Proteomics in Clinical Trials and Practice

PRESENT USES AND FUTURE PROMISE*

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The study of clinical proteomics is a promising new field that has the potential to have many applications, including the identification of biomarkers and monitoring of disease, especially in the field of oncology. Expression proteomics evaluates the cellular production of proteins encoded by a particular gene and exploits the differential expression and post-translational modifications of proteins between healthy and diseased states. These biomarkers may be applied towards early diagnosis, prognosis, and prediction of response to therapy. Functional proteomics seeks to decipher protein-protein interactions and biochemical pathways involved in disease biology and targeted by newer molecular therapeutics. Advanced spectrometry technologies and new protein array formats have improved these analyses and are now being applied prospectively in clinical trials. Further advancement of proteomics technology could usher in an era of personalized molecular medicine, where diseases are diagnosed at earlier stages and where therapies are more effective because they are tailored to the protein expression of a patient’s malignancy. Molecular & Cellular Proteomics 5:1819–1829, 2006.

The genome has been the primary focus of past explorations of the molecular basis of disease. The information coded within genes is transcribed into mRNA, which then is further processed, modified, spliced, and translated to produce a multitude of proteins from the same starting genetic material, an explosion of raw material. The proteome consists of all of the protein products that are derived from an individual’s full genetic code. Proteomics bridges the gap between what is encoded in the genome and its translation into proteins. Proteins, rather than nucleic acids, represent the functional output of a cell. Proteins thus reflect the true status of a cell, and differential protein expression may help define healthy from diseased states (1).

The study of proteomics characterizes all or selected proteins expressed within a given cell and can outline the flow of information within that protein network (2). It is estimated that more than 500,000 proteins comprise the human proteome, derived from ~35,000 genes in the human genome (3, 4). Proteomics offers more complexity but potentially more specificity than examining genes alone. The study of proteomics can generally be divided into two categories: 1) the characterization of protein expression and 2) the characterization of protein function.

Expression proteomics evaluates cellular protein production encoded by those genes active in a cell and present in the target organ. It exposes the differential expression of proteins between healthy and diseased states. The technologies applied to expression proteomics allow investigation of protein expression in multiple sources including tissue as well as serum, urine, and ascites. The most common techniques used in expression proteomics include two-dimensional gel electrophoresis (2DE), mass and other forms of spectrometry coupled with MALDI, SELDI, and other sample presentation platforms.

Expression proteomics has been applied to the discovery and validation of biomarkers of disease. Biomarkers can be grouped into three major classes: diagnostic, prognostic, and predictive. Diagnostic markers can aid classical histopathological diagnoses especially in cases where a tumor has metastasized and the tumor of primary origin cannot be determined. Although validated diagnostic markers may improve early detection of cancer thereby minimizing the need for invasive diagnostic procedures, very few true diagnostic biomarkers exist. β-Human chorionic gonadotropin has been applied as a diagnostic biomarker of pregnancy with outstanding sensitivity and specificity. Others such as prostate-specific antigen (PSA) in screening for prostate cancer or CA-125 for ovarian cancer are less sensitive and/or specific. Prognostic markers, such as hormone receptors, angiogenic markers, and proliferation markers, may provide information about the likely clinical course of a disease or malignancy and may guide treatment planning (5, 6). Finally predictive markers can foretell the course of a disease and the best modality of treatment. For example, breast tumors expressing estrogen

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1 The abbreviations used are: 2DE, two-dimensional gel electrophoresis; PSA, prostate-specific antigen; TCC, transitional cell bladder carcinoma; CSF, cerebrospinal fluid; NAF, nipple aspirate fluid; LCM, laser capture microdissection; CML, chronic myelogenous leukemia; MAPK, mitogen-activated protein kinase; RICK, Rip-like interacting caspase-like apoptosis-regulatory protein; mTOR, mammalian target of rapamycin; SMIR, small molecule inhibitor of rapamycin; RPA, reverse phase array; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; VEGF, vascular endothelial growth factor.
receptor are best treated with an antiestrogen such as tamoxifen or an aromatase inhibitor (7, 8).

Functional proteomics evaluates the activation state of and/or interactions between proteins and can be used to map the extensive network of signaling pathways in a cell. Mapping protein-protein interactions can be useful for several reasons. The discovery of new binding interactions may give insight into new proteins that are involved in cancer as well as novel oncogenes or provide evidence of common downstream events shared by two distinct signaling networks. Further information of how various pathways intersect can help in developing therapeutic interventions directed against these pathway targets (9). Functional proteomics utilizes MALDI/SELDI technology and also incorporates the use of newer protein and tissue microarrays.

