Current status of renal and urinary proteomics: ready for routine clinical application?

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Introduction

Renal and urinary proteomics are among the most rapidly growing subdisciplines of proteomics applied to biomedical research. The rapid growth of this field is evidenced by an increasing number of published articles related to renal and urinary proteome analyses. Using the keywords ‘proteomics’ or ‘proteome’ or ‘proteomic’ together with ‘kidney’ or ‘renal’ or ‘urine’ or ‘urinary’, >1200 articles have been found in PubMed since 1996 to July 2009. This rapid growth reflects much interest of nephrologists and renal physiologists in applying proteomics to address clinical and basic questions. Moreover, urinary proteome analysis offers opportunities for biomarker discovery not only in kidney diseases but also in other organ disorders and systemic diseases [1]. Together, these have accelerated the progress in this field during the past several years.

Commonly used methods for renal and urinary proteome analyses include two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), liquid chromatography coupled to tandem MS (LC-MS/MS), surface-enhanced laser desorption/ionization (SELDI)-TOF MS, capillary electrophoresis (CE) coupled to ESI-TOF MS, and protein microarrays (details of these technologies, including their advantages and limitations, can be found in many recent reviews [2,3], whereas this article focuses only on the current status of renal and urinary proteomics and their future directions). All of these techniques have been applied to renal and urinary proteomics with the ultimate goals being as follows: (i) to better understand the biology and physiology of the kidney;
(ii) to unravel the pathogenic mechanisms and/or pathophysiology of kidney diseases and related disorders; (iii) to identify diagnostic and prognostic biomarkers; and (iv) to define new therapeutic targets, drugs and other treatments. A schematic summary of methodologies and applications of renal and urinary proteomics is shown in Figure 1. From the efforts made during the past 12 years, some achievements have been made, while further developments are also required. Examples for such achievements are provided as follows.

**Better understanding of biology and physiology of the kidney**

Several proteomic studies have been conducted to better understand the biology and physiology of the kidney. In 2002, Arthur and colleagues [4] performed proteome mapping of the renal cortex and medulla. A total of 72 forms representing 54 unique proteins were identified by 2-D PAGE followed by MALDI-TOF MS. Among these, 13 proteins were identified as differentially expressed proteins between cortex and medulla. Another study by Magni and colleagues [5] expanded the 2-D proteome map of renal cortex by identifying 89 proteins using MALDI-TOF MS. Yoshida and colleagues [6] constructed the proteome database for the human glomerulus isolated by sieving technique. Using MALDI-TOF MS and LC-MS/MS, they identified >200 unique proteins in the human glomerular proteome map.

For the urinary proteome, our group identified 67 forms representing 47 unique proteins in the normal human urine using 2-D PAGE followed by MALDI-TOF MS [7]. The human urinary proteome database was expanded by Adachi and colleagues [8]. Using SDS-PAGE followed by highsensitive linear ion trap-Fourier transform (LTQ-FT) and linear ion trap-orbitrap (LTQ-Orbitrap) mass spectrometers, they identified >1500 proteins in the normal urine. Interestingly, a large number of the identified proteins were typical membrane proteins [8]. The most likely source of membrane proteins in the urine is the microvesicle namely, the ‘urinary exosome’ [9], which is derived from renal tubular epithelial cells. After endocytosis from the apical membrane, the endocytic microvesicle, which has an inside-out orientation, can fuse and invaginate into the multivesicular body, making the right orientation of inner and outer membranes. When the multivesicular body fuses with the apical membrane of a tubular cell, these microvesicles are released into the urinary space as exosomes [9]. Using
LC-MS/MS, Knepper and colleagues [9,10] identified >1000 proteins in urinary exosomes, including disease-related proteins, transporters, proteins in endosomal sorting complex required for transport, vacuolar H-ATPase subunits and phosphoproteins.

Investigations of the renal and urinary proteomes during physiological stimuli or experimental interventions would lead to a better understanding of the renal salt-water handling. For example, a gel-based proteomic analysis revealed that sodium loading causes changes in excretion of 45 urinary proteins, some of which play roles in renal tubular transport [11]. Additionally, Dihazi and colleagues [12] utilized 2-D PAGE followed by quadrupole time-of-flight (Q-TOF) MS/MS and observed that osmotic stress causes alterations in abundance levels of 40 proteins in the thick ascending limb of Henle’s loop, a site where the urinary concentrating mechanism occurs. All of the aforementioned and many other studies result in better understanding of the biology and physiology of the kidney.

Unraveling of pathogenic mechanisms and/or pathophysiology of kidney diseases and related disorders

Most of these studies applied proteomics to the investigations of diabetic nephropathy. For example, Tilton and colleagues [13] examined changes in the renal cortical proteome in Type 2 diabetes using a db/db murine model and found alterations in levels of 147 cortical proteins. These altered proteins play significant roles in several biological processes, particularly in metabolic pathways. The investigators also created an interaction map of all the altered proteins and demonstrated that peroxisome proliferator-activated receptor-alpha (PPARα) was the common node of these interactions.

