10 cell equivalents. This level of sensitivity combined with analytical robustness is critical if the starting input material is only a few hundred cells from a biopsy specimen.

The reverse phase protein array has demonstrated a unique ability to analyze signaling pathways using small numbers of cultured cells or cells isolated by laser capture microdissection from human tissue procured during clinical trials (17, 24, 26, 27). Using this approach, microdissected pure cell populations are taken from human biopsy specimens, and a protein lysate is arrayed onto nitrocellulose-coated slides (Fig. 3). Key technological components of this method offer unique advantages over tissue arrays (33) or antibody arrays (34, 35). First the RPA can use denatured lysates so that antigen retrieval, which is a large limitation for tissue arrays, is not problematic. Protein microarrays can also consist of non-denatured lysates derived directly from microdissected tissue cells so that protein-protein, protein-DNA, and/or protein-RNA complexes can be detected and characterized. Each patient sample is printed on the array in serial dilutions, providing an internal standard. When an internal reference standard of known and fixed amounts of the analyte are applied to the same array, a direct and quantitative measurement of the phosphorylated end point can be attained within the linear dynamic range of the assay. Finally RPAs do not require direct labeling of the patient sample as a readout for the assay, which provides a marked improvement in reproducibility, sensitivity, and robustness of the assay over other techniques (36).

The RPA platform has been used to explore a variety of signaling pathways involved in malignant progression and tumor biology (17, 26–29, 37). For example, in a study of prostate tissue, pathway profiling of microdissected cells from normal, stroma, and prostate tumors revealed the preliminary finding that activation of protein kinase C α is down-modulated in prostate cancer progression (26). If validated, this finding could have profound effects on the rationale behind some current therapies (38) and illustrates the importance of

[1] The abbreviation used is: RPA, reverse phase array.

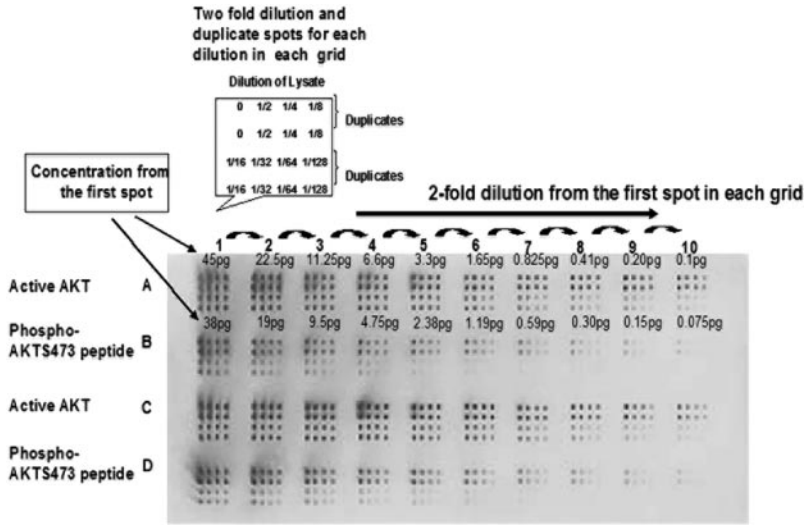

FIG. 2. **Example of sensitivity and reproducibility analysis of the reverse phase protein microarrays.** The first spot of *A1* and *C1* is 45 pg, and the first spot of *B1* and *D1* is 38 pg. *Rows A* and *C* are duplicates from dilutions for recombinant active Akt protein. *Rows B* and *D* are duplicates from dilution for phospho-Akt (Ser-473) peptide. In each grid, the sample was diluted 2-fold from the first spot. Each dilution was spotted in duplicates. From *columns 1* to *10*, each first spot is 2-fold diluted from the previous one. For example, the first spot of grid *A1* is 45 pg, the first spot of grid *A2* is 22.5 pg, and the first spot of grid *A3* is 11.25 pg. The array was stained with phospho-Akt antibody (1:250 dilution).

proteomic technology coupled to signal pathway profiling in providing new and unexpected insights into cellular processes.

MAPPING MOLECULAR NETWORKS IN EPITHELIAL OVARIAN CANCER

With these potentials in mind, we are elucidating the value of RPAs in several types of human cancer tissues to gain insights into potential novel therapeutic strategies. In particular, epithelial ovarian cancer represents a clinical challenge for which much remains to be discovered. Of all gynecological cancers, it carries the worst prognosis primarily due to the late stage at presentation. For the patient with advanced disease, cytoreductive surgery and cytotoxic chemotherapy with taxane and platinum compounds will produce an initial response in the majority of patients, but ultimately most will experience relapse or develop drug resistance and consequently die of their disease (39).