**EXPRESSION PROTEOMICS**

**Techniques**

Proteomics encompasses a broad array of techniques. Bodily fluids and tissues are complex mixtures of proteins that may require separation for further analysis. One of the most common although low resolution techniques is simple 2DE. Proteins are first separated based on charge across a defined pH gradient in one direction and then separated by mass. Proteins are detected by color, immunoanalysis, or prebound fluorescent dyes, and unique spots are quantified by their staining intensity. Spots of interest are excised from the gel and subjected to protease digestion, and peptide fragments can be examined using MS. There are limitations to 2DE analysis. It is a time-consuming process that is difficult to automate and requires a large amount of input protein for most successful applications. Furthermore it cannot adequately resolve the large number of protein modifications within a tumor sample.

Mass spectrometry measures the $m/z$ of ionized proteins as they travel through an electric or a magnetic field. Proteins are analyzed and identified based on unique spectrometric signatures. These features allow identification of structural features such as phosphorylation or methylation. Trypsin digestion of proteins yields peptides that are amenable to MS sequencing. MS is a leading technology for high throughput analysis of expressed tissue or biofluid proteins. Coupling MS with MALDI is one high throughput method by which proteins can be identified. The type of matrix can bias the subset of proteins or peptides flown through the MS system and characterized in the resultant data stream. The use of tandem mass spectrometers allows a more detailed determination of peptide sequences (10).

SELDI-TOF-MS has increased in popularity especially in the examination of clinical samples. SELDI uses chromatographic chip arrays, such as reverse phase, anion exchange, cation exchange, normal phase, or immobilized metal affinity, to
selectively bind proteins (Fig. 1). SELDI, like MALDI, is linked to an automated high throughput system and can examine low molecular mass peptides and proteins below 20 kDa in size (1, 11). More recently, magnetic beads have been introduced for the selective capture of peptides and proteins within test samples such as serum (12). These beads can target different moieties through the use of various normal phase, anionic, cationic, hydrophobic, and metal ion beads. By incorporating the use of MALDI-TOF-MS, the identification of the protein fingerprint can be performed at the same time as evaluating protein-peptide interactions (13).

**Clinical Applications of Expression Proteomics**

**Serum Proteomics**—MS and other advancing proteomic techniques are being applied to identify protein biomarker profiles or protein signatures of specific disease states or phenotypes. We originally posited the hypothesis that the circulation would contain a pattern of expressed proteins that form a signature of organ-confined disease identified with proteomic analysis yielding a highly specific and sensitive signature (14). Once such a biomarker signature or pattern of proteins has been identified, independent studies are necessary to validate and then apply that knowledge to clinical samples with high throughput abilities. Our initial findings that this concept has clinical potential has been confirmed in multiple disease states by a variety of groups. However, until a defined signature or pattern of identified proteins is developed, validated, and demonstrated to retain robustness at an adequate sensitivity and specificity, this approach will neither earn nor maintain the enthusiasm that it has at present. The following examples will highlight some fields in which proteomics may have some clinical utility, but further investigation is needed.

Ovarian cancer is characterized, incorrectly, as a silent disease due to its lack of specific signs and symptoms. Most patients are diagnosed with advanced stage incurable disease making it a logical target against which to apply the new concept of proteomic diagnostic signatures. CA-125, a serum marker for the detection of recurrent ovarian cancer, lacks both sensitivity and specificity and can be elevated in numerous benign conditions. The test case for application of the hypothesis of expression proteomic biomarkers was SELDI-MS proteomic analysis of a series of ovarian cancer and unaffected serum samples (14). Sera were subjected to SELDI-TOF-MS, and the resultant data streams were analyzed. A supervised analysis was done to train an algorithm to recognize cancer and unaffected patients using a set of serum samples obtained from 50 ovarian cancer patients and 50 unaffected women. This discriminatory pattern was applied to an independent masked set of serum samples from which it correctly identified the presence of disease with a sensitivity of 100% and specificity of 95% (14). The concept and general results that a signature can be derived from MS data streams and bioinformatic analysis have been confirmed using a variety of platforms and MS technologies (15, 16).

