Urine Proteomics and Biomarkers in Renal Disease

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Background

Approximately 70% of the urinary proteomes in healthy individuals originate from the kidney and the urinary tract, and therefore urine samples are a valuable source, which can be utilized to identify biomarkers in patients with renal disease [1]. One of the early attempts to define the urinary proteome using liquid chromatography-tandem mass spectrometry (LC-MS/MS) was published in 2001 [2]. Since then, consistent efforts to identify efficient urinary biomarkers using different methods of proteomics have been made. Many studies on urinary proteomes in patients with different categories of renal disease have been conducted in the last decade, and the clinical application of some of the identified biomarkers may not be too far away from reality. A basic understanding of urinary proteomics will therefore be helpful not only for researchers but also for clinicians. The aim of this minireview is to provide concise information about currently available proteomic methods and the clinical application of urinary proteomics in renal disease.

Introduction to Terminology

The ‘proteome’ is the protein complement expressed by the genome. ‘Proteomics’ is the study of the proteome and can be further defined as the analysis of the spatial
and temporal expression of a subset (and ultimately a full set) of proteins in a defined biological system [3].

A major goal of proteomics in the clinical field is to identify biomarkers that can aid in the diagnosis and assessment of prognosis of different conditions. They may also be useful for monitoring therapeutic responses, optimization of treatment and guiding the choice of more appropriate therapies for each patient. Two different approaches in clinical proteomics can be termed ‘discovery proteomics’ and ‘targeted proteomics’ (table 1). In discovery proteomics, also referred to as ‘fishing proteomics’, the proteomes of the same biological samples from different groups of patients or healthy individuals are analyzed and compared to detect significant differences. The most common proteomic methods for this approach are various MS-based techniques. The advantage of this approach is the possibility to detect a novel candidate molecule as a new biomarker. The disadvantage, however, is that this approach is not hypothesis driven. In targeted proteomics, proteins to be investigated are defined beforehand, and appropriate methods, mostly immunology based, are applied for their detection. ELISA and protein microarrays are most commonly used. Efforts have also been made to adapt MS to detect a selective group of proteins [4]. Although this strategy is both powerful and useful to follow the changes in expression levels of specific proteins, it is not suitable for the discovery of a novel molecule involved in physiological processes.

Urine proteomics has traditionally focused on the study of soluble proteins. Recently, however, urinary exosomes have been receiving increasing attention as a new source of potential biomarkers. Exosomes are 40- to 100-nm membrane vesicles of endocytic origin secreted by most cell types. They are found in body fluids and contain membrane proteins such as transporters and ion channels as well as cytosolic proteins. Urinary exosomes are a rich source of biomarkers because they are released from every segment of the nephron, including podocytes. After a purification process using several techniques, MS and Western blot are commonly used for further investigation [5]. In a small study, transcription factors were detected in the urinary exosomes from patients with acute kidney injury and focal segmental glomerulosclerosis [6].

**Table 1. Comparison of discovery and targeted urine proteomics**

|                  | Discovery                                      | Targeted                                      |
|------------------|-----------------------------------------------|-----------------------------------------------|
| Methods          | gel electrophoresis                           | immune-based assays (e.g. ELISA, antibody arrays); modified MS |
|                  | LC, capillary electrophoresis                 |                                               |
|                  | MS                                            |                                               |
| Examples         | hepcidin in lupus nephritis; fragments of collagen I in diabetic nephropathy [12] | CCL2 in proliferative glomerulonephritis [18, 19]; connective tissue growth factor in diabetic nephropathy [20] |
| Advantage        | discovery of novel molecules                  | hypothesis based                              |
| Disadvantage     | not hypothesis based                          | restricted to study of known proteins         |

CCL2 = CC chemokine ligand 2 (also known as monocyte chemoattractant protein-1).

**Overview of Currently Available Urine Proteomic Techniques**

**Methods Used for Discovery Proteomic Approach**

MS-based proteomic methods are in use for this approach, and each method consists of several steps. Initially, urine samples need to be handled by different methods such as centrifugal filtration, ultrafiltration, lyophilization or precipitation. Removal of albumin is sometimes required to improve the identification of low-abundance proteins. For certain MS techniques, protein digestion to peptides is necessary. The second step is the separation of proteins/peptides, and this can be achieved using 2-dimensional gel electrophoresis, LC or capillary electrophoresis (CE). The next step involves the mass spectrometric measurements of separated proteins/peptides. A mass spectrometer consists of an ion source, a mass analyzer that measures the mass-to-charge ratio (m/z) of the ionized analytes, and a detector that registers the number of ions at each m/z value. Different ionization sources include matrix-assisted laser desorption/ioniza-
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The results are statistically analyzed to find the best peptide masses of each protein encoded in the genome. The peptides of the unknown protein to the theoretical masses of the proteins are then theoretically cut into peptides. This method is automated and offers a high sensitivity. Sequential separation using different media in two independent steps provides a multidimensional fractionation that can generate vast amounts of information. In a study of patients with Fanconi syndrome due to Dent’s disease, more than 100 peptides were identified by this approach [9]. The variability on multidimensional separations, however, makes comparative analysis difficult, and the substantial amount of time required makes the technique unsuitable for clinical application. Furthermore, it is very sensitive to interference from lipid or detergent.

