REVIEW ◄

Proteomics and bladder cancer: Opportunities and challenges

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Abstract

Recent progress in life science technology and the availability of much information on genes obtained by genome analysis has enabled us to analyze the changes of proteins on a large scale. Sets of proteins are called proteomes, and proteomics is the scientific field of proteome analysis including differential, post translational modification and interaction analyses. Various proteomic approaches, particularly two-dimensional gel electrophoresis (2-DE), mass spectrometry and protein chip methods, are very useful for acquiring proteomes in cells, tissues and body fluid, for analyzing interactions between a protein and other biofactors including proteins. A proteomic analysis is also useful for determining biomarkers of diseases and key proteins involved in various stages of metabolism such as differentiation, cell cycle and apoptosis. The proteomic approach to revealing urologic diseases, including bladder tumor, has only recently been introduced. The proteomes of malignant and benign samples from bladder cancer have been compared using the 2-DE technique; the disease specific proteins have been identified, and their expressions have been assessed. The aim of this review is to illustrate the proteomic technologies that have emerged for comprehensive and high-throughput protein analysis and to provide more detailed of their application in bladder cancer research and diagnosis.

Introduction

The International Agency for Research on Cancer (IARC) estimated that bladder cancer is the 9th most common cancer diagnosis worldwide, with more than 330.000 new cases each year and more than 130.000 deaths per year [1, 2]. Cigarette smoking is the most important risk factor for bladder cancer, accounting for approximately 66% of new cases in men and 30% of the cases in women in industrialized populations. More than 90% are of the transitional cell carcinoma (TCC) histology. Its natural history is related to a combination of factors that impact on its aggressiveness. Cystoscopy and urine cytology are the currently used techniques for the diagnosis and surveillance of non-invasive bladder tumors. The sensitivity of urine cytology for diagnosis is not high, particularly in low-grade tumors. The combination of voided urine cytology and new diagnostic urine tests would be ideal for the diagnosis and follow-up of bladder cancer. However, in order to have some clinical utility, new diagnostic and/or prognostic markers should achieve better predictive capacity that the currently used diagnostic tools.

Proteomics is the study of protein expression in a tissue or biological fluid. Comparison of protein patterns in biological fluids between healthy individuals and patients with disease is increasingly being used both to discover biological markers of disease (biomarkers) and to identify biochemical processes important in disease pathogenesis [3]. Although currently available tests for urine proteins measure either the total level of urine proteins or the presence of a single protein species, emerging proteomic technologies allow simultaneous examination of the patterns of multiple urinary proteins and their correlation with individual diagnosis, response to treatment or prognosis [3, 4 and 5]. Analysis of the various protein constituents of urine may suggest novel, noninvasive diagnostic tests, therapeutic guidance, and prognostic information for patients and clinicians.
With the emergence of newer technologies, in particular mass spectrometry, it has become possible to study urinary protein excretion in even more detail [3]. A variety of techniques have been used both to characterize the normal complement of urinary proteins and also to identify proteins and peptides that may facilitate earlier detection of disease, improve assessment of prognosis and allow closer monitoring of response to therapy [3, 4 and 6]. Such proteomics-based approaches hold great promise as the basis for new diagnostic tests and as the means to better understand disease pathogenesis. In this review, we describe the current practice of urine protein testing and the emerging technologies that are being used for analysis of the urinary bladder proteome.

The proteomic tools for identifying molecular markers of the bladder

Proteomic analysis can be viewed as an experimental approach to explain the information contained in genomic sequences in terms of the structure, function, and control of biological processes and pathways [6]. Therefore, the proteome reflects the cellular state or the external conditions encountered by a cell. In addition, proteomic analysis can be viewed as a genome-wide assay to differentiate distinct cellular states and to determine the molecular mechanisms that control them [7]. Quantitative proteomic analyses can be used to identify the protein content in complex samples such as serum, plasma, and urine extracts and to determine the quantitative difference in abundance for each polypeptide contained in different samples [7]. Analyses of the proteomic profiles would impact a wide range of biological and clinical research questions, such as the systematic study of biological processes and the discovery of clinical biomarkers for detection and diagnosis. Actually, biomarkers can be defined as cellular,