PSA is a serine protease, the measurement of which has been applied as a biomarker to help identify patients with prostate cancer. Like CA-125, it lacks specificity. It is not consistently accurate at differentiating between benign prostatic hypertrophy and prostate cancer (17). Proteomics may help identify other biomarkers that may be more useful. Serum protein profiles were obtained for 326 men with prostate cancer or benign prostatic hypertrophy or unaffected normal controls in a study conducted by Adam et al. (18). The investigators devised a decision tree algorithm based on these proteomic data and applied it to a separate blinded cohort of patients. Patients with prostate cancer were successfully identified with a sensitivity of 83% and a specificity of 97% (18). Petricoin et al. (19) used SELDI-TOF-MS and a bioinformatic algorithm to devise a proteomic profile of prostate cancer. Their discriminating profile was derived from a training set of 56 patients, 25 of whom had no evidence of prostate disease and a serum PSA concentration of <1 ng/ml, as well as serum from 31 men with biopsy-proven prostate cancer and a PSA concentration greater than 4 ng/ml. The proteomic pattern was then applied to a blinded set of 266 sera to predict prostate cancer from benign disease. It correctly identified 95% of patients with prostate cancer and 78% of patients with benign conditions in this retrospective serum cohort (19). Interestingly, several men who were initially described incorrectly as positive for prostate cancer but whose biopsy showed no cancer later went on to develop prostate cancer. This suggests that there may be potential for this to be predictive of organ-confined subclinical or preclinical disease. Lehrer et al. (20) used SELDI-TOF to examine the serum from 11 men with prostate cancer and compared them with 12 men with benign prostatic hyperplasia. They were able to identify three protein peaks between 15 and 17.5 kDa that were present in the prostate cancer patients but absent in the hyperplasia controls, further showing the potential for this clinical proteomic application (20). Autoantibodies were the focus of a study conducted by Wang et al. (21). Through the use of protein microarray analysis, they examined the serum of 39 prostate cancer patients and 21 controls to develop an algorithm based on the presence of 22 autoantibody markers. This algorithm was then applied to a blinded set of patients and appeared to be superior to PSA screening in correctly identifying affected patients with prostate cancer (21).

Li et al. (22) evaluated the serum from 103 breast cancer patients with stage 0 to stage IV disease and compared them to 41 healthy women as well as 25 women with benign breast disease. Using SELDI-TOF-MS and immobilized metal affinity protein arrays, they identified three serum biomarkers that could differentiate breast cancer patients from unaffected patients with a sensitivity of 93% and a specificity of 91%. These biomarkers, however, could not differentiate between the various stages of disease and thus appeared to reflect presence of malignancy rather than aggressiveness of dis-
nostic signature identified for breast cancer. An algorithm identified as an ongoing issue (24). Our NCI group has differentiated patients with cancer from healthy women with a sensitivity of 76% and a specificity of 90% (23).

Consistency and stability of the proteomic marker has been identified as an ongoing issue (24). Our NCI group has invested detailed attention to quality control. The result of this careful attention to detail is evident in the stability of a diagnostic signature identified for breast cancer. An algorithm was developed in late 2004 using sera from women with varied stages of breast cancer (n = 109) or no breast cancer (n = 109). A quality control algorithm was used first, removing one degraded sample from analysis. The diagnostic algorithm was tested at that time with an independent set of sera and proved highly sensitive and specific for 106 blinded cases. This algorithm was used 6 months later on a separate set of 17 cases of blinded sera from stage I breast cancer patients, correctly identifying 15 as cancer cases. Longitudinal assessment of this algorithm is ongoing.

The BRCA-1 mutation has been linked to an increased risk of breast cancer, but 20% of carriers will remain cancer free and will have no need to undergo prophylactic surgeries (11). Laronga et al. (25) sought to further examine this subset of patients. They followed 30 women with the BRCA-1 gene mutation but no evidence of breast disease until they developed breast cancer (n = 15) or until they remained free of breast cancer for a 7-year follow-up period. MS analysis of the serum from these patients revealed a peak at 5909 m/z that distinguished the two groups (25). A follow-up study conducted by Becker et al. (26) compared the serum samples from this same set of patients and identified 23 specific markers present within the sera of the BRCA-1-positive cancer patients, some of which were overexpressed and others of which were underexpressed. One of the underexpressed proteins was 5.9 kDa, possibly corresponding to the peak seen by Laronga et al. (26). Sequence confirmation and subsequent blinded validation are necessary before this can go further.