As a result of poor outcome and inability to detect disease confined to the organ, a lot of emphasis has been directed at identifying new disease biomarkers, indicators of response to therapy, and novel treatment options for patients with advanced or refractory disease. Newer chemotherapeutic agents including topoisomerase I inhibitors and taxane analogues may offer scope for defeating resistant neoplastic cells (40). Furthermore our increasing knowledge of the molecular biology of ovarian cancer coupled with advances in global expression profiling has led to the development of novel targeted therapies including monoclonal antibodies, small molecule inhibitors, gene therapy, selective hormonal agents, and cytokines (40). For epithelial ovarian cancer, much emphasis has been placed on developing agents that block epidermal growth factor receptor signaling with either monoclonal antibody (cetuximab) (41) or small molecule inhibitors of the receptor tyrosine kinase (gefitinib) (42). Other innovative agents undergoing trials that specifically target signal transduction pathways directly associated with tumor growth and progression include bevacizumab (Avastatin), a monoclonal antibody that inhibits vascular endothelial growth factor receptor (43), and imatinib mesylate (Gleevec), a small molecule inhibitor of three tyrosine kinases, Bcr-Abl, c-Kit, and platelet-derived growth factor receptor (44). However, as a consequence of the heterogeneous nature of ovarian cancer, the efficacy of specific cancer agents will invariably only suit a subset of patients and probably only at a particular stage of their disease. Given the multitude of molecular defects in ovarian cancer, it is therefore necessary to profile multiple signal transduction pathways simultaneously and define a carcinogenic "fingerprint" specific to the patient. Novel agents can then be selectively applied either alone or in combination with other novel or existing treatments.

Our laboratories have a significant interest in defining and tailoring combinations of new specific drug targets for patients with ovarian and other cancers using the reverse phase arrays to characterize the activated state of cellular signaling pathways in human patient biopsy material. At a molecular level, the process of growth, invasion, and migration of neoplastic cells is driven by a substantial number of integrated and interconnecting pathways that can be quantitatively and sensitively detected in human tissue lysates using protein microarray methodology (45). In a previous study, ovarian cancer epithelial cells were microdissected by laser capture (46), and the activation state of prosurvival and mitogenic