biochemical, and molecular alterations by which normal, abnormal, or simply biologic processes can be recognized or monitored [6, 8]. These alterations should be able to objectively measure and evaluate normal biological process, pathogenic processes to a therapeutic intervention. Therefore, proteomic profiling is valuable in the discovery of biomarkers as the proteome reflects both the intrinsic genetic program of the cell and the impact of its immediate environment. Protein expression and function are subject to modulation through transcription as well as through translational and post-translational events. In addition, breast markers can be subtle changes in molecular structures, for instance alterations of post-translation modifications, which often can only be examined at the protein level [9, 10]. Currently investigators are pursuing three different approaches to develop a technology to study biomarkers with increased sensitivity and specificity [4]. The first is to improve on currently used or known biomarkers. The second approach is to discover and validate novel biomarkers with greater sensitivity and specificity. The third approach is to use a panel of biomarkers, either by combining several individually identified biomarkers or by using mass spectrometry to identify a pattern of protein peaks in sera that can be used to predict the presence of cancer [10].

Techniques for bladder proteome analysis

Two-dimensional polyacrylamide gel electrophoresis

In recent years, the combination of 2-DE (two-dimensional electrophoresis) and MS (Mass spectrometry) has been utilized extensively for proteomic research in medicine [6,11]. The power of the 2-DE-based technology was recognized by the research community early on, and scientists from various disciplines were attracted to the field of proteomics [11]. The information obtained by the 2-DE-based approach is high because a number of specific protein attributes can be determined. This method used a combination of isoelectric focusing (IEF) and the SDS–PAGE system of Laemmli. Thousands of proteins can be resolved and visualized simultaneously on a single 2-DE gel; for each protein, the isoelectric point, MW, and the relative quantity can be measured. High-resolution capabilities of 2-DE allow the separation and detection of post-translationally modified proteins. In many instances, post-translationally modified proteins can be readily located in 2-DE gels because they appear as distinctive horizontal or vertical clusters of spots.

There are a variety of sample preparation methods for 2-DE separation. Body fluids such as serum, plasma, cerebrospinal fluid and urine are soluble, liquid samples that can be separated by 2-DE using a simple solubilization buffer**.** Protease inhibitors are sometimes added, but it should be remembered that such reagents can modify proteins, leading to charge artifacts. Albumin and immunoglobulins exist as high abundance proteins and thus can obscure many of the minor component proteins. This can be avoided by removing these proteins on an affinity column, but there remains the possibility that the nonspecific deletion of other proteins can occur. Cells cultured on solid substrates can be harvested by scraping and lysed by the addition of solubilization buffer. Tissue samples that have been frozen in liquid nitrogen can be processed in buffer by using a homogenizer. The heterogeneous nature of tissue samples may cause a problem in analyzing proteomes. To overcome this, laser capture microdissection (LCM) can be usefully employed to obtain a pure population of cells from a tissue section [12]. One of the most difficult challenges of obtaining reproducible, high-resolution separation of proteins is poor solubilization. The best known method for protein solubilization is that originally described by O'Farrell, which uses a mixture of 9.5 M urea, 4% (w/v) of NP-40, 1% (w/v) of dithiothreitol (DTT), and 2% (w/v) of synthetic carrier ampholytes of the appropriate pH range. While this method works well for many types of samples, it is not universally successful, especially with membrane proteins. Many effective detergents and solubilizing agents, such as a CHAPS/urea–thiourea mixture and SDS, are used to improve solubilization. The development of basic IPGs with pH values up to pH 12 has made it possible to analyze very alkaline proteins; the introduction of narrow-range IPGs has enabled the separation of proteins with high resolution. Recent developments in fluorescent technology will help researchers to quantify proteins more precisely. Proteins extracted from the spots of 2-DE gels can also be identified easily by mass spectrometry (MS)-based protein identification systems.

