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Restructuring proteomics through verification

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Abstract

Proteomics technologies have revolutionized cell biology and biochemistry by providing powerful new tools to characterize complex proteomes, multiprotein complexes and post-translational modifications. Although proteomics technologies could address important problems in clinical and translational cancer research, attempts to use proteomics approaches to discover cancer biomarkers in biofluids and tissues have been largely unsuccessful and have given rise to considerable skepticism. The National Cancer Institute has taken a leading role in facilitating the translation of proteomics from research to clinical application, through its Clinical Proteomic Technologies for Cancer. This article highlights the building of a more reliable and efficient protein biomarker development pipeline that incorporates three steps: discovery, verification and qualification. In addition, we discuss the merits of multiple reaction monitoring mass spectrometry, a multiplex targeted proteomics platform, which has emerged as a potentially promising, high-throughput protein biomarker measurements technology for preclinical ‘verification’.

Keywords

biomarker; multiple reaction monitoring mass spectrometry; proteomics; verification

Better biomarkers are urgently needed to improve diagnosis, detect cancers at an early and less aggressive stage, improve treatment outcomes, determine likelihood of recurrence and monitor response to treatment before standard clinical end points become apparent. A significant increase in clinically relevant molecular biomarkers could have an enormous impact on improved patient outcomes and the financial viability of healthcare systems [1–2]. While the genomics community is making significant advances in understanding the molecular basis of disease [3–5], an understanding of the functional consequences that derive from these genetic alterations will be key. Proteomics can facilitate this process as it undoubtedly holds great promise in personalized medicine for cancer in the postgenomic era [6–8]. In the past decade, great strides have been made to characterize a large number of proteins, qualitatively and quantitatively, in a proteome, including sample fractionation, microfluidics and automation, mass spectrometry (MS) and protein microarrays [9–15]. It is believed that differential proteomic analysis of high-quality clinical biospecimen (tissue and biofluids) can potentially reveal protein/peptide biomarkers responsible for cancer by means

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of their altered levels of expression between control and diseased states [16–18]. Multiple reaction monitoring (MRM)-MS, a multiplex-targeted platform that uses stable isotope dilution MS, has emerged as a potentially promising technology in the ‘verification step’ (bridge between biomarker discovery and qualification) for high-throughput protein biomarker measurements as a diagnostic tool for patients [19–21].

A broken pipeline

Discovering potential candidates for inclusion in a multiplex protein biomarker assay is relatively easy. Hundreds or thousands of candidates are typically discovered in a single study using proteomic technologies such as MS. In fact, there are well over 1000 cancer-associated protein biomarker candidates described in the scientific literature [22].

Despite the great number of biomarker candidates discovered, only a handful have been clinically qualified and cleared for use by the US FDA. The lack of FDA-approved biomarkers is caused by a lack of standardized technologies and methods for verifying the candidates, the majority of which are not clinically useful. Culling through the lengthy list of candidates to identify the most promising clinically relevant biomarkers for further qualification has been the biggest rate-limiting step in protein biomarker research (Figure 1A). Therefore, this limitation is largely responsible for the research community’s failure in recent years to provide significant numbers of new protein biomarkers to the clinic in the form of standalone diagnostic tests or companion diagnostics [22].

Biomarker candidates are currently qualified using immunoassays (e.g., ELISA), which rely on the availability of suitable, well-characterized antibodies [23,24]. However, such reagents for novel candidates do not exist, and the time, expense and technical limitations required to generate them provide a strong incentive to develop alternative approaches. In addition, immunoassays are difficult to multiplex and are associated with high development expense (between US\$100,000s and 1,000,000s) and a long lead time (1–2 years) [15]. To overcome this bottleneck of many discoveries awaiting qualification, bridging technologies that accurately and efficiently credential biomarker candidates are needed prior to clinical qualification.

Protein biomarker verification: restructuring the pipeline

To address challenges, the National Cancer Institute established the Clinical Proteomic Technologies for Cancer (CPTC) initiative, with the mission to build a more reliable and efficient biomarker development pipeline. In support of this mission, the CPTC network is pioneering a new method for detecting and quantifying protein biomarkers in body fluids that may, ultimately, make it possible to screen many candidates simultaneously in hundreds of patient samples, ensuring that only the verified candidates will advance into biomarker qualification (Figure 1B). The goal of this endeavor is to reduce the time and cost of developing cancer diagnostic tests.

Candidate biomarker verification is a critical step that must be incorporated into the biomarker development pipeline (Figure 1B). The goal of verification is to provide reproducible, rapid and sensitive quantitative assays that bridge the gap between discovery and qualification, ensuring that only analytically verified candidates advance down the pipeline. Verification steps allow for a cost-effective manner to provide go/no-go decisions for potential biomarkers by systematically addressing the rate-limiting step of translational medicine.

