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Proteomic Tools for Cancer Research: Updating the Oncoproteomics

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Abstract

The search for cancer biomarkers remains a challenge. Proteomic tools for cancer biomarker discovery are limited by the need for highly sensitive technologies to analyze small-sized and rare samples. A amount of data has been obtained using proteomic strategies; however, the putative protein biomarkers for cancer have had no clinical impact until now. Growing evidence has shown that the search for a single biomarker in cancer is very complex, suggesting that an integrative view of protein datasets is required. Studies have focused on the analysis of oncogenic signaling pathways, identifying networks as potential cancer targets. This review discusses the major classical and emerging proteomic strategies used in oncoproteomics studies and how these approaches have contributed to translating basic cancer research to clinics.

Keywords: Proteomics; Cancer; Serum proteomics; Mass spectrometry

Proteomic Technologies in Cancer Research–An Overview

The molecular changes that occur in cancerous cells can be viewed as biochemical modifications orchestrated on a protein level. The human proteome is estimated to contain more than 20,000 proteins. Thus, studying proteins that are differentially expressed in cancer cells can reveal a plethora of important components involved in cell signaling and functionality. Notably, within the last 20 years, there has been a significant increase in proteomic-based studies in cancer research.

The use of applied proteomic studies dates back to the early 2000s [1,2]. Initial reports identified protein datasets that represented the differential analysis of up- and down-regulated proteins, providing a significant amount of data from the analysis of small-sized samples. However, the generation of very large protein datasets quickly demonstrated our inability to understand the crosstalk among several components of healthy conditions and disease. This realization propelled the development of bioinformatics strategies to identify interactions between protein networks, thereby converting lists of proteins into structural and quantitative datasets.

A growing number of scientific advances have enabled the identification of uncommon and low-abundance proteins in very rare materials, increasing our understanding of oncoproteomics [3-5]. As a result of improved instrumentation, data handling, and computer algorithms in conjunction with the integrative understanding of oncogenic signaling pathways, proteomic approaches are increasingly being used in cancer research [6-10].

Producing solely a proteomic dataset is not sufficient in cancer research today. Basic and applied researchers are now forced to refine their findings and direct their efforts towards performing bioinformatics data analyses using a combination of mathematical modeling and several proteomic-based technologies [11]. These collective efforts have resulted in the mapping of protein expression patterns of cells and tissues found in the Human Proteome Atlas (http://www.proteinatlas.org) and Clinical Proteomic Technologies for Cancer Database (http://proteomics.cancer.gov).

The complexity of cancer has turned the initial vision of studies based on protein identification to those based on protein functional analysis [12]. Thus, the focus of cancer research has changed from the search for a single cancer biomarker to the understanding of complex oncogenic signaling pathways [13,14].

The tools currently used in proteomic research are powerful and robust and enable the study of protein-protein interactions, the detection of post-translational protein modifications (e.g., phosphorylation, glycosylation, and oxidation) as well as the quantitation of specific proteins in samples with high confidence. For this purpose, emerging analytical strategies include the label-free proteomics, MALDI-imaging approaches, antibody-based arrays, image-based systems and and single-cell proteomics [12,15-19]. Here, we discuss some of these proteomic-based strategies and demonstrate how the combination of classical and emerging proteomic technologies can serve as the basis for understanding tumor biology and how they may shape the future of clinical cancer research.

Recent Advances in Proteomic-Based Technologies: Building Up the Oncoproteomics

Proteomic analysis of the oncogenic signaling pathways

Currently, proteomics is widely used to understand oncogenic signaling pathways, as well as tumor-derived markers. As reviewed by Jain [18], this field of proteomics focused on cancer research - called oncoproteomics - has grown in recent years and represents a promising area for proteomics-based research breakthrough. The proteomic-based strategies can be useful to predict the behavior of cancer cells and how should they respond to specific cellular interventions. Some newsworthy examples have been pointed out and include the use of 2D-electrophoresis, image analysis and MS as friendly technologies for investigating the differential protein profile between distinct

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cellular profiles, such as responsive versus drug-sensitive cells. These strategies help identify not only the differential expression but also if there are changes in the structure of such resistant-related proteins that support the chemoresistant phenotype. Further aspects have been explored by emerging oncoproteomics strategies and have included the searching for biomarkers discovery by persecuting specific cellular targets. The autoantibody signature method, known as Serological proteomic analysis (SERPA), is one of such technologies that combine proteomic strategies pooled in the MAPPing (multiple affinity protein profiling) strategy. The SERPA associates two-dimensional liquid chromatography followed by immunodetection of proteins. This combination can be performed in serum samples and helps to identifying specific autoantibodies produced in response to tumor antigens.

