Biomarker discovery: proteome fractionation and separation in biological samples

Peter Matt,1,2 Zongming Fu,1 Qin Fu,1 and Jennifer E. Van Eyk1

1Johns Hopkins Proteomics Center and 2Howard Hughes Medical Institute, Johns Hopkins Medicine, Baltimore, Maryland

Submitted 9 December 2007; accepted in final form 18 December 2007

While the genome provides information about the theoretical status of the cellular proteins, the proteome describes the actual content, which ultimately determines the phenotype. Not every gene is transcribed and translated into a single protein, due to complex and regulated processes such as mRNA splicing (producing protein isoforms), RNA editing, co- and posttranslational modifications (PTMs, e.g., phosphorylation, glycosylation, and acetylation), and protein processing, which extends the number of protein species that are possible within a cell (17). Single-nucleotide polymorphism and polymorphism inherent within the biological variation of the gene lead to additional variations between individuals. Interactions between modified proteins and the dynamics of protein expression under specific biological circumstances lead to an enormous molecular complexity, which exceeds that indicated by the genome sequence alone. Gene expression can be studied either at the mRNA level or at the protein level, but full protein characterization is required to truly understand the proteome. Powerful techniques make the rapid screening of mRNA expression possible; however, there is often a poor correlation between mRNA abundance and the quantity of corresponding functional proteins (16). Proteomics is an emerging scientific field that involves identification, characterization, and quantification of proteins in a cell, tissue, or body fluid. The broad application of proteomics in basic science and clinical medicine, with its range of tools, will accelerate our understanding of disease processes and may facilitate the discovery of new drug targets and diagnostic markers (7). Clinical proteomics, defined as the application of proteomics in the field of medicine, has the potential to influence daily clinical practice in providing tools for diagnosis or prognosis, defining disease states, assessing risk profiles and outcomes, and setting up individual therapeutic strategies. With this objective, most clinical applications of proteomics focus on blood (serum or plasma) biomarkers although other body fluids can be used. Unlike cells or tissues, blood does not have a genome, making proteomic techniques one of the few options for discovering biomarkers. Effective biomarker discovery, even in simple biological samples, requires a combination of subfractionation and separation, or targeted protein or peptide enrichments, before identification and characterization of the markers by mass spectrometry (MS) (4). The appropriate technology or combination of technologies to match the biological questions to be answered must be identified to allow for maximum coverage of the selected subproteome and to maximize the...
interpretation and downstream utility of the data. In this review, we describe the current technologies for proteome fractionation and separation from biological samples, based on our laboratory work in the discovery and validation of serum biomarkers.

BIOMARKER DISCOVERY

In general, two different strategies are being used to discover biomarkers using proteomic technologies (Fig. 1). The first strategy is a “targeted” approach based on the more traditional hypothesis-driven evaluation of specific biomarker candidates, selected either on the basis of a biological rationale or from analysis of candidates derived from other sources. The second strategy is a “de novo” discovery approach that uses different proteomic techniques and finally validates potential biomarker candidates. Both strategies are complementary, have advantages and disadvantages, and may be performed in parallel (4). Regardless of the strategy used, we believe that the discovery and development of a robust biomarker candidate, using proteomics, demands a systematic in-depth approach in which discovery and validation are coupled. Figure 2 illustrates such an approach, as adopted in our laboratory for serum/plasma and tissue samples.

SAMPLE PREPARATION AND FRACTIONATION

Before any proteomics analysis, blood samples must be collected and processed according to a standardized tightly regulated protocol, which is of great importance in obtaining reproducible proteomic data (3). Whether plasma or serum should be collected for proteomic analyses is controversial. One study revealed that a large number of highly abundant peptides are detectable only in serum samples, and not in plasma (13). The authors took the view that those peptides must be produced by ex vivo degeneration during the clotting procedure and, therefore, suggested that plasma may be superior to serum, in particular for low-molecular-weight proteins. However, there are considerable technical issues, and many in the field use serum samples, collected and processed with proper control, for biomarker discovery. After all, the majority of clinical immunoassays are performed on serum.

Because of the limitations in the dynamic range of proteomic technology, it is widely accepted that to detect proteins that are low in abundance, blood samples must be depleted of highly abundant proteins. The most common depletion method is affinity chromatography. Several companies market affinity columns that remove up to 12 of the most highly abundant proteins, e.g., the column from Beckman Coulter removes albumin, immunoglobulin G (IgG), IgA, IgM, transferrin, fibrinogen, apolipoprotein A-I and A-II, haptoglobin, alpha-1 antitrypsin, alpha-1 acid glycoprotein, and alpha-2 macroglobulin. Although these columns are thought to have little nonspecific binding, caution is required to prevent carryover (protein stuck to the column and then leaking) between sequential runs on the same column and the loss of proteins present in low abundance during the chromatography or downstream concentration step. Furthermore, these multiple antibody affinity columns are expensive for routine usage in academic laboratories. As an alternative to affinity-based depletion methods, our laboratory has developed a simple, reproducible, and inexpensive chemical method for the depletion of lipids, immunoglobulins, and albumin (2). Following a high-speed centrifugation step to deplete lipids and depletion of IgG with a protein A resin, the serum or plasma is fractionated into albumin-enriched and depleted fractions by ethanol precipitation. Both fractions can be used for biomarker discovery using proteomic techniques. Although the observable proteome is significantly enhanced by such depletion procedures, the remaining proteins and peptides are still present in a wide range of concentrations. Therefore, depletion of serum or plasma samples serves only as a starting point for further proteomic analysis.