Mass spectrometry: MALDI-TOF/MS

Mass spectrometry is an indispensable tool for the identification and quantification of proteins from complex mixtures such as urine, plasma and tissue [12]. For this purpose, it is important to separate the maximum number of proteins. A combination of 2-DE and MS has been identified one of the most widely used strategies [11]. After proteins are separated by 2-DE and quantitated by the intensity of their staining, selected spots are excised, digested, and identified by peptide mass mapping using matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) or peptide sequence analysis using electrospray ionization (ESI)–MS/MS.

A mass spectrometer consists of an ion source, a mass analyzer, and a detector. The ionized analytes in the ion source are separated in the mass analyzer by their mass to charge ratio (m/z) and registered in the detector by the number of ions of each m/z value. Electrospray ionization and matrix-assisted laser desorption ionization methods are currently the principal soft ionization methods for the MS analysis of peptides or proteins. These techniques have been used with high throughput sample preparation techniques such as liquid chromatography (LC). ESI ionize the analytes out of a liquid phase and thus is readily coupled to LC. At a low flow rate of 500 nl/min or less, which is called, a micro- or nanoelectrospray, more sensitive MS spectra can be obtained with less sample consumption. MALDI ionize the analytes out of a co-crystallized dry matrix using energy supplied by a laser. The ion trap, quadrupole, time-of-flight, and fourier transform ion cyclotron (FT-MS) are the major methods used for mass analysis in proteomic research; they can be used either alone or in combination with tandem mass (MS/MS) analysis. A diverse combination of ionization methods and mass analyisis has made possible a number of proteomic approaches of differing sensitivities, resolutions, and applications.

Proteins are identified by matching experimental to expected mass data from sequence databases. Isolated proteins can be analyzed by identifying the accurate mass of peptides derived from specific enzymatic cleavages, while more complex mixture of proteins can be identified by the MS/MS spectra of individual peptides. In general, the former method, known as peptide mapping or fingerprinting, uses MALDI-TOF. The latter method provides a higher level of certainty in the identification of proteins, because, in addition to the peptide mass, the peak pattern in the MS/MS spectrum also provides information about peptide sequence. This method therefore generates information about the type and site of modifications.

Large-scale proteome analysis also requires high-throughput techniques for searching databases. A multiprocessing algorithm for searching databases has been described which increases the search speed for a large number of spectra. Assembling or filtering algorithms have been developed to analyze search results in order to identify proteins with more confidence.

Protein databases

The identification of a protein from its peptide sequence derived from the mass spectrum has been facilitated by the development of proteomics databases. The first major protein database, Swiss-Prot, was established in 1986 and is maintained collaboratively by European institutions. Others have been developed in different countries and with different focuses (eg, cell lines or plant proteins). Major databases include Protein Information Resource (PIR), Protein Research Foundation (PRF) and Protein Data Bank (PDB). Most protein databases are free-access.

The Entrez search and retrieval system is a tool for accessing and searching a large range of databases, including protein databases and literature databases, such as PubMed. It was made available in 1988 by the US National Center for Biotechnology Information (NCBI) at the National Library of Medicine. Protein entries in this system contain amino acid sequences from the protein databases Swiss-Prot, PIR, PRF and PDB, as well as amino acid sequences translated from DNA sequences in genetic databases such as GenBank.

Proteomic biomarkers in bladder cancer

Bladder cancer is the fourth most common malignancy in males, the tenth most common malignancy in females, and the second most common tumor of the urinary tract. With regard to the types of tumors involved, transitional cell carcinoma (TCC) represents >90% of bladder cancers. Cystoscopy represents the gold standard for the detection and monitoring bladder tumors and has a sensitivity of about 70%. This procedure is also useful for resection of tumors and provides specimens for the pathological evaluation of prognostic factors. However, cystoscopy is painful, costly, and therefore it is not suitable as a screening test. Proteomics refers to the study of proteins, including structure and function using technologies such as, high resolution 2-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS).