Ramachandra Rao and colleagues [14] examined changes in membrane and cytosolic subproteomes of human mesangial cells under a high-glucose condition. Among these, calmodulin was identified as one of the increased proteins in the cytosolic fraction. Immunostaining confirmed the increase of calmodulin in mesangial cells under the high-glucose condition. Western blot analysis confirmed the increase of calmodulin not only in mesangial cells but also in rat and murine diabetic kidneys. To address functional significance of calmodulin in the diabetic kidney, the investigators examined effect of calmodulin inhibition on glucose uptake in mesangial cells. Functional analysis revealed that inhibition of calmodulin activity by two compounds, W7 and trifluoperazine, completely abolished the TGF-β-induced glucose uptake in mesangial cells. These data indicated that calmodulin plays a significant role in glucose transport in mesangial cells.

Another study by Barati and colleagues [15] examined changes in the golmerular proteome of db/db mice and found altered levels of 40 glomerular proteins. Among these, they highlighted the increased levels of several proteins involved in redox pathways, particularly superoxide dismutase isoform 1, peroxiredoxin-3 and glutathione peroxidase-1. Our group also performed proteomic analysis of kidney tissue obtained from Type 1 diabetic animals and observed altered levels of 41 proteins in the diabetic kidney [16]. We then created the renal protein trafficking model for Type 1 diabetic nephropathy, based on our findings.

Proteomics has been applied also to kidney stone disease (nephrolithiasis), which is a common disease worldwide. Our group has explored pathogenic mechanisms of this disease focusing on the role of the crystal–cell interaction [17,18]. Using a gel-based proteomic approach, we identified a number of proteins involved in the crystal–cell interaction. Interestingly, changes on the proteome of renal tubular cells adhered with calcium oxalate monohydrate (COM) [17] did not overlap with those observed in cells adhered with calcium oxalate dihydrate (COD) crystals [18]. As COM has much greater adsorptive capability, this difference underscores the pathogenic role of COM crystals in kidney stone disease.

In addition to diabetic nephropathy and kidney stone disease, which are used as examples for the discussion herein, applications of proteomics have also led to a better understanding in pathogenic mechanisms and/or pathophysiology of many other diseases.

Identification of potential candidates for diagnostic and prognostic biomarkers

Among all proteomic applications, the majority relate to biomarker discovery, particularly in the urine because of its availability in almost all subjects and ease of sample collection. CE-MS, which is a powerful tool for proteome profiling, was employed by Mischak and colleagues [19] to differentiate urinary polypeptide profiles of patients with Type 1 diabetes from that of age-matched healthy controls. The urinary polypeptide pattern of patients with diabetes significantly differed from that of the normal controls. They also performed subgroup analysis and found a pattern of potential biomarkers that could differentiate patients with diabetic nephropathy from those without nephropathy. The same group also employed CE-MS to differentiate urinary polypeptide profiles of patients with Type 2 diabetes from those of age-matched healthy controls [20]. Again, they observed specific urinary polypeptide profiles for each subgroup in association with the degree of albuminuria.

Cho and colleagues [21] utilized SELDI-TOF MS to search for potential biomarkers in sera of streptozotocin-induced diabetic rats compared to control animals. They observed eight potential biomarkers in the serum, one of which was identified as C-reactive protein. The increased serum level of C-reactive protein was confirmed by ELISA. In another study, Dihazi and colleagues [22] performed SELDI proteome profiling of urine samples obtained from type 2 diabetic patients with nephropathy, compared to those without nephropathy, proteinuric patients from non-diabetic causes and healthy controls. They identified three SELDI spectra with mass/charge (m/z) of 6188, 11 744 and 14 766 of urinary proteins that were differentially excreted among groups. Interestingly, these spectra could differentiate diabetic patients with nephropathy from those without nephropathy. Subsequently, they were identified as ubiqui-
In addition to diabetic nephropathy, proteomics have been also applied to many other diseases and a large number of biomarker candidates have been identified [1, 2]. One of the most successful stories is the study conducted by Decramer and colleagues [24] to identify biomarkers for neonatal ureteropelvic junction (UPJ) obstruction. The patients were classified into three groups; No_OP (no operation was required), OP_Poss (might possibly undergo operation), and OP (with severe UPJ obstruction that required surgery rapidly after birth). Using CE-MS, they successfully identified a panel of potential biomarkers containing 19 urinary polypeptides that could distinguish the OP group from the healthy newborns and No_OP group. With this molecular signature, a blinded validation in independent sample sets could differentiate OP from No_OP patients with a sensitivity of 94% and a specificity of 80–100%. More importantly, they could also prospectively predict the clinical outcome of patients in the OP_Poss group (i.e. to progress further to the OP group or to emit as the No_OP group).

Another successful study was done by Zhou and colleagues [25] to identify potential biomarkers for acute kidney injury (AKI). Using 2-D difference gel electrophoresis followed by mass spectrometry, a number of potential biomarkers were identified in urinary exosomes derived from a rat model of cisplatin-induced AKI. Among these identified proteins, they validated the increased level of exosomal fetuin-A in cisplatin-induced AKI rats using Western blot analysis. Also, the increased level of exosomal fetuin-A was validated in animals with bilateral renal ischaemia and reperfusion injury, as well as in ICU patients with AKI. These data underscore the promise of proteomics in biomarker discovery for diagnostics and prognostics of kidney and other diseases.