The verification technology being tested by CPTC is based on existing technology, MRM-MS, which has been used for decades in clinical reference laboratories to accurately measure

small molecules in plasma, such as drug metabolites. MRM-MS has only recently been proven to be suitable for use in preclinical studies for rapidly screening and measuring large numbers of candidate proteins in complex patient samples, allowing verification of these candidates. MRM-MS provides a rapid way to measure the abundance of a particular candidate and determine whether changes in abundance correspond to the presence or stage of a disease.

Prior to MRM-MS-based workflows, precursor peptide ions are selected from previous experiments, combining empirical MS data, database searches of peptide libraries and software predictions, to develop the best peptide targets to monitor. Figure 2 demonstrates the general components of MRM-MS-based approaches for targeted protein quantitation on a triple quadrupole mass spectrometer. The first quadrupole is set to transmit only the selected precursor peptide ion into the second quadrupole. Collisionally induced dissociation in the second quadrupole yields a signature fragment ion of particular m/z value or several fragment ions that are then allowed into the third quadrupole and subsequently measured by the detector. The quantitation of peptides is achieved by measuring the intensity of the product ion(s). Therefore, MRM-MS can precisely detect a wide variety of peptides and proteins via peptide sequencing. This approach has the potential benefits that make it a desirable alternative to immunoassays, including, but not limited to, short assay development timeline, lower cost, high multiplexing capability, potential for high specificity, inclusion of internal standards, and particular advantages in detecting post-translational modifications, mutations and other variants of a protein.

However, potential drawbacks of MRM-MS assays include: the complex selection process for determining target precursor peptide ions to monitor, relatively low resolution of the triple quadrupole mass spectrometer (usually unit resolution) in resolving components in complex biofluids and the possibility of interference from in-source fragmentation of abundant peptides that prevent analysis of desired precursor peptides. In terms of sensitivity, MRM-MS assays alone are, at best, in the range of microgram per milliliter of biofluids. However, when coupled with immunoaffinity enrichment (i.e., Stable Isotope Standards and Capture by Antipeptide Antibodies [SISCAPA]) [25], it elevates the sensitivity of detection by several orders of magnitude [26,27]. With improvements in generating monoclonal antibodies against target peptides, instrument designs and workflow automation, MRM-MS may potentially provide a very reliable go/no-go decision point in the new biomarker development pipeline.

To determine the reliability and transferability of this technology, the CPTC network conducted a first-of-its-kind seminal study to assess the reproducibility and transferability of this approach across multiple laboratories [28]. Data show that the MRM-MS-based platforms can consistently identify and measure a large number of candidate proteins across laboratories, and proteins can be measured, sensitively and quantitatively, in a high-throughput fashion on instruments already deployed in clinical diagnostic laboratories [27]. This represents the first critical step toward potential widespread implementation of assays for the verification of candidate biomarkers. Follow-up studies are ongoing and include targeting cancer-specific proteins, enhancing sensitivity, lowering the coefficients of variation, increasing multiplex level and assessing reproducibility across a larger network of institutions.

Interested investigators can obtain the raw data sets, reagents, standard reference materials and detailed experimental protocols through the CPTC website [101]. The CPTC maintains an open-access policy for their data as advancements in science and healthcare are made possible through widespread access to results from cutting-edge research, enabling scientists to use and build on this knowledge. Although the emphasis of the CPTC program is on

cancer biomarkers, the workflows being developed (e.g., technologies, software and reagents) are broadly applicable to a range of human diseases.

Regulatory science

The addition of protein biomarker multiplex tests to the arsenal of cancer diagnostics will greatly advance personalized cancer care, but the success of any effort to translate scientific findings into the clinical setting depends on FDA approval. Fixing the biomarker pipeline will be fruitless if the end products – clinical diagnostic tests – are not approved.

Navigating the regulatory process is daunting enough, and this intensifies when new technologies are brought under review. The advanced technologies being used in the field of clinical proteomics are relatively new to the FDA, and this creates uncertainty both on the part of researchers in how their findings should be presented in a submission to the agency and on the part of the FDA in evaluating the data.

The lack of requirements for analytical validation of protein-based multiplex technologies prompted participants of the National Cancer Institute-FDA Interagency Oncology Task Force on Molecular Diagnostics workshop, held in October 2008, to recommend that the CPTC network leads efforts in the development of two ‘mock’ 510(k) submissions to the FDA [29]. The submissions, containing information from hypothetical experiments, described two protein-based platforms: multiplex immunoaffinity MS for protein quantification and immunological array for quantifying glycoprotein isoforms [30]. The FDA, in turn, agreed to review and provide comments.