An extensive 2-DE study on the protein expression profiles of bladder tumors including transitional cell carcinoma and squamous cell carcinoma (SCC) has been implemented by Celis et al. [13]. By examining more than 63 TCC cases, they identified four proteins that are expressed in normal urothelium and low-grade TCCs but not in high-grade TCCs. These are adipocyte-type fatty acid binding protein (A-FABP), glutathione S-transferase (GST-μ), prostaglandin dehydrogenase (PGDH), and keratin [13]. Six of 150 bladder tumors were diagnosed as SCC, and their proteome analyses showed different expression patterns that depended on the degree of differentiation. All six SCC patients secreted the protein psoriasin in the urine although this protein was identified only in frozen sections of the more-differentiated SCCs [14]. Moreover, non-keratinizing metaplastic lesions that exhibit a spectrum of abnormalities were identified and analyzed immunohistochemically using antibodies against the proteins differentially expressed between normal urothelium and SCCs [15].
NMPs (Nuclear matrix proteins) are the best examples that bladder cancer markers can be identified initially through 2-DE and then developed into conventional ELISA tests [16]. However, this proteomic approach is technical skill, its reproducibility is moderate and it cannot detect hydrophobic proteins. The technique is intensive and time consuming. SELDI (surface enhanced laser desorption/ionization) is a preferred MS-technique used to identify and quantify biomarkers form complex biological fluids such as urine [4]. Proteins are captured by a selective surface and the mass of each protein is measured based on its velocity through the time-of-flight analyzer, after proteins are ionized by a laser. The SELDI-TOF measures mass-to-charge ratio and quantifies the amount of each protein present in a biological sample. The advantages of SELDI include, rapid protein profiling to screen large numbers of samples in a clinical research setting. Bladder cancer is amenable to SELDI-based biomarker development since many tumor-associated molecules are secreted in urine [17]. From such studies, several markers have been identified but only a small number have undergone rigorous testing with clinicopathological correlation and study. Some of those will be reviewed here, including CXCL-1, TATI (tumor associated trypsin inhibitor), and MMPs (matrix metalloproteinase). CXCL-1 is a member of the CXC chemokine family noted to be associated with tumorigenesis, angiogenesis and metastasis [18, 19].

Recently, Kawanishi et al. [20] evaluated proteins that could differentiate between normal controls, non-invasive, and invasive bladder cancer patients [20]. Using cell culture from known bladder cell lines and mass spectroscopic analysis, they identified CXCL1 as a protein with significantly increased expression in invasive bladder cancer. Subsequently, they evaluated its in vivo utility by assaying urine samples from normal individuals, patients with non-invasive tumors, and patients with muscle invasive disease. CXCL1 showed sensitivity of 70.1% and specificity of 80.6% [18, 19].

A second newer set of proteomic markers undergoing further testing are matrix metalloproteases (MMPs). While MMPs have been studies for many years, recent advances in proteomics have allowed for more specific evaluation and identification of MMP related complexes and their utility in bladder cancer [21, 22]. Roy, et al. [23] reported on their findings of tumor specific urine MMP complexes including the MMP-2, MMP-9/TIMP-1 complex, MMP-9 dimer, and ADAMTS-7 [23]. They report that using a combination of MMP-2 and MMP-9 dimer in multivariate regression and binary analyses, they were able statistically significantly differentiate bladder cancer from controls. However, the study compared only normal versus bladder and prostate cancer patients. Thus, the sensitivity and specificity of any MMP to detect cancer (bladder or prostate) between 75% and 80% will need to be confirmed in a larger cohort of patients that include patients with benign urological conditions [21,22 and 23].

One SELDI study for the proteome analysis of urine from TCC patients has been performed in a relatively wide range of participants (n = 94: 30 TCC, 34 normal, 30 benign urologic diseases) [24]. Among the five potential markers that were expressed differentially in TCCs, one was identified by SELDI immunoassay as defensin [24]. The main function of defensins is thought to be a direct immediate antimicrobial action and the protection of the host against invading bacteria. Also, defensin has been shown to play a role in processes of neovascularization that are tightly related to tumor invasion and metastasis.

