Proteomics-driven cancer biomarker discovery: Looking to the future

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Truncated title: Cancer biomarker discovery

Summary

Availability of a suite of biomarkers for early detection, stratification into distinct subtypes and monitoring progression or response to therapy promises significant improvements in clinical outcomes for cancer patients. However, despite the recent progress in proteomics technologies based on mass spectrometry (MS), discovery of novel clinical assessment tools has been slow. This is, in part due to the inherent difficulties in working with blood as the biospecimen for candidate discovery. A better

understanding of the limitations of blood for comparative protein profiling and a better appreciation of the advantages of cancer tissue or cancer cell secretomes has the potential to greatly enhance progress.

Introduction

The genomic revolution brought in its wake the promise of an exciting era of biomedical discovery, especially in relation to early diagnosis of cancer and identification of cancer risk. In this context, much effort has been directed towards integration of multidisciplinary technologies to identify blood-based cancer biomarkers. This led to the establishment of the National Cancer Institute's Early Detection Research Network (URL: http://edrn.nci.nih.gov/). Much of the focus of the EDRN is based upon the well-used aphorism "prevention is better than a cure" and it was anticipated that collective efforts of members of the EDRN would swiftly translate basic research findings from laboratory to clinic. However, to date, the anticipated range of new diagnostic tools has been slow to emerge

The central hypothesis underlying the quest for blood-based cancer biomarkers is that organs secrete specific proteins (including those harboring characteristic post-translational modifications, PTMs) that collectively constitute a molecular fingerprint reflecting physiological function. In disease (e.g., cancer versus normal) this unique blood fingerprint of secreted proteins should reflect gene mutations contributing to the cancer phenotype. Discovery of such specific molecular fingerprints for cancer will be vital to track the nature and progression of the disease. Ability to read and understand the fingerprints in blood, which can be obtained non-invasively, will provide a unique window into disease status.

Significant advances have occurred in identifying genes mutated in cancers for identifying cancer risk [1,2], and assessing transcriptome differences (mRNA levels) between normal and tumor cells for stratifying cancer sub types [3,4]. Although transcriptome differences are easier to study than proteome (protein levels) differences, RNA expression profiling studies do not always address the changes in biological function of encoded proteins. Moreover, transcript expression levels do not always correlate with protein abundance. Thus, there is no *a priori* guarantee that increased mRNA expression of genes encoding proteins predicted by *in silico* means to be secreted [5], are actually secreted. Therefore, mRNA expression profiling studies aimed at identifying secreted cancer signatures must be complemented by direct identification of secreted proteins in the microenvironment. Further, it is well recognized that the pathogenic signaling pathways involved in the initiation and progression of cancer are not confined to the cancer cell itself, but can extend to the tumor-host interface and induce dramatic alterations of the surrounding stroma (microenvironment) [6]. Furthermore, tumor-related events, not included in the cancer cell signature *per se* may still be indicative of disease (e.g., cancer related alterations in basement membranes result in the release of prostate-specific antigen into seminal fluid and blood). It is reasonable to expect biomarkers emanating either from the tumor itself or its microenvironment to be present in tissue interstitial fluid and blood. Hence, proteomics, in contrast to other 'omic'-based disciplines such as genomics and metabolomics, must play a key role in clinical biomarker discovery – especially, given the fact that only proteomics can truly address alternative splicing and PTMs, which are seminal events in complex biological processes associated with cancer.

In this review, we discuss strategies for identifying cancer protein signatures and briefly examine pertinent proteomic technologies related to biomarker discovery.

Challenges of protein biomarker discovery: Is blood the best place to look?

Because the driving goal of biomarker discovery is to develop blood-based assays for early detection and prediction of therapeutic response, blood has been the logical biospecimen and most-used biomarker discovery matrix to date [7]. However, while blood is a very appropriate noninvasive fluid for monitoring biomarkers it poses many challenges from the perspective of discovery. For a recent review of blood-based strategies for the proteomic profiling of cancers, see [8].

Current proteomics technologies limit our capacity to directly interrogate the blood proteome for the purpose of biomarker discovery. One reason for this is the extraordinary dynamic range in blood protein abundances, from albumin (~40 mg/mL) to cytokines (~5 pg/mL), with 22 proteins accounting for 99% of blood protein content. This range and complexity creates extreme difficulties in the use of many existing two-dimensional protein separation tools (e.g., 2-DE is ~ 10^4 [9], free-flow electrophoresis/RP-HPLC ~10^5 [10] etc) for identification of low-abundance markers directly in blood. One method that addresses this problem is immunological and/or bio-specific depletion of high-abundance proteins [11]. However, although efficiency of immunodepletion ranges from 96.0 to ~99% , the remaining concentration of albumin, for example, would still be ~ 50 μg/mL, about 10^4 fold higher than blood CEA levels (~ 5 ng/mL) and $5x10^6$ fold higher than blood IL-6 levels (~ 10 pg/mL). Hence, MS-based detection of most already known biomarkers in blood requires deployment of additional separation/enrichment technologies.