Two-Dimensional Gel Electrophoresis-Mass Spectrometry

After separation of proteins by 2-dimensional gel electrophoresis according to their isoelectric point and molecular weight, and ‘in-gel’ digestion of gel spots of interest by a protease, the produced peptides are commonly analyzed by matrix-assisted laser desorption/ionization-TOF. This technique allows the study of large molecules and enables the estimation of actual molecular weights and quantification of proteins [8]. This is the method of choice for the comparative analysis of unmodified medium-size or large proteins (10–200 kDa) in the discovery phase of biomarker definition. However, there are several limitations such as low throughput, low reproducibility, high intensity of time and labor, and automation difficulties. Therefore, the method is not suitable for regular clinical application.

Quantitative Comparison of Proteomes by Labeling Methods

Labeling methods have been developed to compare the proteomes between samples quantitatively. Isotope-coded affinity tags (ICAT) were used in earlier studies. The ICAT reagents label cysteine amino acid residues. The ICAT reagents contain reporters with different molecular mass reporters. Then, the labeled peptides from lipid or detergent.

Improved methods based on the principle of ICAT have been developed. For example, isobaric tags for relative and absolute quantification may be used. These isobaric tags contain reporters with different molecular mass reporters. Then, the labeled peptides from different urine samples may be mixed in equal proportion for analysis by LC-MS/MS. After fragmentation during MS, the reporters with different molecular masses are separated from the balance region and become distinguishable by MS. The relative quantity of
peptides from different samples will be quantified by the intensities of different reporter ions [10].

Surface-Enhanced Laser Desorption/Ionization-Time-of-Flight

This technique incorporates chromatographic and MS principles on a single platform (fig. 1). An activated surface on a ‘chip’ binds proteins on the basis of their chemical and physical properties; unbound proteins are washed off. A subset of the proteome is thus selected, and the chip plugs directly into the MS for analysis. This is a useful high-throughput screening technique, and the relative speed and simplicity give it the potential for clinical use [11]. However, only a very small fraction of all proteins in a sample binds to the chip surface, and binding to the different chip surfaces varies depending on sample concentration, pH, salt content and the presence of interfering compounds. Furthermore, it offers only a low resolution. Using this technique, our laboratory has detected a combination of urinary peptides which distinguishes patients with active lupus nephritis from those in remission (part of the figure reproduced from Mosley et al. [11] with permission).

Capillary Electrophoresis-Mass Spectrometry

CE separates proteins in a silica capillary in a single step with a high resolution based on their migration through a gel in the electrical field. It is commonly combined with ESI-MS (ESI-TOF). This technique is robust and fast, uses inexpensive capillaries instead of expensive LC columns and generates comparable high-resolution data sets [13]. The data sets from individual analyses can be compiled to generate a typical proteome pattern that can be based on >100 individual analyses. This method is generally not suited for larger molecules (>20 kDa); however, this is generally of less significance for urine samples as the urinary proteome contains a high percentage of low-molecular-weight proteins. Sequencing of protein can be achieved by directly interfacing CE with MS/MS instruments, or by subsequent targeted sequencing using LC-MS/MS. CE-MS is therefore a good alternative to the commonly used proteomic technologies. In a study of patients with diabetes, a panel of 40 biomarkers was found to distinguish a subgroup of diabetic patients with different severities of nephropathy. It is interesting that many of these biomarkers are fragments of abundant proteins such as collagen type I. The mechanistic basis for
and importance of using these abundant proteins as biomarkers need further investigation [14].

Methods for Targeted Proteomic Approach

While the MS-based techniques are applied for the detection of novel molecules (discovery proteomics), targeted proteomic methods are suitable for the validation of these molecules. They are mostly immunology-based and very powerful methods if the antibodies for the proteins under study are specific and readily available. Commonly used methods are ELISA, Western blot and protein microarray-based methods [15]. These methods are well established and widely applied in clinical diagnostics as well as research.