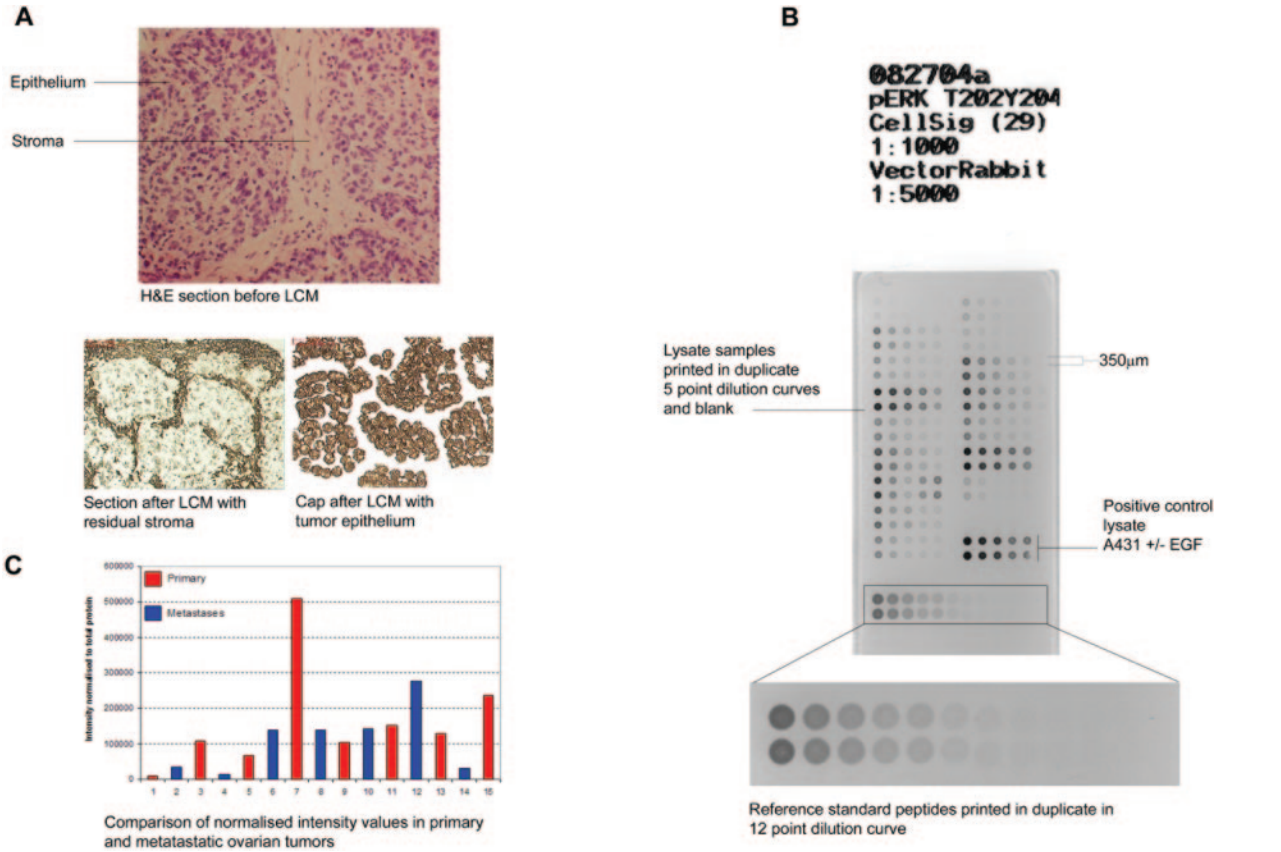

FIG. 3. **Application of reverse phase arrays in mapping molecular networks of ovarian cancer.** *A*, using laser capture microdissection (*LCM*), ovarian cancer epithelial cells were isolated under direct microscopic vision from stained tissue sections leaving residual stroma on the slide. Approximately 25,000 cells were dissected for each case and lysed directly on the laser capture microdissection cap with extraction buffer. One hundred arrays were printed on nitrocellulose-coated slides. *H&E*, hematoxylin and eosin. *B*, example of an ovarian cancer reverse phase array probed for active extracellular signal-regulated kinase (*ERK*) signaling using a phosphospecific antibody detected with a tyramide-based avidin/biotin amplification system. Samples are printed in two columns: the *left column* represents primary tumors, and the *right column* represents metastatic lesions. Cases are printed in duplicate, five-point dilution curves to ensure the linear detection range for the antibody concentration is achieved. The sixth point represents a negative control consisting of extraction buffer alone. A positive control lysate (A431 squamous carcinoma cell line) is printed on the array for monitoring immunostaining performance. Phosphorylation-specific reference peptides are printed in a 12-point dilution curve on the *bottom* of the array for comparative, precise quantification of patient samples between arrays. *pERK*, phosphorylated extracellular signal-regulated kinase; *EGF*, epidermal growth factor. *C*, stained slides for the multiple phosphorylation-specific end points were scanned using Adobe Photoshop. Following total protein estimation with a Sypro Ruby stain, the intensity values of each antibody were normalized to total protein, and dilution curves were generated using Microvigene software. Histograms could then be generated to compare alterations in cell signaling between the primary and metastatic samples.

signaling pathways was evaluated to assess the profiles in primary tumors at different stages of disease progression (27). While the levels of phosphorylated extracellular signal-regulated kinase 1/2 were higher in advanced stage tumors and in those with endometrioid morphology in conjunction with Akt, expression levels tended to be more patient-specific rather than stage-specific (27). The findings exemplify the need to develop patient-tailored therapy that blocks the signaling driving neoplastic progression at the time of treatment irrespective of the stage.

TARGETING THE METASTATIC OVARIAN TUMOR

Unlike other solid epithelial tumors, ovarian cancer spreads initially by surface shedding into the peritoneal cavity followed by invasive implantation. Approximately 70% of patients with ovarian cancer present with International Federation of Gynecology and Obstetrics Stage III or IV disease, indicating that they have metastatic dissemination to the peritoneum beyond the pelvis (47). Accordingly prognosis of the majority patients with ovarian cancer is governed by the behavior of the disseminated metastatic cells and not by the primary tumor. Our understanding of the signaling events that facilitate the detachment, migration, and survival of neoplastic cells from the primary tumor is not fully known, and whether these changes are a cause or consequence of the metastatic process remains to be determined. These answers are critical in identifying specific targets for therapeutic intervention of advanced metastatic disease.

