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Quantitative Proteomics and Biomarker Discovery in Human Cancer

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Abstract

“Early diagnosis and prevention is a key factor in reducing the mortality and morbidity of cancer. However, currently available screening tools lack enough sensitivity for early diagnosis. It is important to develop noninvasive techniques and methods that can screen and identify asymptomatic patients who have cancer. Biomarkers of cancer status can also serve as powerful tools in monitoring the course of cancer, and in determining the efficacy and safety of novel therapies. Thus, discovery of novel specific biomarkers are needed that may provide informative clues for early diagnosis and treatment of cancer. Recently, remarkable progress has been made in the development of new proteomics technology. The progress that has been made in this field is helpful in identifying biomarkers that can be used for early diagnosis of cancer and improving the understanding of the molecular etiological mechanism of cancer. This article describes the current state of the art in this field.”

Keywords

Biomarker; quantitative proteomics; cancer

Early diagnosis and prevention is crucial for reducing mortality and morbidity of cancer. Current clinical examination such as mammography and invasive needle or surgical evaluation for breast cancer; chest X-ray for lung cancer, etc., is often not sufficiently sensitive for early diagnosis. In order to detect cancer in its early stages, it is necessary to identify biomarkers for those asymptomatic patients who may have cancer. Biomarkers could be proteins, metabolites or electrolytes whose differential expression indicates the presence of disease [1]. Biomarkers of protein origin are powerful both in monitoring the development of cancer and in determining the efficacy and safety of drugs. Novel biomarkers are urgently needed for early diagnosis and treatment of cancer. Nowadays, the remarkable progress in proteomics technology has offered unprecedented opportunity for biomarker discovery.

Traditionally, two-dimensional gel electrophoresis (2-DE) is the standard method for comparing protein expression between normality and disease-perturbed state. However, it is later known that 2-DE lacks resolution and sensitivity. Only abundant proteins can be resolved on a 2-D gel. Biomarkers, which are usually low in abundance, are rarely found with 2-DE method. Gel-free, or mass spectrometry (MS)-based, proteomics techniques are emerging as

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the choice for quantitatively measuring protein levels due to better sensitivity and reproducibility over 2-DE-based methods [2-3]. However, quantitative proteomic profiling of complex biological samples for the purposes of biomarker discovery remains a challenge in proteomics. Multiple innovative profiling techniques have been introduced with the aims of comprehensively identifying and quantifying the proteins. These can be roughly divided into two categories: isotope-labeled and label-free mass spectrometry.

ISOTOPE-LABELED MASS SPECTROMETRY

Isotope-labeling methods have been developed that introduce stable isotope tags to proteins via chemical reactions using isotope-coded affinity tags (ICAT [4-5] and iTRAQ [6]), enzymatic labeling (e.g., using ^{18}O water for trypsin digestion [7]), or via metabolic labeling [8] (SILAC). The pioneering ICAT technology selectively targets peptides containing a specific amino acid (cysteine) with a stable isotope-coded internal reference or standard [9]. The extracted proteins from treatment and control samples are labeled with either light or heavy ICAT reagents by reacting with cysteinyl thiols on the proteins. Peptides containing the labeled and unlabeled ICAT tags are recovered by avidin affinity chromatography and are then analyzed by LC-MS/MS. Differential protein expression is determined by the isotope peak ratio of the peptide. Enrichment of low-abundance proteins can be performed through cell lysate fractionation [10]. ICAT technology has been widely used for protein identification and quantification in mammalian, liver and breast tumor cells [11]. However, disadvantages of ICAT analyses are obvious: it is only applicable to proteins containing cysteine; it can only identify 300-400 proteins, far fewer than 2-DE method; the peptides contain large labels, which makes database searching more difficult, especially for short peptides [10].

Isobaric tag for relative and absolute quantitation (iTRAQ) is another labeling technique first developed by Ross et al. [12], which use special isobaric tags to label proteins extracted from samples for comparison. The proteins from control and treatment samples have the same mass after reacting with the iTRAQ reagents. These peptides give four ion species of different masses upon collision induced dissociation. These ions allow samples to be quantified in MS/MS mode [12]. The amine specificity of the labeling reagents makes most peptides amenable to this labeling strategy with no loss of information. This is especially important for proteins with post-translational modifications, such as phosphorylation and glycosylation. In addition, the multiplexing capacity of these reagents allows for comparison among multiple cellular states. However, as a chemical labeling method, iTRAQ may generate side products during labeling, and cause some loss of analytic sensitivity because chemical strategies involve a derivatization step that might not be complete.