Developing these mock submissions provided a mutually beneficial way for members of the scientific and regulatory communities to identify the analytical issues that need to be addressed when developing protein-based multiplex clinical assays. It is anticipated that these documents will serve as a springboard for education to the clinical proteomics community in the regulatory clearance of protein-based multiplexed *in vitro* diagnostic tests.

Bibliography

Papers of special note have been highlighted as:

- of interest
- of considerable interest

1. Etzioni R, Urban N, Ramsey S, et al. The case for early detection. *Nat. Rev. Cancer* 2003;3:243–252. [PubMed: 12671663]
2. Feero WG, Gutmacher AE, Collins FS. Genomic medicine – an updated primer. *N. Engl. J. Med* 2010;362(21):2001–2011. [PubMed: 20505179]
3. Workman P. Strategies for treating cancers caused by multiple genome abnormalities: from concepts to cures? *Curr. Opin. Investig. Drugs* 2003;4(12):1410–1415.
4. Nibbe RK, Chance MR. Approaches to biomarkers in human colorectal cancer: looking back, to go forward. *Biomark. Med* 2009;3(4):385–396. [PubMed: 20161020]
5. Jolly Graham A, Potti A. Translating genomics into clinical practice: applications in lung cancer. *Curr. Oncol. Rep* 2009;11(4):263–268. [PubMed: 19508830]
6. Anderson L. Candidate-based proteomics in the search for biomarkers of cardiovascular disease. *J. Physiol* 2005;563:23–60. [PubMed: 15611012]
7. Rifai N, Gillette MA, Carr SA. Protein biomarker discovery and validation: the long and uncertain path to clinical utility. *Nat. Biotechnol* 2006;24:971–983. [PubMed: 16900146]

8. García-Foncillas J, Bandrés E, Zárata R, Remírez N. Proteomic analysis in cancer research: potential application in clinical use. *Clin. Transl. Oncol* 2006;8(4):250–261. [PubMed: 16648100]
9. Wang H, Wong CH, Chin A, Kennedy J, Zhang Q, Hanash S. Quantitative serum proteomics using dual stable isotope coding and nano LC–MS/MS. *J. Proteome Res* 2009;8(12):5412–5422. [PubMed: 19817497]
10. Lee J, Soper SA, Murray KK. Microfluidic chips for mass spectrometry-based proteomics. *J. Mass Spectrom* 2009;44(5):579–593. [PubMed: 19373851]
11. Pierobon M, Calvert V, Belluco C, et al. Multiplexed cell signaling analysis of metastatic and nonmetastatic colorectal cancer reveals COX2–EGFR signaling activation as a potential prognostic pathway biomarker. *Clin. Colorectal Cancer* 2009;8(2):110–117.
12. Ramachandran N, Raphael JV, Hainsworth E, et al. Next-generation high-density self-assembling functional protein arrays. *Nat. Methods* 2008;5(6):535–538. [PubMed: 18469824]
13. Beirne P, Pantelidis P, Charles P, et al. Multiplex immune serum biomarker profiling in sarcoidosis and systemic sclerosis. *Eur. Respir. J* 2009;34(6):1376–1382. [PubMed: 19541722]
14. Kelleher MT, Fruhwirth G, Patel G, et al. The potential of optical proteomic technologies to individualize prognosis and guide rational treatment for cancer patients. *Target Oncol* 2009;4(3):235–252. [PubMed: 19756916]
15. Wang P, Whiteaker JR, Paulovich AG. The evolving role of mass spectrometry in cancer biomarker discovery. *Cancer Biol. Ther* 2009;8(12):1083–1094. [PubMed: 19502776]
16. Gromov P, Gromova I, Bunkenborg J, et al. Up-regulated proteins in the fluid bathing the tumour cell microenvironment as potential serological markers for early detection of cancer of the breast. *Mol. Oncol* 2010;4(1):65–89. [PubMed: 20005186]
17. Levin Y, Schwarz E, Wang L, Leweke FM, Bahn S. Label-free LC-MS/MS quantitative proteomics for large-scale biomarker discovery in complex samples. *J. Sep. Sci* 2007;30(14):2198–2203. [PubMed: 17668910]
18. Neubert TA, Tempst P. Super SILAC for tumors and tissues. *Nat. Methods* 2010;7(5):361–362. [PubMed: 20431548]
19. Paulovich AG, Whiteaker JR, Hoofnagle AN, Wang P. The interface between biomarker discovery and clinical validation: the tar pit of the protein biomarker pipeline. *Proteomics Clin. Appl* 2008;2(10–11):1386–1402. [PubMed: 20976028]
20. Makawita S, Diamandis EP. The bottleneck in the cancer biomarker pipeline and protein quantification through mass spectrometry-based approaches: current strategies for candidate verification. *Clin. Chem* 2010;56(2):212–222. [PubMed: 20007861]
21. Anderson L, Hunter CL. Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. *Mol. Cell. Proteomics* 2006;5(4):573–588. [PubMed: 16332733]
22. Polanski M, Anderson NL. A list of candidate cancer biomarkers for targeted proteomics. *Biomark. Insights* 2007;1:1–48. [PubMed: 19690635]
23. Backen AC, Cummings J, Mitchell C, Jayson G, Ward TH, Dive C. ‘Fit-for-purpose’ validation of SearchLight multiplex ELISAs of angiogenesis for clinical trial use. *J. Immunol. Methods* 2009;342(1–2):106–114. [PubMed: 19174166]
24. Fu Q, Zhu J, Van Eyk JE. Comparison of multiplex immunoassay platforms. *Clin. Chem* 2010;56:314–318. [PubMed: 20022982]
25. Anderson NL, Anderson NG, Haines LR, Hardie DB, Olafson RW, Pearson TW. Mass spectrometric quantitation of peptides and proteins using Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA). *J. Proteome Res* 2004;3(2):235–244. [PubMed: 15113099]
26. Kuhn E, Addona T, Keshishian H, et al. Developing multiplexed assays for troponin-I and interleukin-33 in plasma by peptide immunoaffinity enrichment and targeted mass spectrometry. *Clin. Chem* 2009;55(6):1108–1117. [PubMed: 19372185] • Describes in detail careful planning and design of multiplexed assays for cardiac biomarkers troponin-I and IL-33 in plasma by peptide immunoaffinity enrichment (Stable Isotope Standards and Capture by Anti-Peptide Antibodies [SISCAPA]) and targeted multiple reaction monitoring mass spectrometry.
27. Hoofnagle AN, Becker JO, Wener MH, Heinecke JW. Quantification of thyroglobulin, a low-abundance serum protein, by immunoaffinity peptide enrichment and tandem mass spectrometry. *Clin. Chem* 2008;54(11):1796–1804. [PubMed: 18801935]