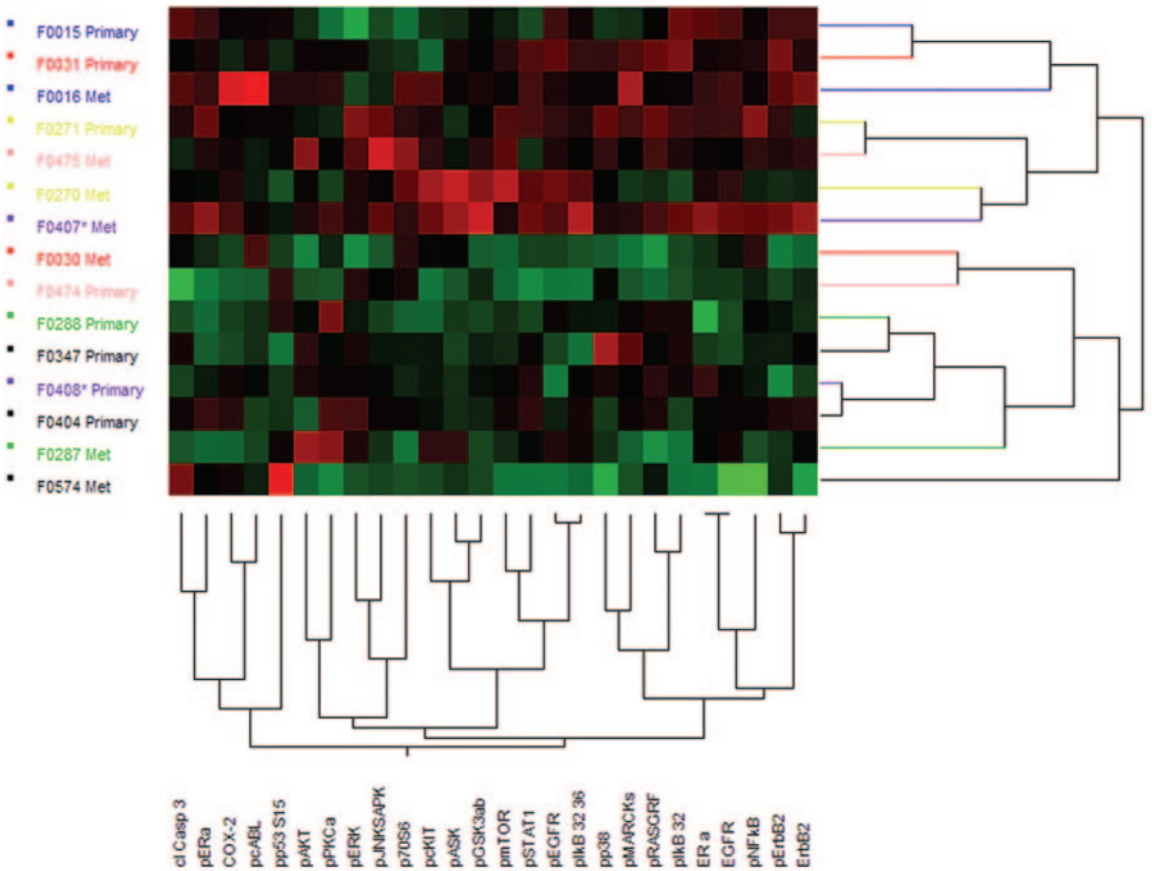

FIG. 4. **Unsupervised hierarchical clustering analysis of multiplexed kinase substrate end points.** The multiple different kinase substrates are outlined on the *horizontal axis*, and the tissue phenotype is on the *vertical axis*. Higher relative levels of a phosphorylated substrate are represented in *red*; lower levels are in *green*. Matched cases are labeled in the *same color type* on the *vertical axis*, and unmatched cases are represented in *black type*. *Met*, metastatic tissue; *cl Casp 3*, cleaved Caspase 3; *p*, phosphorylated; *ER*α, estrogen receptor α; *COX-2*, cyclooxygenase-2; *PKC*, protein kinase C; *JNK*, c-Jun N-terminal kinase; *SAPK*, stress-activated protein kinase; *GSK3*, glycogen synthase kinase-3; *mTOR*, mammalian target of rapamycin; *STAT*, signal transducers and activators of transcription; *EGFR*, epidermal growth factor receptor; *MARCKS*, myristoylated alanine-rich C kinase substrate; *ERK*, extracellular signal-regulated kinase.