SAMPLE SEPARATION

Because of its complexity, the serum or plasma proteome cannot be resolved completely using a single proteomic technology. Multiple proteomics techniques for protein separation must be combined to analyze and cover a large spectrum of the proteome (Fig. 3). Which methods are needed depends on the underlying biological and clinical questions to be answered. Core technologies for protein separation are one- and two-dimensional gel electrophoresis (1-DE, 2-DE) and, for protein or peptide separation, one- and two-dimensional liquid chromatography (1DLC, 2DLC), all coupled with mass spectrometry (MS).

2-DE

The first technology to be used in proteomics was 2-DE, which was developed independently in the laboratories of O’Farrell and Klose more than three decades ago (6, 10). In standard 2-DE, proteins are separated in the first dimension, known as isoelectric focusing, by their molecular charge (pI). The second dimension separates the proteins according to their molecular mass (or molecular weight, MW). The MW separation is done in a polyacrylamide matrix in a sodium dodecyl-sulfate (SDS) milieu; the most common procedure utilizes an acrylamide gradient of 10–20%. Proteins can be visualized in 2-D gels using different detection methods. The more common protein staining methods include Coomassie blue and silver
staining, use of fluorescence dye (e.g., Cy dyes, LAVAPurple, Sypro dyes), radiolabeling, and immunodetection. Using standard-format SDS-gels for 2-DE, it is possible to routinely separate up to 2,000 protein spots from serum/plasma or tissue extracts, which reflects ~100–300 different proteins, depending on the pH gradient used in the first dimension.

Although 2-DE is an important and popular protein separation technique, it is limited by the solubility and mass of the separated proteins. Differential in-gel electrophoresis (2D-DIGE) is a recent improvement of the 2-DE technology. It improves gel reproducibility, minimizes alignment issues, and allows better quantitative comparison between samples. In 2D-DIGE, proteins from different disease states are separately labeled with different fluorescent dyes, and an internal pooled standard is labeled with another dye. The labeled samples are then combined and subjected to 2-DE, and the gel is scanned at different emission wavelengths generating multiple images that can be overlaid. Figure 4 shows an example of a 2D-DIGE, which allows the differentially regulated proteins to be viewed as changed in color.

2-D gel images are evaluated and analyzed using specialized software packages. The software stores all of the relevant information on each and all of the spots of a 2-D gel in a database, compares gel patterns using complex algorithms, and highlights differences between gel images. 2-D image analysis can be time-consuming and difficult, particularly if there are marked differences between samples. Software packages can be purchased and used in-house for analysis, or companies will now provide image analysis on a contract basis. However, by using strict inclusion and exclusion criteria one can sieve out the high probability markers (or protein spot changes).

**MULTIDIMENSIONAL LIQUID CHROMATOGRAPHY**

Recently, other methods for separating proteins have been exploited in proteomic analysis. Many involve liquid chromatography technologies that use solid- and liquid-phase media to separate proteins and/or their peptide fragments. The basic principle is a soluble sample that is separated in a liquid phase through a column, which is usually a tube packed with small particles of specific surface chemistry (9, 15). The sample is resolved as it traverses the length of the column based on protein- or peptide-specific chemical or physical interactions with the solid-phase. The time when the separated sample is detected at the end of the column (e.g., by UV absorbance at 210 nm, which essentially measures the number and quantity...
of peptide bonds) is the retention time and is quantitative if the peak contains a single protein/peptide (which in proteomics is rare, and therefore, peak volume or intensity in this case is semiquantitative).

1DLC can be used to separate proteins according to their molecular mass, isoelectric point, or hydrophobicity, which are the three chemical characteristics that define any given protein. The most commonly used 1DLC is reversed phase chromatography, in which proteins are separated based on hydrophobicity. Reversed phase chromatography can also be used to concentrate and/or desalt samples. In 2DLC, proteins are separated in the first dimension by chromatographic focusing (pI) and in the second dimension by reversed phase chromatography (hydrophobicity). Thus, 2DLC increases the extent of protein fractionation, which facilitates analysis of a larger spectrum of the proteome, including specific isoforms, PTMs, and low-abundance proteins. As with 1DLC, this method has been used in proteomics primarily for peptide separation before MS analysis (due to its compatibility with ESI instruments); however, it is increasingly used to separate complex intact protein mixtures, which are then enzymatically digested for LC or MALDI (matrix-assisted laser desorption/ionization) MS/MS analysis. 2DLC requires a larger quantity of sample for a single run (>2.5 ml) compared with 1DLC (50–100 μl), which can be a difficulty if available sample volumes are small (e.g., from mouse models). It is important both to quantify and to identify proteins present in fractions generated by 1DLC or 2DLC. One strategy is to normalize, overlay, and compare elution profiles between different samples using specialized software packages (for which there is currently a need especially when analyzing a large number of samples) and analyze, using MS, only the fraction that varies between samples.

