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Targeted proteomics: a bridge between discovery and validation

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Abstract

New technologies in mass spectrometry are beginning to mature and show unique advantages for the identification and quantitation of proteins. In recent years, one of the significant goals of clinical proteomics has been to identify biomarkers that can be used for clinical diagnosis. As technology has progressed, the list of potential biomarkers has grown. However, the verification and validation of these potential biomarkers is increasingly challenging and require high-throughput quantitative assays, targeting specific candidates. Targeted proteomics bridges the gap between biomarker discovery and the development of clinically applicable biomarker assays.

Keywords

biomarker; LC–MS; MRM, PRM; proteomics; SRM; targeted proteomics

One of the goals of clinical proteomics has been the discovery of protein biomarkers, which could translate into relatively cost–effective assays for the early detection, diagnosis and even prognosis of disease. The field has produced thousands of publications outlining potential biomarkers based on discovery experiments [1,2], yet there are relatively few biomarker assays available for clinical use [3]. Only about 100 US FDA-approved protein assays exist, 80% of which were developed before 1993, few of them contain panels of proteins [3]. There is a growing consensus among leaders in the field that the development of quantitative high-throughput assays for the candidate proteins represents a bottleneck between biomarker discovery and fully validated assays for use in the clinic [4–10]. Targeted proteomics affords the potential for the development of assays that are sensitive, reproducible, quantitative and possess a high enough throughput that statistically relevant conclusions can be drawn about the potential clinical value of a biomarker [5,11–15].

Selected reaction monitoring

The pipeline for evaluation of potential biomarkers after initial discovery requires the verification and validation of biomarkers through analysis of hundreds and even thousands of biological samples [9]. This pipeline requires the development of assays that are sensitive, reproducible, quantitative and high throughput enough that a statistically significant

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evaluation can be made concerning the clinical value of each target [6–10]. To address these needs, assays have been developed using a technique known as selected reaction monitoring (SRM), which is a mass spectrometry technique that has recently been applied to the evaluation of quantitative differences between biological samples [5,11,12,16–18].

This SRM technique has been used widely for the analysis of small molecules [19]. It normally involves the coupling of in-line chromatography [20,21] with electrospray ionization for introduction into a triple quadrupole mass spectrometer [22–26]. In a triple quadrupole format, the mass spectrometer analyzes a (normally tryptic) peptide of interest by isolating a 'precursor' ion in the first quadrupole, after which the precursor ion is delivered to the second quadrupole where the ion is fragmented, resulting in 'product' ions that are delivered to the third quadrupole where they are scanned one at a time in separate scanning events [27–29]. Each precursor–product ion pair can be thought of as a separate scanning event, known as a 'transition', which must be programmed for mass spectrometry acquisition prior to analysis, and normally at least three daughter ions per target are recommended to insure optimum selectivity [30]. Verifying more than one transition per peptide and multiple peptides from the target protein is also important for controlling for matrix effects [28,31,32]. It has become common to use standards containing stable isotopes as internal standards in a technique known as isotope dilution mass spectrometry [33]. This technique allows for normalization and controls for variability and allows for relative quantitation based on the ratio of 'heavy' isotope containing standards to 'light' endogenous molecules, thus improving reproducibility and allowing for quantitation [23–26]. Another advantage of this technique is the relatively large dynamic range and sensitivity associated with isolating unique precursor-product ion transitions one at a time [34–38]. This strategy has also been used with SRM in tissue culture experiments through the use of stable isotope labeling by amino acids [39], which has benefited SRM by being used to quantitate proteins and to achieve absolute quantitation in biological samples by use of addition of a known amount of stable isotope synthetic peptide [40].

Advantages of SRM

Other techniques exist for targeted analysis, such as western blot and ELISA. Although western blot techniques have low throughput and are less quantitative, SRM and ELISA assays have shown a good degree of correlation [41]. Though ELISA assays can achieve a very high degree of sensitivity and specificity, the degree of sensitivity and specificity is dependent on the quality of the paired antibodies used. In addition, the development of new antibodies normally requires a large investment of money, time and effort for the development of antibodies if they are not already available and has a high failure rate [42–44]. ELISA assays, once developed, have a high degree of reproducibility and sensitivity on the order of sub-ng/ml. Though systems such as XMAP-Luminex and others have the ability to multiplex more than 40 proteins, they are not feasible for verification of a large number of potential biomarkers. One advantage of SRM is that, in contrast to the effort involved in developing an antibody, the development of an SRM assay is faster and more cost–effective [43,44].

Another significant advantage that SRM has over ELISA is the ability to multiplex large numbers of SRM assays with a high degree of reproducibility. The ability to multiplex SRM assays, known as multiple reaction monitoring (MRM), has allowed the targeted quantitation of potentially hundreds of proteins in a single analysis by scanning for specific peptides only during specific scanning windows, based on retention time [42]. This technique requires prior knowledge of retention time, but has allowed for the quantitation of hundreds of targets in a single analysis, though SRM remains the basis of this trend [45–47].