As an adjunct to and extension of our previous study of primary ovarian carcinoma (27), we utilized RPA technology to profile a matched cohort of primary and metastatic ovarian carcinomas. In characterizing signal pathway alterations between the two tissue microenvironments, we hoped to gain insights into the aberrant signaling that maintains shed neoplastic ovarian cells at secondary sites. In collaboration with the National Ovarian Cancer Early Detection Program (Northwestern University Hospital, Chicago, IL), 15 frozen tissue samples were obtained from nine patients with a diagnosis of Stage III or IV epithelial ovarian cancer. Six patients had matched primary ovarian tissue and omental metastases obtained during cytoreductive surgery. The histological diagnoses comprised papillary serous, endometrioid, and mixed carcinomas and one primary peritoneal carcinoma. Epithelial cells were microdissected from frozen tumor sections and printed on the arrays as described previously (27) (Fig. 3). The slides were probed with 26 phosphospecific antibodies to proteins involved in mitogenesis including growth factor receptors, signal transducing proteins, and nuclear transcription factors to profile the phosphoproteomic signal pathway circuitry.

Analysis of multiple different kinase substrates detected by phosphorylation-specific antibodies revealed a striking degree of patient heterogeneity in the activity of the signaling cascades within each patient. Unsupervised hierarchical clustering analysis revealed that the samples were divided into two large groups: one in which the majority of end points were activated and the other in which they were not (Fig. 4). This division was not based on primary or metastatic tissue origin or by histologic type. Interestingly the primary peritoneal carcinoma did not have a significantly different phosphoproteomic portrait than the primary ovarian tumors. The second observation was that comparison of cell signaling within the primary group itself or the metastatic group demonstrated considerable variation in the level of signal pathway activation; there was no common pattern specific to either of the tissue microenvironments.

Finally perhaps the most intriguing finding was that the metastatic signatures were dramatically changed compared with their matched primary counterparts with entirely different portraits emerging. Each patient's proteomic pattern had evolved as the tumor spread to a secondary site. In part, these results are similar to our previous work in primary ovarian cancers in that the patterns of activation in human ovarian tumors may indeed be patient-specific. The additional discovery that metastatic cell signaling is so dissimilar to the primary tumor highlights the critical need for patient-tailored therapy that is designed to specifically target the disseminated cells as it is these aberrant pathways that most likely reflect the behavior of the disease within the patient. In this small cohort, each of these patients may have responded quite differently to conventional chemotherapy despite being of similar disease stage. The acquired change in the tumor proteome may indeed be associated with drug resistance. The question currently being addressed by our group is whether metastasis to different secondary sites, *e.g.* liver or lung, shows the same degree of signaling heterogeneity, demonstrating organ- or patient-specific phosphorylation patterns.

The expression profile produced by the microarray experiments represents a snapshot of the proteomic state of the tissues from two distinct microenvironments. To pursue the identification of specific targets that may separate primary and metastatic tumors, we then applied principle component analysis to identify end points that will segregate them from each other. Principle component analysis is an analytic method that identifies a subset of variables that is responsible for the majority of the observed differences among data sets (48). It has been used previously for data mining of both cDNA (49) and tissue microarrays (50) to determine transcriptional fingerprints underlying phenotypic variations. In our cohort, principle component analysis identified several phosphorylated proteins that represented most of the variation between primary and metastatic tissue expression patterns. These included the phosphorylated forms of c-Kit, Ask, myristoylated alanine-rich C kinase substrate, IκBα, and Ras-GRF. Using partition analysis (51, 52), we then found that most of the primary and metastatic tumors could be distinguished from each other by phosphorylated c-Kit expression alone; 13 of the 15 tissue samples could be categorized as either primary or secondary origin (Fig. 5).