**Urine Proteomics**—Urine provides an alternative source to blood for the detection of potential biomarkers. Urine proteomics is an attractive technique because it is noninvasive, and urine is easily collected. The urine proteome also may reflect disease, especially urinary tract diseases due to proximity. It also may provide information related to drug metabolism effects (27). Bladder cancer is diagnosed by cystoscopy, which is both expensive and invasive. Urine cytology is the current non-invasive gold standard but lacks sensitivity. Numerous studies have focused on urine proteomics as a source for a potential biomarker. Vlahou et al. (28) applied SELDI-TOF-MS to urine samples to detect transitional cell bladder carcinoma (TCC). They evaluated 30 patients with TCC, 34 healthy controls, and 30 patients with other benign disease. Five potential biomarkers and seven protein clusters were identified with mass ranges between 3.3 and 133 kDa. Sensitivity for the detection of TCC based on these markers individually ranged from 43 to 70% with specificity of 70–86%. When the protein biomarkers and clusters were combined, sensitivity increased to 87% with a loss of specificity to 66% (28). 2DE urine protein analysis was used by Kageyama et al. (29). They identified increased calreticulin in bladder cancer urothelium through proteomic analysis of cancer tissue. Western blot analysis was used to confirm the finding using 22 malignant and 10 benign surgical specimens (p = 0.0003). Given the finding of calreticulin as a potential biomarker, further evaluation of urine samples was performed. Analysis of urine specimens from 70 patients with bladder cancer and 181 patients with benign and other malignant conditions supported the use of urinary calreticulin as a biomarker for bladder cancer, yielding a sensitivity of 73% and a specificity of 86% (29).

**Cerebrospinal Fluid**—Cerebrospinal fluid (CSF) bathes the central nervous system and protects the brain from trauma. Although containing small concentrations of protein in normal patients, CSF protein increases with most brain diseases, leaving the CSF as a possible rich source of proteomic information (31). Jiang et al. (32) studied the CSF of schizophrenic patients through 2DE coupled with MALDI-MS analysis. Protein analysis revealed decreased expression of apolipoprotein A-IV in schizophrenic patients (32). Castaño et al. (33) compared the cerebrospinal fluid from 43 patients with pathologically confirmed Alzheimer disease with that of 43 non-demented control subjects. Proteins differentially expressed by 2DE were excised and subjected to MS analysis and sequencing. Apolipoprotein A-I, cathepsin D, and transthyretin were significantly reduced in the CSF of Alzheimer disease patients, whereas hemopexin and pigment epithelium-derived factor were increased (33). Further validation of these findings is needed. The evaluation of CSF may also prove to be useful in neuro-oncology. Zheng et al. (34) identified the differential expression of N-MYC oncprotein and low molecular weight caldesmon in the CSF of normal subjects as compared with patients with primary brain tumors. The utility of using these markers for prognosis and to monitor response to chemotherapy is currently under investigation.

**Nipple Aspirate/Ductal Lavage**—Most studies have examined serum for proteomic analysis because of its ease and...
availability, but information may be gleaned from tissues and fluids near the source of a tumor. Fluids such as nipple aspirate or breast ductal lavage may bathe local ductal pre-malignant or carcinoma cells and are being exploited for their potential protein expression. In a study conducted by Sauter et al. (35), 114 nipple aspirate samples were examined from 27 cancer patients and 87 control women. They identified three MS peaks that distinguished between the two groups using multivariate logistic regression models. Based on their data, the use of clinical predictive factors and the detection of an 11.8-kDa protein appeared to be the most sensitive and specific model for identifying breast cancer patients (35). Pawlik et al. (36) made use of SELDI-TOF to analyze nipple aspirate fluid (NAF) in 23 women with stage I or II unilateral breast cancer and compared them to five healthy control subjects. Two distinct peaks were noted to be overexpressed in NAF of breast cancer patients, and one was underexpressed ($p < 0.0027$) in comparison with the healthy patients (36). In a study by Alexander et al. (37), NAF was examined by 2DE in 10 breast cancer patients and 10 nonmalignant patients. Through MALDI-TOF-MS, three peaks were up-regulated in breast cancer patients and were identified as gross cystic disease fluid protein, apolipoprotein D, and $\alpha_1$-acid glycoprotein. Further work is needed to ensure that these potential biomarkers are indeed up-regulated given the variability of expression in some samples (37). However, other studies have been performed that show significant heterogeneity within the nipple aspirate samples, confounding the interpretation of some of these results. Pawleitz et al. (38) examined nipple aspirate samples from 12 cancer patients and 15 control women and noted considerable heterogeneity among the women in the group. Despite this, there were two peaks at 4233 and 9470 m/z that appeared to be specific to the cancer group and two peaks at 3415 and 4149 m/z specific to the control group (38).