This integrative view of proteomic technologies has been applied to oncoproteomics studies, revealing pivotal information regarding tumor biology in several cancer models. Examples include the down-regulation of phenol sulfotransferase (SULT1A1) as indicative for the early detection of small-size hepatocellular carcinoma and the OVA1 predictive test for ovarian cancer screening [20]. This integration is determinant for improving the clinical application of oncoproteomics and recognition of proteomics as a tool clinically feasible.

In this context, recent cancer studies have looked to oncoproteomics-derived datasets searching for understanding the role of some signal transduction pathways [21]. Researchers have actually focused on understanding the protein networks interactions and its post-translational modifications [22], in part due to the advances in sensitivity and accuracy of mass spectrometry-based technologies. The use of fine procedures aiming to purify protein complexes before mass-spectrometry analysis has provided impacting findings regarding tumor biology at the level of protein-protein interactions [18].

Some oncogenic pathways have been targeted by functional proteomic analysis are reviewed by Kolch and Pitt [23]. The authors review the three major cancer pathways – epidermal growth factor receptor (EGFR), breakpoint cluster region (BCR)-ABL1 and ERK pathway under the prism of proteomic-based approaches. These oncogenic signaling pathways are responsible for triggering multiple downstream effectors that are enrolled in the malignant transformation of cells. The understanding regarding the EGFR interactome is notable and has helped to understand its role in cancer-related responses. Functional proteomics has provided evidences based on MS-quantitative proteomics that reveal intrinsic properties of interaction regarding the epidermal growth factor family, such as the ErbB members [24]. These findings showed relevant information about the actual knowledge concerning the specific preferential partners for ErbB members' interaction during its dimerization, which is proportionally linked to the power of transformation of ErbB dimers in cancer cells. Further studies employing SILAC-based quantitative proteomics determined modifications concerning ERK interactome and single cell proteomics profiling in BCR-ABL/chronic myeloid leukemia [25]. This type of study has given rise to the kinome-based proteomics, due to the need for the discovery of kinase targets and its related protein interactome.

The impact of such oncogenic signaling pathways is in part regulated by post-translational modifications (PTMs). Studies focusing phosphoproteomics have also shown successful results for cell signaling knowledge [26]. A phosphoprofiling study recently conducted by Andersen et al. [27] reported novel putative markers related with the response of prostatic cancer cells against PI3K-mTOR-AKT-PDK1 inhibition. This study employed the immunoaffinity precipitation strategy in association with mass spectrometry stable isotope labeling by amino acids in cell culture (SILAC) and identified a network enrolling 375 phosphopeptides related to the PI3K signaling. The use of this approach revealed pivotal points of such inhibition on impairing cell spreading processes and proposed the phospho-Thr246-PRAS40 residue as a possible biomarker during AKT inhibition. Further, using an antibody against this residue it was established that high expression of the phospho-Thr246-PRAS40 represented a good predictor of lung and breast cancer cells to AKT inhibitors. Thus, the use of combined proteomic strategies can serve as a platform for discovering new cell signaling-related markers.

Another signaling sensor that has emerged in cell biology is the study of the global thiol-cysteine state. Alterations in the cysteine residues consist in one of the most important post-translational modifications, however, it is not been applied for cancer research until now. The mapping of the thiol content can be investigated by using capture tags and enrichment strategies in association with fluorescence and mass spectrometry approaches. More recently, specific thiol-trapping techniques have been introduced, as the OxiCAT method. OxiCAT strategy identifies relevant targets of hypochlorite and hydrogen peroxide-driven oxidative stress *in vivo* [28]. This technology precisely identifies the redox-sensitive cysteine residues of proteins by using a combined mass spectrometry global approach with isotope-coded affinity tag (ICAT) technology in association with the thiol-trapping technique, after the modification of the accessible cysteine residues by the thiol-trapping reagent iodoacetamide. The OxiCAT approach provides a reliable picture regarding the redox state of the cell and can be employed for analyzing both plasmatic and tissular changes in the thiol content. Enhanced oxidative stress occurs in several cancers [29,30] and is the major source of cysteine oxidation; therefore, the study of the redox proteomics constitutes a promising tool for cancer research in the future.