The c-*kit* gene encodes a transmembrane receptor tyrosine kinase, a family of receptors that play important roles in the regulation of cellular proliferation and differentiation (53). Its expression and activation are well documented in a variety of human tumors including gastrointestinal stromal tumors, leukemias, germ cell tumors (53), and breast tumors (54). The expression of c-Kit has been also described in epithelial ovarian carcinoma, and although the data vary considerably, the majority have shown elevated expression compared with normal ovary (55). Furthermore c-Kit correlates with advanced stage and chemotherapy resistance in serous ovarian carcinomas (56). c-Kit expression represents one of the fine examples of how expression profiling has led to the development of a selective tyrosine kinase inhibitor, imatinib mesylate (Gleevec) (44).

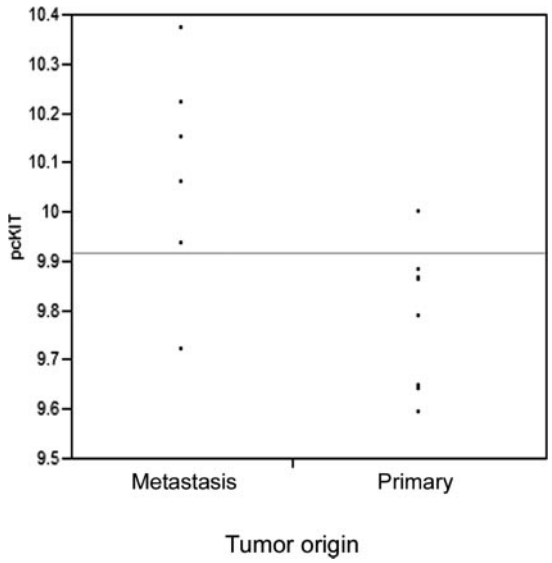

FIG. 5. **Analysis of phosphorylated c-Kit by tissue origin.** Shown is the one-way analysis of normalized relative intensity values for phospho-c-Kit (*pcKIT*) demonstrating segregation of primary and metastatic tissue origins by this end point.

To our knowledge, comparisons of phosphorylated c-Kit expression in primary and metastatic ovarian tumors have not been evaluated previously. Our results have revealed potentially important insights into the most optimal and therapeutic formula for advanced ovarian cancer. In this small study set, the transition from primary to secondary site correlated with activation of c-Kit; therefore these patients may specifically benefit from Gleevec therapy. However, because the individual patients had such a heterogeneous pattern of activated signals in other pathways, combination therapy with other specific kinase inhibitors would have to be selectively applied based on each phosphoproteomic fingerprint.

Clinical trials of Gleevec therapy are already underway for ovarian cancer (40). RPA technology could be an effective method of stratifying patients who may benefit from therapy or for comparing pre- and post-treatment proteomic patterns in patients who are c-Kit-positive. For patients with resistant or refractory disease, an entirely different therapeutic regime may then be chosen based on their activated kinome state.

It is clear, based on these aforementioned case studies, that reverse phase protein microarray technology can generate important and enabling data. However, circumspection is clearly warranted as the translation of intriguing research findings into clinical implementation is fraught with the challenges of reproducibility, standardization, and clinical validation. What technological advances and method developments are required before phosphoproteomics-based molecular network analysis of patient tissue is being routinely performed at

the bedside and the results are used for therapeutic decision making?

CHALLENGES FOR PROTEIN MICROARRAY USE AT THE BEDSIDE

The field of gene expression profiling was catalyzed by the ease and throughput of manufacturing probes with known, specific, and predictable affinity constants. In contrast, the probes (*e.g.* antibodies, aptamers, ligands, and drugs) used for protein microarrays cannot be manufactured with predictable affinity and specificity. The availability of high quality, specific antibodies or suitable protein binding ligands is a major limiting factor and starting point for the successful utilization of this technology (4). Post-translational modifications or protein-protein interactions of an individual protein will contain critical biological meaning that cannot be ascertained merely by measuring the total concentration of the analyte. Consequently a significant challenge for protein microarrays is the need for antibodies or similar detection probes that are specific for the modification or activation state of the target protein.

Antibody specificity must also be thoroughly assessed and validated by Western blot prior to use in any protein array format, and appropriate standards for specificity should be established. Such standards should include evidence of a single, appropriate sized band in immunoblot analysis of complex biological samples similar to those planned for array analysis such as cell lines or whole or microdissected tissue samples. Assessment of a phosphospecific antibody might also require evidence of a differential signal between control and treated samples known to activate the pathway or end point of interest. These same positive and negative controls are also printed directly on each array to ensure real time assessment of specificity. A web posting of our validated antibodies can be found at home.ccr.cancer.gov/ncifdaproteomics/. A significant challenge for cooperative groups, funding agencies, and international consortia is the generation of large comprehensive libraries of fully characterized specific antibodies, ligands, and probes. Fortunately some large initiatives such as those of the internationally based Human Proteomic Organization and the Human Proteome Resource in Sweden and individual investigators are beginning efforts to provide the scientific community with critical antibody resources (57–59).