Expert commentary

It has long been thought that circulating blood contains molecular markers for disease due to the access this biological fluid has to every organ in the body. In addition, within the field of clinical chemistry, blood is the predominate biological sample procured from patients being diagnosed by medical clinics. Consequently, the plasma proteome is considered an attractive source of potential biomarkers, which contains as many as 10,000 unique proteins [48]. The clinical relevance can be seen in the assays that have been developed for quantitation of specific targets in the plasma proteome, for example blood levels of cardiac troponin after myocardial infarction [49,50]. In addition, cancer diagnostic biomarkers have been discovered, for example, thyroglobulin detection for metastatic thyroid cancer after thyroid removal [51]. These biomarker assays, which are now routinely used in a clinical setting, demonstrate proof of principle and inspire hope that many more biomarker tests may be validated for routine clinical use [3].

Five-year view

Despite these successes, the field of proteomics has relatively few fully validated assays for clinical use. Though SRM has greatly increased the feasibility of targeted assay development, hurdles remain due to high throughput and reproducible sample preparation to generate peptides from targeted proteins, appropriate transitions specifically designed for each protein target, the cost associated with the development of assays using mass spectrometry and stable isotope peptides, the development of highly multiplexed SRM/MRM assays and the enrichment reagents and methods for low-abundant targets [27,29,52]. To address the first hurdle, there is need for sample preparation and automation [53]. To address the second hurdle, publically available tools are available, such as PASSEL, NIST peptide library and SRMAtlas, which list spectral libraries, which can be used to develop SRM assays [5,54,55]. However, there is still a need for accurately predicting the fragmentation patterns and collision energies that will yield the most sensitivity in a triple quad instrument. This has been addressed in large part by the development of high-resolution scan of product ions which has allowed for the development of a technique known as multiple reaction monitoring high resolution [56]. The multiple reaction monitoring high-resolution technique scans for precursor ions performs fragmentation and detects daughter ions in a high-resolution unit, allowing for the collection a full spectrum of the daughter ions, eliminating the need for the determination of precise collision energy and optimum daughter ion charge state [56]. To address the third hurdle of expense associated with stable isotope internal standards, effective and inexpensive ways, at least for tagging large numbers of peptides, have been developed such as mass differential

tags for relative and absolute quantitation [57,58], tandem mass tag [59] and ^{18}O labeling [60,61] and dimethyl labeling [62], which also allow for normalization and quantitation, thus eliminating the need to purchase stable isotope standards. In addition, mass spectrometers have been developed with specific goals for targeted analysis to reduce the cost of using high-end mass spectrometers for targeted analysis. To address the fourth issue of multiplexed SRM/MRM, methods for using retention time standards have been developed, which allow the transfer of retention times from one chromatography platform to be transferred to another [63,64]. However, there is a concern with the number of targets to analyze in a single liquid chromatography–mass spectrometry analysis, and data independent acquisition approach such as energy dependent fragmentation and sequential window acquisition of all theoretical fragment-ion spectra techniques provides a solution [65,66]. To address the last issue, the low-abundant targets were enriched using upfront target enrichment followed by SRM analysis [67,68].

In the next 5 years, it is conceivable to imagine that these tools and others like them could be used to rapidly create fully quantitative targeted assays that are transferable and cost–effective. It is hoped that this will lead to the verification of many potential biomarkers with greater degrees of confidence. If this possibility is realized, then full FDA validation of novel biomarkers using targeted proteomics may be on the near horizon.

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Key issues

- The field of proteomics has produced many publications outlining potential biomarkers based on discovery experiments, yet there are relatively few biomarker assays available for clinical use.
- There is a growing consensus among leaders in the field that the development of quantitative high-throughput assays for the candidate proteins represents a bottleneck between biomarker discovery and fully validated assays for use in the clinic.
- Targeted proteomics affords the potential for the development of assays that are sensitive, reproducible, quantitative and possess a high enough throughput that statistically relevant conclusions can be drawn about the potential clinical value of a biomarker.
- To address these needs, assays have been developed using a technique known as selected reaction monitoring (SRM), which is a mass spectrometry technique that has recently been applied to the evaluation of quantitative differences between biological samples.
- The advantage of SRM assays is faster development time, the ability to multiplex large numbers of assays and cost–effectiveness in assay development.
- It has long been thought that circulating blood contains molecular markers for disease due to the access this biological fluid has to every organ in the body and is hoped that many more biomarker tests may be validated for routine clinical use.
- Though SRM has greatly increased the feasibility of targeted assay development, hurdles remain due to issues such as sample preparation, assay development costs and sensitivity.
- Tools such as SRMAtlas and retention time standards have been created to address these challenges. It is conceivable to imagine that these tools and others like them could be used to rapidly create fully quantitative targeted assays that are transferable and cost–effective, leading to validation of biomarkers for clinical use.