Activity-based profiling (ABPP) platforms have highlighted some of the most important cancer-related enzymes, providing advances on cancer biochemistry and deregulated signaling networks. ABPP are specific probes that link covalently to a given protein (enzyme) by electrophilic and photoreactive groups. ABPP platforms provide a picture about the enzymatic activity of the targeted protein, and are based in proteomic technologies such as gel-based and LC-MS methods. Successful examples employing ABPP have been reported by the literature. Some metabolic enzymes as KIAA1363, monoacylglycerol lipase (MAGL) and serine hydrolase retinoblastoma-binding protein 9 (RBBP9) are reported as up-regulated proteins in cancer cells [31]. Moreover, the ABPP strategy serves as a reliable assay for screening potential inhibitors of some serine hydrolases, frequently expressed in tumor proteomes. Using this approach, selective inhibitors of KIAA1363 [32] and MAGL [33] were successfully identified.

Another recent focus of proteomic-based strategies in cell signaling is the metadegradomics, a functional proteomic approach based on the proteolysis-induced protein modifications. Technically, the degradomics analysis is based in the combination of the ABPP approach with MS-analysis, which can include several technologies such as ICAT, iTRAQ and SILAC labeling that allows identifying specific cleavage sites of protease-generated protein fragments (N-terminome analysis) [34]. In cancer, this method explores the fact that the oncogenic processes induce the enhanced expression of

proteases, which disturb important signaling pathways and alter the tumor microenvironment. The degradomics approach has been used mainly to identify the substrates, inhibitors and interactors of proteases in cancer aiming to differentiate its physiological and pathological role, consisting in a valuable tool for biomarker studies, protease biology research and drug target validation in cancer research.

Tissue proteomics

Antibody-based proteomics is especially relevant in the high-throughput validation pipeline of biomarkers discovery [35]. Strategies such as tissue microarray and chromogenic-based immunohistochemistry and fluorescence-based immunohistochemistry are examples of useful methods for the in situ study of tumor proteins. Advantages and drawbacks of the most used proteomic tools for serum and tissue proteomics are listed in the Table 1.

Tumor heterogeneity is the main challenge for proteomic-based studies [36] and determines several hallmarks of cancer such as angiogenic potential, motility, senescence, drug response and signaling pathways [37].

To overcome tumor heterogeneity, the laser capture dissection (LCM) technology has been employed for extracting more homogeneous tumor areas for antibody-based studies [38,39]. As example, the microproteomic analysis of laser capture microdissected breast tumor cells have been performed by using SDS-PAGE and porous layer open tubular (PLOT) LC-MS/MS strategies. This microproteomic workflow allows analyzing sample sizes of 10,000 cells with high sensitivity [40].

Tissue microarray (TMA) technology has been widely employed in the last years to generate the large-scale profiling of protein expression simultaneously in multiple cancer samples, using the principle of immunohistochemistry assay [41]. This high-throughput platform has improved the manual immunohistochemistry [42] by using automated image scores that offer a reliable quantitation of antibody labeling from cancer tissues [43].

Image analysis technologies are actually available and further allow identifying the cellular location of the target antigen. The use of immunofluorescence in association with digital image analysis algorithms strongly increases the capacity of detection of TMA assay, as the fluorescent-based automated quantitative analysis method (Histo-Rx TMA-AQUA platform). The AQUA strategy is a method for precise quantitative measurement of protein expression in paraffin-embedded tumor sections. After a fluorescent-based immunohistochemistry, TMA sections are scanned and analyzed by software, which evaluated specific cell compartments to calculate the AQUA scoring. The scores represent the exposure time-adjusted pixel intensity density of biomarker proteins within the tumor compartment area of each core (nucleus, cytoplasm, membrane). The AQUA scores can be expressed as a relation between the expression of the protein of interest in the normal tissue versus its expression in the tumoral tissue. Further, the median tumor AQUA scores for each protein can be useful to dichotomize patients into low or high expression groups for specific protein. Thus, a protein biomarker can be defined in this system if the tumor score exceeds the normal tissue scores [44]. The use of the AQUA method for image analysis has provided new information regarding cancer prognosis [45].