**Tumor Specimen Analysis**—Diagnostic techniques for cancers have evolved during the past century from simple histology through many advances, including immunohistochemistry and genomic testing such as microarrays, fluorescence in situ hybridization, and chromosome karyotype analysis. Diagnosis is most commonly made by a pathologist using a complex, but subjective, analysis of cell morphology, tissue architecture, and staining patterns. These techniques are not precise as pathologists can and do disagree on a diagnosis. Some poorly differentiated tumors cannot be further distinguished using standard techniques as seen in patients with carcinoma of unknown primary. The difference between a benign and malignant condition is often based on cell behavior in the specimen (i.e. invasion), such as the difference in ductal carcinoma in situ versus infiltrating ductal carcinoma. Accordingly the diagnosis is subject to sampling error in the specimen and thus may lead to over- or undertreatment of the patient.

In addition, there is a spectrum of cellular aberration and dysplasia that has different malignant potentials in many cancers such as colon cancer and breast cancer. Morphologic or genetic changes (i.e. BRCA mutation) may herald a higher likelihood of developing cancer, but these patients may not have the same outcome as described for a more general population. Additional tools are needed to overcome these deficits and to complement current diagnostic and prognostic approaches. Tissue proteomics has the potential to be a powerful new tool in the diagnostic armamentarium as proteins are the effectors of the molecular aberrations that cause cancer.

Varambally et al. (39) elegantly tested this hypothesis using benign and malignant prostate tissue extracts. Their group found 64 proteins that were altered in prostate cancer compared with benign prostate tissue and 156 more proteins that were different in metastatic disease relative to localized carcinoma using SDS-PAGE technology and high throughput immunoblotting. They found a 48–64% concordance between the genome and proteome of the tissues when compared with genomic analysis of the same tissues using a prostate cancer transcriptional database, demonstrating the limitations of genomic-only analyses of malignancies. Proteomic changes between localized and metastatic cancer that were concordant with differences in gene transcription did predict clinical outcome, indicating a potential integrative role of genomics and proteomics in prognosis (39).

Two groups have also shown that MS analysis of lung tissue samples can yield information to differentiate between normal lung and non-small cell lung cancer. Yanagisawa et al. (40) examined 42 lung tumors and eight normal samples using MALDI-TOF and constructed a normal and malignant lung tissue proteome model based on over 1600 protein peaks. They then tested a validation cohort of 37 lung tumors and six normal samples and found near perfect classification of tumor tissue. They compared localized and metastatic disease and found 64 proteins that were altered in prostate cancer compared with benign prostate tissue and 156 more proteins that were different in metastatic disease relative to localized carcinoma using SDS-PAGE technology and high throughput immunoblotting. They found a 48–64% concordance between the genome and proteome of the tissues when compared with genomic analysis of the same tissues using a prostate cancer transcriptional database, demonstrating the limitations of genomic-only analyses of malignancies. Proteomic changes between localized and metastatic cancer that were concordant with differences in gene transcription did predict clinical outcome, indicating a potential integrative role of genomics and proteomics in prognosis (39).

Chen et al. (41) analyzed 682 distinct proteins in 90 lung adenocarcinoma specimens using two-dimensional PAGE followed by MS. A 20-protein profile was shown to predict survival in stage I lung cancer patients ($p = 0.01$). One protein in the glycolysis pathway, phosphoglycerate kinase 1, was associated with survival when found in the serum (using ELISA) as well, suggesting that applying proteomic techniques to analysis of tissue specimens may also help identify potential serum biomarkers (41). Recent work in cell line models of lung, colon, and breast cancer has indicated that phosphoglycerate kinase 1 expression may facilitate hypoxia-induced apoptosis and thus may be important in cancer cell survival pathways (42–44). A similar analysis of colon cancer tissue compared with normal colorectal tissue found 35 proteins to have different expression in malignant versus benign tissue. The protein secretagogin was identified by MS and was expressed only in colon cancer cells with a neuroendo-
crine morphology and thus could be used to help subclassify malignant colonic tumors (45).