Absolute Quantification (AQUA), uses synthesized isotopically labeled peptides that mimic native peptides as internal standards [13-14]. The method has the potential for high throughput and multiplexed sample analysis [15]. Stable isotope labeling of amino acids in cell culture (SILAC), which takes a similar strategy but utilizes different media containing light- or heavy forms of particular amino acids, has emerged as a popular label-based quantification techniques [16]. It was first developed by Mann et al. [8] based on metabolic incorporation of 'light' or 'heavy' form of amino acids into the proteins in living cultured cells. Usually, heavily labeled (^{13}C or ^{15}N) arginine or lysine or both are used in culture medium to ensure complete labeling of every trypsinized peptide fragment. In experiments, one cell population is fed with regular amino acids and the other fed with ^{13}C or ^{15}N labeled amino acids. After several rounds of cell division, heavy amino acids will be incorporated into newly synthesized proteins. In the mass spectrometry spectrum, the light and heavy peptides will show up as two distinct peaks separated by the incremental mass of the labeled amino acids. By comparing the signal intensity, relative quantification can be achieved. Because of its simplicity in principle, SILAC is widely used for biomarker discovery [17], cell signaling dynamics [18], identification of

posttranslational modification sites [19-20], protein-protein interaction [21-23], and subcellular proteomics [24].

The dynamics of protein turnover is another key feature to the understanding of regulation of protein expression in cells [25-26]. Recently, we developed a general method for determination of protein synthesis rate using labeling of amino acids with deuterium or ^{15}N at low enrichment [27-28]. This method, “modified SILAC (mSILAC)”, can measure protein synthesis rate quantitatively based on analysis of mass isotopomer distribution (MIDA) [28]. Once precursor enrichment is known, protein synthesis is determined from isotopomer distribution. In experiments with 30-50% enriched ^{15}N amino acids, incorporation of ^{15}N amino acids result in sufficient mass shift in the new protein. The ^{15}N enrichment can be estimated from the mass shift by curve fitting and the expected isotopomer distribution of the new peptide can be generated by the concatenation function. Synthesis rate is then calculated by multiple linear regression analysis of the observed peptide spectrum on the expected new and the old (unlabeled) spectra [28]. This method obviates the need for the use of a 100% newly synthesized protein as a reference as in Vogt’s methods [29-30]. The concatenation function provides an ideal 100% labeled spectrum and multiple regression analysis uses all the information from the mass spectrum. Our mathematical algorithm represents a major improvement in the calculation of protein synthesis rate, permitting the use of isotope labeling of protein through the pathways of amino acid metabolism with low cost isotopes [27-28].

For small peptides, another method, multiple reaction monitoring (MRM), attempts to monitor pre-specified ions (and their daughter product ions after MS/MS) as specific signatures for a particular protein instead of aiming to measure everything in a sample. In this method, initial selective scanning for a particular precursor ion in the first MS is followed by scans of a particular transition. Only one of the ions produced during collision induced dissociation is selected based on prior knowledge of precursors and transition state [31]. Due to limitation in mass spectrometer dynamic range and resolution of chromatography separation, MRM is frequently combined with immunodepletion, size exclusion chromatography [32], or enrichment of stable isotope standards with capture by anti-peptide antibody (SISCAPA, [33]). These hybrid methods are particularly attractive because they can measure precisely the quantity of low abundant proteins, which are otherwise bypassed in other conventional methods. MRM is comparable to ELISA with regard to sensitivity but obviate the necessity of antibody, which is costly to obtain and sometimes unavailable. MRM is usually more accurate than ELISA because it does not have propensity of cross reaction as antibody has.

LABEL-FREE MASS SPECTROMETRY

With the advances of new instrumentation, computing power, and advanced bioinformatics, generic label-free LC-MS shotgun screening methods such as Multidimensional Protein Identification Technology (MudPIT) have been alternatives for relative and absolute protein quantitation in biological samples. Without requiring modification of the analytes, the label-free approaches were based on the observation that MS intensity is linearly proportional to ion concentration even in complex samples like serum [34]. However, it was later observed that not all peptides are equally detectable because of competition between ions, dynamic range limitation, and sensitivity of MS instrumentation [35]. A new technique called spectral counting circumvented these pitfalls by not looking at differences in ion peak areas or intensities, as used in isotope-based methods [35]. Nevertheless, the main bottleneck for label-free LC-MS still lies in the computer softwares and sample preparation. Although many software tools are available, there is huge space for improvement, such as user interface and calculation efficiency of workflow. Moreover, the accuracy of label free quantitation depends largely on experimental set-up like protein extraction and sample stability, which is illustrated by the fact that the quantitative reproducibility of technical replicates is much better than that

of experimental replicates [36-37]. With improvements in the efficiency of data analysis workflow, label-free mass spectrometry will be potentially widely used for biomarker discovery and validation.

CONCLUSION

The recent development of mass spectrometry based strategies for absolute protein quantification offers great opportunity for biomarker discovery. While the prospects of this technology are exciting and promising, the current methodology is far from perfect. First, most current methods require complicated sample preparation, such as immunosubtraction, multidimensional LC separation, immunoaffinity and solid phase extraction, in order to enhance the analytical dynamic range and detection sensitivity. To establish high throughput pipeline, we should have ideally one-step preparation. Secondly, useful and validated biomarkers are still rare based on these developed methods because low abundance biomarkers are always immersed in large quantities of routine proteins especially in plasma samples. There is huge space for improvement of sensitivity.

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