The tissue-based proteomics is usually supported by IHC analysis. Notwithstanding the limitations regarding this method, studies have proposed cancer classification [46], diagnosis [47] and prognosis [48,49] in association with data obtained from gene expression studies. A successful example of the use of IHC strategy for biomarker searching is the case of the estrogen receptor (ER) and the human epidermal growth factor receptor 2 (HER2) in breast cancer.

Recently, an outstanding proteomic technology has emerged, based on molecular imaging studies or imaging mass spectrometry (IMS) using matrix-assisted laser desorption ionization (MALDI). IMS allow visualizing the spatial location of proteins in a given sample based on the molecular mass of the molecule. This strategy includes a range of technologies, such as the secondary ion mass spectrometry (SIMS), MALDI imaging and desorption electrospray ionization (DESI). Once the laser focuses on sample, mass spectra of each part of the tissue is analyzed. This approach has been described as an excellent tool for studying complex biological samples such as tumor tissue sections, localizing spatially specific small molecules *in situ* [50]. IMS enable to analyze intact tumoral tissues, providing a result known as histology directed profiling, which allow correlating clinical and pathological data without sample destruction [51].

IMS strategy has been used to identify differentially expressed peptides/proteins in diagnostic and prognostic cancer studies, as well as lipid analysis and drug discovery. Some successful examples include

Approach	Advantages	Drawbacks
AQUA platform	- Indicates the subcellular location of the protein - Accurate quantitative scoring	- Quality of antibodies - Cost
Immunohistochemistry	- Low cost	- Quality and cross-reactivity of antibodies - No reliable quantitative scores
MALDI Imaging	- In situ imaging - Allows predicting the protein subcellular location	- Technically painstaking - High cost
Protein microarrays	- In situ protein screening on a large scale	- Quality of antibodies
Tissue Microarrays	- Screening of a wide of samples	- Poor pathological evaluation
2D-Electrophoresis	- Detection of several proteins in a single run	- Restricted capacity of proten separation
Mass spectrometry	- High throughput screening of samples	- Technical complexity - Cost is not very acessible
ELISA	- Analysis of large sample datasets - Low-cost technology	- Limit of detection
Western Blotting	- Easy implementation	- Not always reprodutible

Table 1: Advantages and drawbacks of the proteomic approaches available tooncoproteomics.

the definition of tumor margins in renal carcinoma [52], differing normal tissue from prostate cancer [53], defining benign/malign ovarian cancer [54], glioma outcome [55] and breast cancer response to taxanes [56], among others.

In addition, automated morphometric pattern recognition image analysis algorithms have improved data analysis when performing tissue proteomics. Such tools can reduce sample variability and guide the choosing of high-quality specimens for biomarker discovery studies by recognizing histologic tumors and nontumor tissue areas in cancer tissue samples [57].

Serum proteomics

A promising approach in proteomic-based studies is the use of high-abundant samples obtained by less-invasive procedures [17]. For this end, studies have focused their efforts in serum and tissue-based proteomics. The serum contains a picture of the systemic status of the individual in healthy and pathological conditions [3]. This sample aggregates information concerning proteins and peptides secreted from tissues, which is especially desirable for cancer biomarker studies by using mass-spectrometry approaches.

Serum proteomics consists in a very powerful strategy for biomarker study and uses a less-invasive procedure. Despite this easy accessibility for sampling, the analysis of serum proteome is still a challenge, mainly due to the fact that the intra-tumoral heterogeneity can determine its secretome [37]. Pre-analytical issues are critical when performing a serum analysis by proteomics and include sample collection, handling process and storage conditions [36]. Major complicating factors for serum analysis include its biological complexity, dynamic range of protein concentration, high levels of interfering compounds and variations among individuals [58].

The main challenge of serum proteomics is to eliminate the high abundance proteins, as albumin, immunoglobulins, complement and coagulation chain proteins, without harm the analysis of other serum components. These abundant proteins respond for about 99% of the total circulating proteins and can potentially impair the analysis of the low-abundance proteins [59]. This fact is particularly important because tumors may be the secreting source of these low-abundance proteins to plasma.