It should be stressed that depletion of abundant plasma proteins is not without risk as these proteins may act as carriers for low-abundance molecules. For instance, albumin depletion has been suggested to lead to a concomitant loss of physiologically important proteins such as cytokines [12]. A further example is the candidate cancer marker, CRISPP (immunodefense suppression, and serine

protease protection peptide), the 35-residue peptide non-covalently bound by the circulating protease inhibitor, α-1-antitrypsin (α1AT). Using a multidimensional FFE/RP-HPLC proteomics strategy, we recently discovered that CRISPP is a processed form of active α1AT that remains bound after cleavage by the protease elastase via a 'suicide substrate' inhibitor mechanism [13].

The issue of the high dynamic range of protein abundances in blood can also be addressed by extensive fractionation using orthogonal three-dimensional protein separations [14]. However, while such approaches can be very useful for the discovery phase of biomarker research, they involve extensive processing and, typically, lack necessary reproducibility and quantitation for clinical validation. Other strategies for overcoming dynamic range protein concentrations in plasma rely on targeted enrichment of specific subpopulations (e.g., glycoproteins or cysteine-rich proteins). Widely-used strategies include lectin affinity chromatography [15], hydrazide coupling [16], cysteine peptide capture [17], or combinations thereof [18]. The efficacy of these profiling approaches was demonstrated by the identification in blood of low-abundance proteins such as angiotensinogen (50-70 μg/mL), epidermal growth factor receptor (1.3-3.5 μg/mL), or the hepatocyte growth factor activator (400 ng/mL) [15-18].

Other important biological shortcomings of direct protein profiling from blood include variations in the plasma proteome due to genetic polymorphism, gender, age, ethnicity, life style, dietary influences, diurnal factors and co-morbidities. Additional factors are variations arising from blood collection, processing time, sample preparation and storage temperature [19], as well as variable release of platelet contents [20].

Does the blood 'peptidome' reveal the cancer?

Matrix assisted laser desorption/ionization-mass spectrometry (MALDI-MS) or the closely related surface enhanced laser desorption/ionization (SELDI)-MS, can allow the surface upon which ionization takes place to provide a degree of fractionation due to variable absorbance of peptides [21,22]. These high-throughput techniques have been widely deployed for cancer biomarker discovery, especially for the measurement of low-M_r protein/peptide fragments (M_r<20K) in blood. These proteolytic fragments including those derived from abundant plasma proteins, are often referred to as the blood peptidome [23]. Non-identity based SELDI-MS involves comparative profiling (pattern-matching) of MS-derived peptide ion patterns purportedly derived from *in vivo* proteolytic cleavage of plasma proteins, low-abundance tumor-derived proteins and/or products of tumor-derived proteases sequested by albumin. These blind-screening methods involve a comparative analysis of polypeptide peaks of different mass/charge ratio that differ in intensity between the blood of patients with cancer and those of healthy individuals. Biomarker discovery studies of this nature have drawn cautionary notes due to problems in experimental design and data analysis or biases related to blood collection, processing and/or storage protocols [24,25]. Without identification of the peptides/proteins accounting for the MS-derived ion patterns using SELDI-TOF-MS it is very difficult to interpret the findings. More recently, it has been proposed by Villanueva and colleagues that tumor-derived proteases initiate cleavage of plasma proteins that can continue *ex vivo* and this phenomenon combined with the physiological action of coagulation enzymes produces serum peptidome patterns diagnostic for specific malignancies [26, 27]. However, it has been argued that these peptidome findings could be simply reflect the hypercoaguable state of the blood of cancer patients [28] first observed by Trousseau in 1865, and not necessarily a specific cancer signature.

Tissues versus fluids

Because of the shortcomings of profiling proteins in blood, other biospecimens such as tumor biopsy tissues are currently being considered as alternative sources for biomarker discovery [29]. It has been hypothesized that concentrations of potential biomarkers are highest in the tumor and its immediate microenvironment (i.e. tissue interstitial fluid) and this will be significantly diluted (~1500 fold) upon passage to the circulatory system via the lymphatics [30]. To reduce micro heterogeneity within tumor tissues, manual micro dissection or laser capture microdissection (LCM) [31] can be implemented to select for tumor cells. However, a major problem with LCM for standard 2-DE based proteomics profiling has been the requirement for ~40,000-70,000 cells (~30-40 μg protein) if using silver stain for protein visualization [32]. This shortcoming has recently been overcome with the introduction of the two-dimensional fluorescence difference gel electrophoresis (DIGE) saturation labeling technique coupled with quantitative image analysis software DeCyder™ [33,34]. Using this approach, Meyer and colleagues report new molecular markers for pancreatic intra-epithelial neoplasias using ~1000 micro-dissected cells (~5 μg protein) from snap-frozen pancreatic ductal adenoarcinoma tissue [29]. Using the same proteomics approach, Meyer and colleagues identified tropomyosin and microfibrillar-associated protein (MFAP) in human cirrhotic liver tissue; they subsequently validated these candidate markers in tissue by immunohistochemistry and in sera of diseased patients suffering liver fibrosis induced by infection of HCV or HBV (Meyer, unpublished data). These methods have now been extended to incorporate Triton X-114 phase partitioning to study scarce membrane proteins obtained from LCM-captured cells [35].