- ELISA and Western Blot
  The mechanism of both methods is the same. A specific antibody for the unknown amount of targeted molecule is applied to the surface so that binding of antibodies and targeted molecules can occur. This antibody is linked to an enzyme, and in the final step, a substance is added that the enzyme can convert to a detectable signal. The signal can be a color change, fluorescence or chemiluminescence. In Western blot, proteins in a sample need to be separated in a gel and blotted on a membrane before the detection procedure.

- Protein Microarray-Based Techniques
  A protein microarray provides an immunology-based technique for detecting multiple proteins. Specific antibodies or antigens are printed on a surface at separate locations in an ordered manner forming a microscopic array. The array surface can be a chip, bead, membrane or slide. The most common protein microarray is the antibody microarray, where antibodies are spotted onto the array surface and used as capture molecules to detect proteins from samples. Capture molecules commonly are antibodies; however, antigens can also be used if antibodies need to be detected in a sample. In our laboratory, we have used a membrane-bound antibody array to identify increased amounts of a profibrotic chemokine, CCL18, in the spent dialysis fluid from patients with long-term complications of peritoneal fibrosis [15] (fig. 2). The application of this technique to patient urine has been more difficult because of the lower concentrations of cytokines in urine. In this situation, it is more appropriate to use the more sensitive method of glass-based antibody arrays or Luminex bead arrays. With their extensive multiplexing capabilities, Luminex beads are becoming increasingly popular, and commercial kits are available for the detection of many different molecules, such as the multiplex cytokine array. These are very powerful methods as the technique is relatively simple and large numbers of samples can be investigated within a reasonable time. The limitations of these targeted proteomic methods are, however, the need for a specific probe for every protein to be analyzed. Furthermore, this kind of method may cause bias as the investigator chooses the set of antibodies to be used.

- Modified MS
  Recently, there have been developments in MS allowing the measurement of selected target proteins quantitatively, such as multiple reaction monitoring (MRM)-based assays [16, 17]. Specific antibodies are not needed for this technique. Efforts are being made to improve the reproducibility and throughput of these MRM-based assays in analyzing specific groups of proteins in biological fluids, so that they may be used in clinical situations.

Fig. 2. Detection of cytokine proteins using a membrane-based multiplex cytokine antibody array. In this membrane-based array, 60 specific cytokines were tested in duplicate. Each dark spot represents a semiquantitative signal according to the cytokine concentration. Both positive and negative controls were included in the same array. In this experiment, high concentrations of CCL18, monocyte chemoattractant protein (MCP)-1 and interleukin (IL)-6 were detected in the spent peritoneal dialysate from a long-term peritoneal dialysis patient. (Reproduced from Ahmad et al. [15] with permission).
Application of Urine Proteomics: Workflow of Clinical Proteomics to Discover Urine Biomarkers

The ideal sequence of urine proteomics to discover new biomarkers would be MS-based discovery of candidate molecules from small, well-defined patient populations, followed by a validation process with samples from large patient cohorts using immunology-based proteomic techniques and, subsequently, clinical application of biomarker assays. However, this is easier said than done, and modifications to this sequential approach are often made in reality. For example, the targeted candidate biomarker for a validation step can be derived from different sources such as in vitro and in vivo studies (fig. 3). These molecules can be investigated in samples from large patient groups using targeted proteomic methods. As they are already known to be involved in the pathogenesis of a disease from previous in vitro or in vivo research, the chance of validation is greater than for newly found molecules by MS discovery. For example, urinary CCL2 (also known as monocyte chemoattractant protein-1) was shown to be elevated in patients with proliferative glomerulonephritis [18, 19]. In patients with diabetic nephropathy, we also found that urinary connective tissue growth factor and CCL2 are prognostic of progression of diabetic nephropathy [20].

Alternatively, it is possible to combine discovery and targeted proteomics using MS. For example, Quintana et al. [21] used LC-MS to discover that amounts of specific fragments of uromodulin and kininogen were lower in the urine of patients with chronic renal allograft dysfunction than in control subjects. These 5 specific protein fragment ions were measured using MRM-based LC-MS/MS to validate the discovery.

It is critical to have adequate validation of any recently discovered biomarkers. This often involves validation of the reproducibility of the assays and clinical validation in large-scale clinical studies across multiple clinical centers (fig. 3). These are not simple tasks [22].

Future Perspectives

With continuous developments and improvements in proteomic methods and clinical trials regarding the validation of the candidate biomarkers, there will be more and more biomarkers or a battery of biomarkers available in the clinical field. These will aid in making diagnoses and prognoses and also in monitoring treatment responses, leading to an improvement in patient management. In the clinical research field, they can be used as surrogate markers, providing additional possibilities of assessing outcomes.

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