The putative cancer biomarkers are probably the very rare low-abundance proteins. Thereby, for biomarker discovery studies, serum samples are incredibly rich, containing thousands of proteins that represent a picture of the systemic status of cancer disease [60]. On the other hand, it is difficult to determine the source of the main circulating proteins, since it can be originated from both host and tumor cells. Thus, until now it is not known any specific protein selectively produced only by tumor cells.

Depletion procedures can eliminate the high-abundance proteins of serum and plasma; however, studies suggest that this step also removes some of the interesting low-abundance proteins, resulting in loss of potentially relevant information [61]. The use of mass spectrometry label-free technologies has overcome some of the limitations for serum analysis found in the conventional proteomic approaches, improving the identification of less abundant proteins. On the other hand, limitations such as the interference of high-abundant proteins remain a challenge, since depletion procedures are not recommended for plasma/serum analysis, as described above. Such techniques provide further advantages for serum proteomics studies as high-sensitivity, real-time measurements, faster quantification and enhanced reproducibility [59]. In this context, studies employing the MS-label-free strategy have provided a new scenario of circulating proteins, not before described in cancer [29,30].

Classical proteomic strategies can be very useful for designing a biomarker study. The enzyme-linked immunosorbent assay (ELISA) in one of the main methods for measuring a circulating protein marker, using a single or multiplexed strategy. Actually, the ELISA method yet represents the gold standard strategy for validating a putative circulating biomarker [43]. The use of multiplex protein analysis for serum analysis has emerged as a rapid and sensitive approach, providing an overview of circulating proteins, as well as identifies the post-translational modifications of such proteins [62]. Commercial kits have been developed for this end, and the multiplexed kits present several advantages when compared to the single-strategy ELISA, as the requirement of reduced sample volume, high-sensitivity and multiple detection of antigens [43].

Aptamer-based proteomic technologies have demonstrated good results for large-scale application in biomarker discovery studies. Aptamers are peptides that can selectively bind specific proteins with high affinity, presented as complex high-performance proteomic arrays. This strategy simultaneously measures thousands proteins by using small samples, with a pM detection [63]. This technology has been applied to the early detection of lung cancer, suggesting that this approach is superior in comparison with broad serum proteome profiling platforms such as mass spectrometry and antibody arrays [64].

Challenges and Concluding Remarks

The main emerging proteomic technologies that promise to improve the oncoproteomics are summarized in Table 2. There are still many challenges related to the use of proteomics for the discovery and validation of cancer biomarkers. Even with numerous recent and ongoing efforts made to identify cancer biomarkers, no specific cancer biomarker has been identified and validated by proteomics [58,65]. This failure may be due to the lack of globally standardized proteomic platforms, the use of poor criteria for the design of studies and sample collection, the type of proteomic technology employed, and tumor heterogeneity [66]. Undoubtedly, tumor heterogeneity and serum proteomics are still major challenges to be addressed in cancer biomarker studies. Furthermore, the limited dynamic range, low-throughput, and high cost of MS-based proteomics are significant obstacles to their use [59].

Proteomic studies have provided a large amount of data; however, only a small amount of useful information has been extracted from it. In the future, the use of integrated proteomic-based methodologies and bioinformatics may result in the identification and validation of relevant clinical cancer biomarkers. The initial screening of cancer samples using highly sensitive technologies provides a non-invasive opportunity for large-scale disease screening, which can be validated using antibody-based panels to identify potential markers for disease prognosis, diagnosis, and treatment. Strong statistical parameters as well as deep biological and biochemical knowledge are also required for data analysis and translation. Together with advances in bioinformatics, data management systems, and biobank resources, proteomic-based technologies can be used to identify reliable biomarkers in cancer research.

Proteomic approach	Technical Considerations
SERological proteomic analysis (SERPA)	
	culture (SILAC)
OxiCAT Thiol-mapping proteome	the thiol-trapping reagent iodoacetamide
(ABPP) platforms	association with gel-based and LC-MS methods Useful to metadegradomics studies.
TMA-AQUA platform	This tissue microarray analysis allows the *in situ*
Imaging mass-spectrometry (IMS)	Enables analyzing intact tissue samples without sample destruction. IMS allow visualizing the spatial location of proteins in a given sample based on the molecular mass of the molecule. This strategy includes a range of technologies, such as the secondary ion mass spectrometry (SIMS), MALDI imaging and desorption electrospray ionization (DESI).
Aptamer-based strategy	

Table 2: Emerging approaches for oncoproteomics.

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