Another issue faced in research using human tissues is small tumor size and limited availability of specimens for study. While techniques such as laser capture microdissection and RNA amplification allow one to utilize biopsy specimens and small tumors for study, it will be important that future clinical trials incorporate tissue accrual components to allow for the facile evaluation by molecular techniques.

REFERENCE STANDARD DEVELOPMENT

Perhaps the biggest challenge facing the widespread use of the RPA in the clinical laboratory setting is the standardization of each step and process involved in array production, bioinformatic analysis, and ensuring that tissue is processed and handled rapidly and optimally. A recent assessment of the gene expression microarray literature found that there is little standardization in the field with regard to the methods, analysis, and controls used and also data validation (60). These issues often prevent productive data comparisons between laboratories and platforms and between experiments separated in time. This is a particularly relevant issue with regard to the analysis of clinical trial specimens by protein arrays where accrual of samples may occur over extended periods of time and analysis may require the use of multiple arrays to accommodate all samples for a particular study. In addition to the standardization of tissue handling techniques and sample preparation protocols, development of a universal reference standard that could be deposited onto every array would allow one to identify and compensate for variation in instrumentation, reagents, samples, and operators in different laboratory settings and across time.

Ideally an RPA reference standard would serve as a universal positive control for the staining process and antibody validation and also be incorporated into data analysis. A good quality reference standard should be renewable, reproducible on a large scale, reliable across a broad range of end points, and stable over long periods of time. A reference standard should also resemble the test samples as closely as possible (61). Our group has assessed various types of source materials for use as a reference standard on our reverse phase protein array platform. Human tissue extracts, although essentially identical to our test samples, are not renewable or routinely available in large enough quantities for large scale reference standard production. We also found that any particular tissue extract often does not provide reliable signal for all phosphospecific end points of interest in our profiling studies. Theoretically multiple tissue extracts could be combined into a reference standard to overcome this problem, but this only serves to compound the problems of large scale production and renewability. Alternatively extracts from a wide variety of treated cell lines can be purchased or produced in large quantities, but they share the same problems of long term reproducibility and stability found with tissue extracts.

In our laboratory, the use of tissue extracts or lysates from treated cells as reference standards has proven most useful as controls for antibody validation and as positive controls for antibody staining on reverse phase arrays (Fig. 3*B*). Ultimately we have chosen to develop a reference standard containing phosphorylated peptides specific for each of the antibodies routinely used in our profiling studies (Fig. 6). Phosphopeptides represent an invariant, renewable, scalable, and reproducible source material for a high quality reference lysate. Since they are often the immunogen source itself, they bind probe antibodies with high affinity. The peptides can be printed in mixtures on arrays in extended dilution curves and