Other authors have used similar approaches to identify potential markers. Smalley et al. [25] used an innovative approach of analyzing subcellular particles released by urothelial cells by mass spectormetry (LC-MS/MS) to identify eight potential biomarkers of bladder cancer. These included Resistin, GTPase NRas, mucin-4, and Retinoic acid-induced protein [25]. While these proteins were found to be differentially expressed in the urine of bladder cancer patients and normal individuals, the sample size was too small. Therefore, these potential biomarkers will need to be validated in larger studies [25].

In other studies, Kageyama et al. [26] were able to identify a potential tumor marker, calreticulin, which is found in the urine of patients with bladder

carcinoma. The authors used a differential display method of bladder cancer vs healthy urothelial tissue and mass spectrometry to identify proteins that are increased in cancer tissue. In addition to calreticulin, an endoplasmic reticulum chaperone, they found nine other candidate proteins that could constitute new biomarkers for bladder carcinoma. The authors confirmed their data with quantitative Western blot analysis and immunohistochemistry. Their reported sensitivity and specificity were 73% and 86%, respectively, similar to the values reported for other biochemical bladder markers. However, the diagnostic accuracy of their test was vulnerable to urinary tract infections [26].

Perspectives

The application of proteomics to the early diagnosis of bladder diseases and their monitoring is a difficult challenge. Owing to the progress in related techniques, the process of biomarker development using proteomics has recently been introduced. Until now, many proteomic technologies, such as 2-DE, SELDI, isotope-coded affinity tags (ICAT), free flow electrophoresis (FFE), and two-dimensional protein fractionation (PF-2D), have been introduced for clinical proteome profiling studies; however, their usefulness is still limited. Thus, the development of new technologies is necessary for the study of protein expression and function.

The 2-DE technique is a powerful method of analysis which can simultaneously resolve up to several thousand proteins. In addition, new methods for the automated characterization of proteins resolved by 2-DE have been developed and are continuously being improved. The automation of 2-DE gel image analysis, protein spot excision, and protein identification by MS-based methods is under development. The rapidly growing commercial interest in this field is responsible for the production of high-performance image analysis, spot-picking robots, and on-line MS/MS analysis technologies. Multidimensional LC and MS/MS spectrometry, mainly interfaced by ESI, is a new strategy for protein identification. The digestion of proteins creates a hugely complex mixture of peptides, making the resolution of the peptides by high-performance separation techniques necessary prior to entering the MS/MS spectrometer. Various combinations of separation schemes for multiple fractionation have been explored. At present, two-dimensional chromatographic separation, consisting of strong cation exchange and reverse-phase C18 chromatographic methods, is often used for the separation of peptide mixtures, which are frequently pre-fractionated by protein separation method such as 1DE or size exclusion chromatography.

A mixture of peptides from the same proteins of different origins can also be quantitated by the stable-isotope dilution method. The stable isotope tags are introduced to proteins via metabolic labeling, enzymatic transference, or chemical reactions.

For an MS-based protein profiling technique such as SELDI to be used more widely and safely, an even and uniform platform (chip) surface is essential so that reproducible data are obtained. Recently, proteomics has been accepted as a useful tool in pharmaceutical research and toxicology. Proteome databases can be queried for changes in the concentrations of proteins presenting responses to a group of pharmaceuticals or toxic substances. Such proteins are useful as markers for specific responses. The power of marker proteins in detecting the diagnostic features of a protein expression profile depends largely on the quality of the proteome database. Many pharmaceutical companies have constructed comprehensive databases regarding the molecular effects of drugs. They also offer a service by which anyone can have access to the database. This kind of proteomics application will be expanded to also include urologic diseases.

Conclusion

The proteomic approach to revealing urologic diseases, including bladder tumor has only recently been introduced. Many biological targets remain to be studied using this new technology. The 2-DE method coupled with MS-based protein identification is a very powerful tool for proteomic analysis. The proteomes of malignant and benign samples from various urologic diseases have been compared using the 2-DE technique; the disease specific proteins have been identified, and their expressions have been assessed. SELDI, an MS-based protein profiling approach, has been demonstrated to provide a high-throughput technology for urologic proteomics research. Computational analyses of the protein profiles from patients have resulted in the discovery of biomarkers. However, obstacles remain to be overcome for the further development of current technologies for proteome analysis.

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