Mass spectrometry of the proteome has also been used to find proteins that may have diagnostic benefit. In prostate cancer, various prostatic tissues (normal tissue, high grade intraepithelial neoplasia, and carcinoma) were analyzed. Growth differentiation factor 15 was absent in benign tissue, of low intensity in high grade intraepithelial neoplasia, and of high intensity in carcinoma (46). Accordingly growth differentiation factor 15 could possibly be used as an adjunct to standard pathologic diagnosis of prostate cancer. Proteomics thus has the potential to aid diagnosis through the discovery of protein expression unique to a disease as well as through the creation of complete protein profiles for a given disease or for a particular prognostic category.

FUNCTIONAL PROTEOMICS

The field of genomics concentrates on delineating the nucleotide code for each protein, but 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 overexpressed proteins researchers will be able to target cancer therapy based on the mechanism of the disease-sustaining 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.

Techniques

Tumors and tumor lysates are analyzed rather than unproven surrogate tissues or blood to study proteins in their microenvironment. Immunocapture through immunoblotting, precipitation, and histochemistry and protein and tissue microarrays are tools applied to clinical samples. Immunoprecipitation can identify interactions between proteins and can be applied if the clinical sample is of adequate size and stability. For example, unknown partner proteins in a multiprotein complex can be identified using SDS-PAGE followed by 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.

Protein and tissue microarrays are powerful tools with which to analyze quantity and post-translational modifications of proteins. Two protein microarray designs have been developed: forward phase arrays and reverse phase arrays (RPAs). Forward phase arrays use protein chips where antibodies are arrayed on a slide, and then the array is probed with cell lysates. In contrast, in RPAs the cell lysates are arrayed and then probed with known anti-protein antibodies (Fig. 2). These techniques allow researchers to scan the proteome simultaneously for many proteins, their possible interactions with

![Tissue lysate microarray schematics.](image-url)
are thought to be druggable have been targeted for therapy (47). The study of the proteome can both identify new targets as well as help clarify known drugs. Many small molecule agents are heralded as specific for a certain protein target based on in vitro assays. It has become clear that these agents can be quite promiscuous and affect many proteins in vivo. Imatinib is a classic example of a drug that was thought to be targeted to the pathologic chronic myelogenous leukemia (CML) fusion protein BCR-ABL but on further testing was found to also target other members of the type III receptor tyrosine kinase family, the tyrosine kinases c-KIT and platelet-derived growth factor receptor. Its ability to target and inhibit c-KIT and preferentially mutant c-KIT has led to its effectiveness in gastrointestinal stromal tumors. SDS-PAGE and MS can be used to isolate different proteins that interact with drugs by using known drugs as bait in protein arrays and accordingly identifying new potential therapeutic targets. For example, proteomic analysis of a MAPK inhibitor, SB 203580, and the protein kinase C inhibitor GF109203X in in vitro cell line studies revealed multiple other potential targets of the agents including RICK (Rip-like interacting caspase-like apoptosis-regulatory protein) and cyclin-dependent kinase 2 (48, 49). Cells can be treated with the drugs, and comparison of tumor growth curves with the post-treatment proteome can be used for proof-of-concept validation of the protein targets.

This technique can also be applied to evaluate the mechanism of resistance of tumor cells to drugs. Here the story of imatinib becomes even more interesting. The majority of CML patients who were resistant to imatinib were found to be sensitive to treatment with pyrido[2,3-d]pyrimidine tyrosine kinase inhibitors. After immobilizing pyrido[2,3-d]pyrimidine as bait with various cell culture lysates, MS was performed and identified over 30 separate kinases that interacted with the molecule. Two of the strongest targets were the serine/threonine kinases RICK and p38α; this was the first indication that the pyrido[2,3-d]pyrimidine compounds could inhibit both tyrosine and serine/threonine kinases (50). The dual selectivity was found to be due to a preserved amino acid in the ATP-binding pocket, which was found to be mutated in imatinib-resistant cells. Imatinib-induced apoptosis is dependent on p38α activation; therefore, inhibition of p38α would be expected to have some prosurvival effect on CML cells; this could be a mechanism of resistance (51). Reducing p38α inhibition by manipulating the structure of the pyrido[2,3-d]pyrimidine drugs or combining treatment with p38α activators could improve their therapeutic benefit in CML (52).