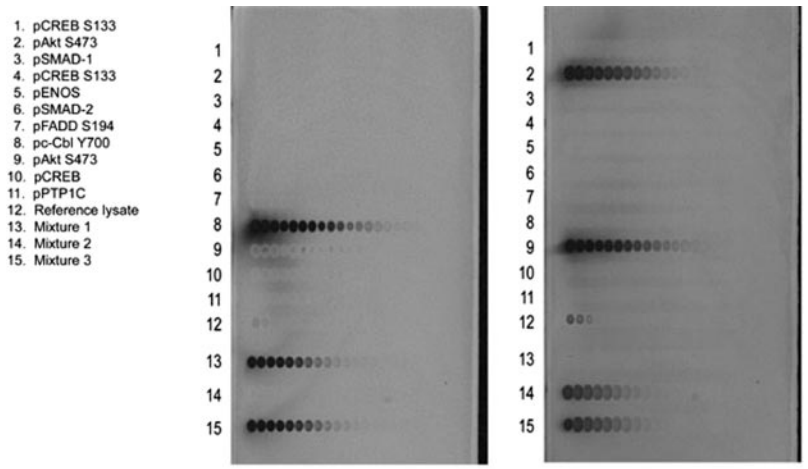

FIG. 6. **Phosphopeptide reference standard development.** Antibody-specific phosphorylated peptides can be used as components of a reference standard for reverse phase protein arrays. This example shows phosphorylated peptides printed in 24-point dilution curves on the arrays at a starting concentration of 1 μg/ml. Peptides were printed alone or in mixtures. The reference lysate sample was composed of whole prostate tumor tissue or peripheral blood lymphocytes. Three different mixtures of peptides were added to determine whether the antibodies could detect peptides arrayed in mixtures. *Mixture 1*: pCREB, pSMAD, pFADD, pPTP1C, and pc-Cbl; *Mixture 2*: pCREB, pAkt, pSMAD-1, and pENOS; *Mixture 3*: all nine peptides. The *left panel* represents an array probed with anti-phospho-c-Cbl Y700 antibody (BD Transduction Laboratories Inc.), and the *right panel* represents a duplicate array stained with anti-phospho-Akt Ser-473 antibody (Cell Signaling Technology Inc.). *p*, phosphorylated; *CREB*, cAMP-response element-binding protein; *FADD*, Fas-associated death domain; *PTP*, protein tyrosine phosphatase; *ENOS*, endothelial nitric-oxide synthase. *SMAD*, human homolog of mothers against decapentaplegic; PTPIC, protein tyrosine phosphatase.

be used to develop a reference calibration curve (Fig. 6). For routine clinical implementation, much like how an ELISA is used, each phosphospecific analyte measurement for any experimental sample that falls into the linear range of the calibrant can be converted into reference standard units based on the calibrant and the dilution factor. The use of a universal reference standard on every array produced in a single facility or a number of different laboratories will facilitate normalization and comparison of test samples across time, platforms, studies, and sites.

COMBINATORIAL TARGETED MEDICINE: THE FUTURE OF PATIENT STRATIFICATION AND "THERANOSTICS"

The use of proteomic profiling to determine the activation status of many dozens of phosphorylation end points and identification of the key signaling nodes in individual tumor tissues could lead to dramatic and significant improvements in therapeutic efficacy and patient survival. Armed with the information about which signaling pathways are being utilized by the primary tumor cells, surrounding stroma, and metastatic lesions will allow us to build specific function interaction maps for each patient. Data produced from our patient-matched ovarian cancer primary/metastasis series indicates the potential need to evaluate the metastatic signaling portrait prior to treatment selection. The metastatic signatures were clearly very different from the primary tumor taken at the same time at surgery, and these fingerprints appear to be virtually patient-specific (Fig. 4). The use of bioinformatics may eventually allow us to understand how this information changes and fluxes as a consequence of disease stage and treatment choice not just within the tumors but in the surrounding cellular milieu. These protein interactions are interdependent on each other, and kinase activity at one location will affect other kinases and substrates within a circuit. We can take advantage of this fact so that we can imagine a future in which targeting a number of critical nodes along the entire deranged signal pathway can result in an outcome with a higher potential for efficacy and lower toxicity (62). Recent mathematical modeling experiments have indicated that, through the selection of multiple and specific signaling interconnections, a large supra-additive synergy can be achieved by inhibiting specific interdependent "nodes" simultaneously (63, 64). This finding is significant based on the patient-specific signaling heterogeneity revealed in actual patient tissue portraits as outlined previously.

The phosphoproteome profiled using RPAs may play a dominant and key role in personalized medicine as the aberrant function of protein kinases are often at the center of many diseases, including cancer (65–73). The ability to develop a portrait built on the activation states of the drug targets themselves represents the power of theranostics (therapeutic drug targets that also are diagnostic biomarkers, such as ErbB2) for cancer and disease treatment. Drug discovery efforts focusing on the development of small molecular weight compounds and biologic agents that can mitigate and modulate specific kinase activity is an intense area of focus for industry due to their key roles in cancer and biology (74–79). On the basis of proteomic and genomic portraits of the disease, an individualized selection of therapeutic combinations that best

target the protein network for that specific patient can be selected and used, resulting in a paradigm shift in patient treatment and disease management.

* The views expressed here are expressed solely by the authors and should not be construed as representative of those of the Department of Health and Human Services, the U. S. Food and Drug Administration, or SAIC, Inc. Moreover, aspects of the topics discussed have been filed as U. S. Government owned patent applications. Drs. Petricoin and Liotta are co-inventors on these applications and may receive royalties provided under U. S. Law.

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