Current data suggest that using multiple proteomic technologies dramatically increases the number of proteins detected, especially of those present in the sample at very low abundance (4). 2-DE, 1DLC, and 2DLC are synergistic separation techniques that, coupled with MS identification, expand the observable proteome and will provide a large dynamic protein spectrum for biomarker discovery. In fact, we recently compared 2-DE and 2DLC by creating a large database for serum and isolated inner mitochondrial subproteome and revealed that only ~12% of identified proteins were common to both platforms (8, 14).

PROTEIN IDENTIFICATION

MS. MS techniques have greatly advanced proteomics and proteomics-based biomarker discovery in recent years. 2-DE coupled with MS is widely used for tissue and serum analyses. Spots from 2-D gels are excised, the proteins are subjected to in-gel digestion, and the resulting peptide fragments are identified by MS. For identification of 2-D gel spots MALDI-time
of flight (TOF) MS is commonly used (4). If information on protein isoforms, PTMs or “absolute” identification of proteins in complex mixtures such as fractions generated by 1DLC and 2DLC are needed, then tandem MS (commonly referred as MS/MS) is required. MS/MS spectra are usually generated by an ion trap or quadrupole TOF mass spectrometer, which allows to generate de novo sequencing and exact localization of PTMs. For protein quantitation in MS analysis, several isotopic labeling techniques (e.g., iTRAQ, 18O/16O, SILCA) and, recently, label-free methods have been developed, though these are not further described in this review.

Biomarker validation. Biomarker candidates have traditionally been evaluated with quantitative immunoassays (e.g., ELISA) that are unique for one analyte (3). With the rapid development of new potential biomarkers, it is important to develop quantitative assay platforms that can simultaneously measure many proteins in many samples at a small sample volume. A variety of multiplex immunoassays have been developed in recent years that offer some advantages over traditional quantitative assays (11). Multiplex immunoassays are essentially the same as an ELISA except that multiple analytes are quantified simultaneously. Thus, many biomarkers can be evaluated at one time under the same standardized conditions, quantitative information can be obtained in a highly parallel analysis, and reagent costs are substantially reduced. The most common multiplex assay used is an array of antibodies printed on slides/or plates at high density. It is now possible to print hundreds of antibodies, although issues with analyte and antibody cross reactivity and matrix affects make smaller numbers (<20) the preferred choice of many. The current issues with multiplex arrays are their inter- and intra-assay reproducibility, matrix affects, background limits, and the specificity and sensitivity of the antibody assay. There are many other quantitative and semiquantitative multiplex immunoassays, such as miniature sandwich immunoassays, bead-based multiplex immunoassays and assays for specific signaling pathways, but investigators must take care to ensure the specificity and reproducibility of each array within the multiplex (5, 12, 13). The ultimate success of a multiplex assay depends upon its ability to quantitatively detect proteins at concentrations likely to be present in serum samples, which range from <1 pg/ml to >1 mg/ml. Multiplex assays can be used as powerful validation tool for candidate biomarkers identified by a de novo proteomic discovery approach. In addition, multiplex assays are often used for evaluating a variety of candidate biomarkers in a targeted approach. In either case, the multiplex assay requires the added flexibility of allowing the investigator to mount their own analytes. To test whether or not a newly discovered biomarker is of clinical utility, we recommend evaluating all candidates in relation to existing biomarkers if such exist. Multiplex immunoassays again are a desirable platform for this approach as it provides quantitative information in a higher-throughput format (3).

PERSPECTIVES

Proteomic technologies applied in basic science will complement genomic-based and physiological approaches. Proteomics will not only reveal new insights into complex molecular processes underlying diseases but will provide tools to develop novel diagnostic and prognostic biomarker(s) that include unique information about the patient. Such biomarkers could have tremendous benefits for patient management and may accelerate the development of new therapeutic strategies. In this context, it may be important to integrate proteomic biomarker information with that available from genetic biomarkers, which could provide a powerful integrated risk stratification (1). Proteomics is a rapidly changing field because of extensive advances in the underlying technologies including the fractionation, separation, and identification of proteins in biological samples. Although proteomics is evolving quickly and providing extensive protein databases with potential biomarkers, the translation of promising disease markers from bench to bedside is another challenge. This requires both close collaboration between basic scientists and clinicians and well-designed studies with appropriate statistical power, blinding, and validation. With the application of such an endeavor proteomics could lead to an optimized and more “personalized” medicine.

ACKNOWLEDGMENTS

Peter Matt thanks the Hippocrates Foundation Basel and the Howard Hughes Medical Institute Johns Hopkins Medicine, Baltimore, MD, for financial support.

GRANTS

Jennifer Van Eyk is supported by grants from the National Heart, Lung, and Blood Institute Proteomic Initiative (contract NO-HV-28120) and the Daniel P. Amos Family Foundation.

REFERENCES