New drug targets can also be identified using proteomic techniques and existing drug libraries. The mammalian target of rapamycin (mTOR) pathway, which is important in nutrient signaling and survival, is dysregulated in many malignancies. Rapamycin is a powerful inhibitor of the mTOR pathway. An existing drug library was screened for drugs that changed the growth curves of rapamycin-treated yeast and identified two classes of drugs, those that inhibit (small molecule inhibitors
of rapamycin or SMIRs) and those that enhance the efficacy of rapamycin (small molecule enhancers of rapamycin or SMERs). When the SMIRs were tested with yeast protein microarrays, their targets included a mammalian homologue of PTEN, a known indirect inhibitor of mTOR (53).

Therapeutic targets can also be found by focused analysis of the proteome for up-regulated proteins compared with normal tissue with emphasis on finding up-regulated proteins that have known inhibitors. For example, Hu et al. (54) found that heat shock protein 70 was up-regulated in ovarian cancer cell lines using SELDI-TOF-MS, and treatment with a heat shock protein 70 inhibitor stunted growth of the cell line (54). Similar studies in vitro and in mouse models have been performed in other malignancies, including acute lymphoblastic leukemia, acute myeloid leukemia, colon cancer, hepatocellular carcinoma, and breast cancer (55–58).

**Monitoring Response to Targeted Therapy**—Proteomics has the potential to revolutionize the monitoring and focusing of a patient’s response to anticancer therapy. The traditional method of following disease has centered on serial imaging studies. These studies are subject to error due to differences in imaging techniques and in reading between radiologists as well as differences in patient positioning resulting in different imaging cuts, especially true with small changes in tumor sizes. Tumor markers measured by standard techniques, such as CA-125, carcinoembryonic antigen, and CA-19-9, have been used as adjuncts to following patient disease but are fraught with often unacceptable sensitivity and specificity. The rapidly expanding presence of molecularly targeted therapies in treating cancer may also need to change our clinical set point for effective therapy as many of these agents are cytostatic rather cytotoxic, and thus we may not see the same degree of regression of disease, if any at all. Cancer patients with prolonged stable tumor while on treatment with molecularly targeted agents are likely having their disease held in check by the agent. This concept can be addressed with proteomic approaches. Proteomic analysis of the serum and/or of tumors post-treatment can validate target and pathway inhibition in the patient and thus improve therapeutic management.

RPA technology is presently being applied to multiple phase II clinical trials in ovarian and breast cancers in the intramural program of the NCI, National Institutes of Health. Biochemical events in tumor samples obtained immediately prior to treatment with a molecular targeted agent were compared with tumor samples taken after the initiation of therapy for target validation in our trials of imatinib and gefitinib for advanced ovarian cancer patients. Arrayed tumor biopsy samples were analyzed for the levels of EGFR, phospho-EGFR, and downstream signaling molecules AKT/phospho-AKT and ERK/phospho-ERK. Proteomic results confirmed the effectiveness of gefitinib at inhibiting its intended target and downstream signaling as would be expected, but no clinical benefit was observed in this patient population. Another trial in the same patient population tested the efficacy of imatinib in relapsed ovarian cancer. Again RPAs of pre- and post-treatment tumor biopsies revealed an association between target inhibition and toxicity rather than disease response; phospho-c-KIT and EGFR prior to treatment predicted nausea/vomiting, whereas post-treatment levels of EGFR and platelet-derived growth factor receptor were linked to fatigue (59). Proteomic analysis is pending on a phase I study of bevacizumab and sorafenib in combination and will be correlated with observed clinical, radiographic, and vascular imaging responses for proof of concept.

Proteomics can also be used to supplement radiographic studies in the new field of molecular imaging. Tumor density and heterogeneity on a scan can be linked to particular proteomic profiles to determine their biological composition with non-invasive radiographic testing. Hobbs et al. (60) describe four patients with glioblastoma multiforme who had different areas of their tumors biopsied based on their radiologic appearance on MRI. They found that each type of tumor heterogeneity on the MRI corresponded with a particular proteomic profile (60). Proteomic profile libraries coupled with characteristic image profiles could decrease the need for invasive biopsies in some tumor types and serve as an alternate method to follow patient responses to therapy.