An emerging method for plasma proteomics is the use of *differential mass spectrometry* to detail changes in peptide abundances based on alignment of *m*/*z* versus time [36] [23]. This method enriches for the low-molecular weight (<20K) component of body fluids such as plasma and sera [20], and CSF [37], thereby overcoming the potential protein abundance dynamic range issue associated with complex proteomes (reviewed in Schulte et al. [38]).

Using cancer cell lines as tumor surrogates

Soluble-secreted proteins and shed membrane proteins from tumor cells (i.e., the 'secretome') present a further promising source of cancer biomarkers. Although large-scale efforts have been initiated to identify secreted and transmembrane proteins using a combination of genomics and bioinformatics (e.g., Secreted Protein Discovery Initiative (SPDI) [5]), these approaches should be complemented by direct protein profiling of the tumor microenvironment tissue interstitial fluid ([30]). Alternatively, cancer cell lines have been used as surrogates defining potential biomarkers such as cathepsin D for prostate [39] and colon cancer [40], A-33 alpha chain for colon cancer (Simpson, unpublished data), elafin for breast cancer [41] , cathepsins B and Z for leukemia [42] , and 14-3-3θ in lung cancer adenocarcinoma cell lines [43].

Conclusions: Where to from here?

The apparent lack of biomarker success suggested by the paucity of new FDA-approved 'commercial' cancer biomarkers in the past decade is not necessarily due to the lack of proteomics-based candidate biomarker discovery. Indeed, one could argue that proteomics has yielded a 'glut' of candidates, which has created a 'logjam' early in the pipeline (Figure 1) connecting biomarker discovery with the necessary verification, assay optimization , validation and commercialization required to take discovery to the clinic ([7]). One of the major blocks to clinical translation is the need to generate analyte-specific reagents, especially antibodies for sensitive clinical blood tests such as enzyme-linked immunoabsorbent assays (ELISAs) or flow cytometry-based assays (e.g., Luminex™). However, efforts to produce antibodies for a large range of candidate proteins (i.e., several hundred) and develop assays are time consuming and expensive. While ELISAs are considered the 'gold standard' in clinical assays, newer

orthogonal MS-based clinical assays (multiple-reaction-monitoring, MRM-MS [44] [45]) that can target a number of candidate biomarkers simultaneously may accelerate clinical translation, but these methods must first be developed and then validated on a case-by-case basis in the clinical setting. While, direct quantitation of biomarkers in plasma by MRM-MS is limited to µg/mL levels [44], ng/mL levels can be achieved using isotope-coded peptide antibody capture technology [46]. In parallel, quantitative immunohistochemistry using tissue and cell microarrays [47] can help triage candidates to select cancer-specific, or ideally cancer-subtype specific, markers. Finally, as candidate proteins move down the biomarker pipeline towards clinical application, development of affordable nanotechnology platforms to provide reliable, sensitive, high-throughput multiplexed identifications [48] will be required for cancer biomarkers to be effectively deployed in a personalized medicine setting [49].

Acknowledgements

The authors gratefully acknowledge Donna Dorow for discussions and critical reading of the manuscript. This work was supported in part by the National Health and Medical Research Council (Program grant 280912) to RJS), a Peter Doherty Fellowship (NH&MRC 381411) to OKB and a University of Melbourne Post-Graduate Student Scholarship to DWG.

Figure legend

Pipeline for Discovery and Validation of Biomarker Candidates. Biomarker development occurs primarily in five consecutive phases, each with selective aims. [a] Test population, indicated is the type of sample required for each phase and the relative quantities. Pictograms stand for human sample, animal model-derived sample and *in vitro* samples such as cell lines or biopsies. One pictogram indicates that a small

number of samples is needed, five pictograms indicate that 1000s of samples are needed; [b] Description and aims' gives a brief description of the aim of each step in the biomarker discovery process; [c] Biomarker candidate numbers, indicates the estimated number of biomarker candidates under investigation in each step of the biomarker discovery process; [d] Estimated cost, indicates the relative cost estimated for the phases of the biomarker discovery process; [e] Relative time investment, gives an estimation of the time needed for the different phases of biomarker discovery in relation to each other. Adapted from [7].

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