28. Addona TA, Abbatiello SE, Schilling B, et al. Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma. *Nat. Biotechnol* 2009;27(7):633–641. [PubMed: 19561596] •• First-of-its-kind ‘round robin’ study to demonstrate acceptable inter-laboratory analytical variability of multiplex multiple reaction monitoring mass spectrometry assays on different mass spectrometry instrument platforms by standardization. This sets the stage for future preclinical or clinical studies using this targeted proteomic approach.
29. Rodriguez H, Tezak Z, Mesri M, et al. Analytical validation of protein-based multiplex assays: a workshop report by the NCI-FDA interagency oncology task force on molecular diagnostics. *Clin. Chem* 2010;56(2):237–243. [PubMed: 20007859] •• Details the experience and mock presubmissions of multiplex protein-based assays in collaboration with the US FDA. It lays the foundation for assay developers using proteomic tools to understand the regulatory pathways that, ultimately, translate research into the clinic.
30. Regnier FE, Skates SJ, Mesri M, et al. Protein-based multiplex assays: mock presubmissions to the US Food and Drug Administration. *Clin. Chem* 2010;56(2):165–171. [PubMed: 20007858] •• Details the experience and mock presubmissions of multiplex protein-based assays in collaboration with the FDA. It lays the foundation for assay developers using proteomic tools to understand the regulatory pathways that, ultimately, translate research into the clinic.

Website

101. National Cancer Institute. <http://proteomics.cancer.gov>

Figure 1. The envisioned National Cancer Institute-Clinical Proteomic Technologies for Cancer biomarker initiative development pipeline from biomarker discovery to biomarker qualification (A) The gap in the current biomarker pipeline, and (B) the introduction of verification between biomarker discovery and biomarker qualification in the NCI-CPTC biomarker pipeline.

NCI-CPTC: National Cancer Institute-Clinical Proteomic Technologies for Cancer.

Figure 2. Multiple reaction monitoring mass spectrometry

A schematic of a triple quadrupole mass spectrometer commonly used in multiple reaction monitoring mass spectrometry (MRM-MS) analysis; Q represents a quadrupole in a triple quadrupole mass spectrometer. Relative intensities of measured transitions from three targeted peptides eluting at different retention times are monitored by MRM-MS (colored in red, blue and green). MS/MS in Q2 illustrates the fragments in the second quadrupole Q2 (collision cell) for one of the three peptides (blue). An MRM-MS assay offers multiplexing capability of many target analytes in a single high-pressure liquid chromatography run.