**Tailoring Individualized Therapy**—The dictum “no two cancer patients are alike” is borne out across all types of malignancies and demographic groups and conceptually all other nonmalignant disease states. Patients diagnosed with the same malignancies, sites of disease, and precipitating factors can and do respond differently to treatment with a particular agent. Proteomics offers characterization of a particular tumor to enable personalized molecular treatment based on the protein targets that are awry in each patient (61). Differences in the genome of the malignant clone may be partially responsible for the differences in susceptibility to anticancer treatment. There are randomized trials now underway testing genomic signatures that have been developed and validated, such as the 21-gene microarray that is being applied prospectively to stratify patient treatment in breast cancer trials (62, 63). However, often the genetic code of a cell does not reflect the aberrant protein production downstream of DNA transcription; instead the cancer-causing cellular dysfunction occurs at the RNA translational or post-translation modification levels, leading to abnormal protein function in critical cellular functions including differentiation, proliferation, apoptosis, invasion, metastases, and survival. Protein kinases and phosphatases are key proteins in regulating the cell cycle as well as intracellular molecular signaling networks; their dysfunction plays a cardinal role in the development and maintenance of cancer and its response to therapy.

Irish et al. (64) demonstrated this principle with their analysis of acute myelogenous leukemia cell samples before and after treatment with chemotherapy. The patient samples were analyzed using intracellular phosphospecific flow. They found that important signal transduction pathways including STAT
and MAPK were variably phosphorylated among the specimens and could be classified into four different phosphoprotein profiles that each had a characteristic prognosis and response to chemotherapy. Each cluster also had similar cytogenetic abnormalities, including Flt3 mutations, showing how the proteome can be used with genomics to attain a more robust picture of tumor biology (64).

Extensive protein profiles of an individual tumor can direct choices in combinatorial molecular therapy. If a signaling pathway is up-regulated or overactive in a particular patient, targeting the pathway in combination may allow for more effective control of its activity, and at potentially lower doses of the agents. We are presently exploring this hypothesis with our phase I study of bevacizumab, a monoclonal antibody to vascular endothelial growth factor (VEGF), combined with sorafenib, a RAF kinase inhibitor. RAF kinase is a downstream effector of VEGF; we are targeting the VEGF pathway in series rather than in parallel to potentiate the anti-VEGF effects of both agents. Initial clinical results of the two agents in combination show good activity in tumors known to have increased VEGF pathway signaling such as ovarian cancer but with synergistic toxicity at doses below the standard single agent treatment doses (65). Proteomic analysis of the tumors prior to treatment will investigate whether the VEGF pathway, including RAF kinase, is up-regulated in patients who had a good response and could provide further justification for tailoring therapy to the proteomic profile of a tumor.

CONCLUSIONS

The clinical utility of proteomics is an area of still unrealized potential. It offers the promise of early diagnosis with new biomarkers using serum or fluid proteome analysis and of novel therapies discovered by analyzing the specific protein
abnormalities in a particular disease. Clinical proteomics may define disease prognoses that will aid in determining the need for more or less aggressive therapy, possibly curtailing treatment that may be unnecessarily toxic or, on the other hand, helping patients decide whether there is a need for high risk treatment. In addition, clinicians may soon use a patient’s specific proteomic profile to rationally select molecular therapy and then use proteomic analysis to monitor disease response as an adjunct to traditional radiologic and serologic studies (Fig. 4). However, there are many challenges to traverse as we strive toward incorporating proteomic study into our diagnostic and therapeutic paradigms. Proteomic techniques need to be further refined to fully tackle the sheer vastness of the human proteome as well as standardized across laboratories so that results are reliable and reproducible. When these hurdles are overcome, proteomics offers the possibility of a revolutionary way to practice medicine with therapy individualized for maximum benefit and the least toxicity for patients.

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REFERENCES


42. Luo, F., Liu, X., Yan, N., Li, S., Cao, G., Cheng, Q., Xia, Q., and Wang, H.
46. Cheung, P. K., Woolcock, B., Adomat, H., Sutcliffe, M., Bainbridge, T. C.,
38. Paweletz, C. P., Trock, B., Pennanen, M., Tsangaris, T., Magnant, C., Liotta,
36. Pawlik, T. M., Fritsche, H., Coombes, K. R., Xiao, L., Krishnamurthy, S.,
30. Celis, J. E., Gromov, P., Cabezon, T., Moreira, J. M., Ambartsumian, N.,
24. Godl, K., Wissing, J., Kurtenbach, A., Habenberger, P., Blencowe, S., Gut-
15. Itoh, K. (2004) Two proliferation-related proteins, TYMS and PGK1, could
2. Itoh, K. (2004) Two proliferation-related proteins, TYMS and PGK1, could
1. Itoh, K. (2004) Two proliferation-related proteins, TYMS and PGK1, could

Molecular & Cellular Proteomics 5.10

Molecular & Cellular Proteomics 5.10

Molecular & Cellular Proteomics 5.10

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