### Identification of new therapeutic targets

Although most of the recent applications of renal and urinary proteomics have focused mainly to biomarker discovery, some of these studies also revealed potential new therapeutic targets. For example, elastin [16] and collagen fragments [26] as the potential targets for prevention/treatment of fibrogenesis in diabetic nephropathy, urinary trefoil factor 1 (TFF1) in prevention of kidney stone formation [27], and many others. More recently, Beck and colleagues [28] applied an immunoproteomics approach (more details of immunoproteomics can be found in [29]) and successfully identified M-type phospholipase A2 receptor (PLA2R) as a glomerular target antigen of idiopathic but not secondary membranous nephropathy. The result was confirmed by using recombinant PLA2R, which also reacted to sera obtained from patients with idiopathic membranous nephropathy. Additionally, IgG (mainly IgG4) eluted from these patients’ sera, but not from those with secondary membranous nephropathy, recognized PLA2R. These data will lead to further development of new drugs or therapies for treatment and/or prevention of kidney diseases and related disorders.

### Summary and outlooks

The current status of the renal and urinary proteomics arena is summarized in Table 1. We have partially achieved the goals to better understand the biology and physiology of the kidney, and to unravel pathogenic mechanisms and/or pathophysiology of kidney diseases and related disorders. For biomarker discovery, although a large number of biomarker candidates have been identified, they are neither validated in a large cohort nor ready for routine clinical practice at present. Similarly, a number of new therapeutic targets have been identified, but no new drugs or therapies have been developed based on such therapeutic targets identified by the proteomic approach. However, these aims will probably be achieved soon. Finally, at the moment, personalized medicine seems very far away, but it may be possible in the future.

<table>
<thead>
<tr>
<th>Goals for applications of renal and urinary proteomics</th>
<th>Current achievement by proteomics approach</th>
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<tbody>
<tr>
<td>Better understanding of biology and physiology of the kidney</td>
<td>Yes (partial)</td>
</tr>
<tr>
<td>Better understanding of pathogenic mechanisms and/or pathophysiology of kidney diseases and related disorders</td>
<td>Yes (partial)</td>
</tr>
<tr>
<td>Identification of biomarker candidates</td>
<td>Yes (many)</td>
</tr>
<tr>
<td>Defining novel diagnostic and prognostic biomarkers (validated in a large cohort and ready for ‘routine’ clinical practice)</td>
<td>Not yet (but will probably be achieved soon)</td>
</tr>
<tr>
<td>Identification of new therapeutic targets</td>
<td>Yes (some)</td>
</tr>
<tr>
<td>Development of new drugs or therapies based on the therapeutic targets identified by proteomics approach</td>
<td>Not yet (but will probably be achieved soon)</td>
</tr>
<tr>
<td>Personalized medicine</td>
<td>Too far from now</td>
</tr>
</tbody>
</table>

**Table 1. Summary of the current status of renal and urinary proteomics**
Even with these achievements, it should be emphasized that many of the previous studies applying renal and urinary proteomics had some drawbacks, which are similar to those of other subdisciplines of clinical proteomics [30,31]. Ideally, a renal and urinary proteomics study should address the following issues of concern:

- Clear clinical and scientific questions.
- Appropriate study design.
- Sufficient sample size.
- Sufficient details of patients/subjects (number, gender, age, definitive diagnosis, disease classification/staging, related laboratory tests, treatment/medication, complications, comorbidity, etc.) and clinical samples (number, replication, time/phase of collection, storage, preservation, preparation, variability, etc.).
- Appropriate/sufficient controls; not only normal-controls but also disease controls (which have clinical and laboratory profiles similar to those of the disease of interest) should be included to define the ‘disease-specific’ biomarkers.
- Appropriate and high-quality methodology.
- Functional analysis to link proteomic results to renal physiology and pathophysiology.
- Appropriate statistics.
- Analysis of pooled samples should not be done without subsequent validation in individual samples.
- Validation in an independent (blinded) sample set.
- Confirmation or translation of the data obtained from cell lines and animal models to humans.
- Translation of the data obtained from proteomic platforms to the easier tests for ‘routine’ clinical use. There is no doubt that mass spectrometric analyses can be applied to identify potential biomarkers and to validate them. However, mass spectrometry-based analysis is considerably sophisticated for ‘routine’ clinical applications. Therefore, translation of mass spectrometric data to the much easier formats (e.g. diagnostic kits) would be definitely desirable.

To gear up for further progress in this field, we have to rethink all the analytical processes, from sample collection to clinical application (starting from the initial steps, which are sample collection and preparation). One of the major obstacles in renal and urinary proteomics is the lack of standards in this field at present, which limits robust comparability and reduces the significance of the data obtained previously. Marked variability has been observed in several aspects of methods and protocols used previously. Therefore, the standard protocols and guidelines for renal and urinary proteomics are urgently required. This task has been taken by an international network, namely ‘Human Kidney and Urine Proteome Project’ (HKUPP) (www.hkupp.org) [32], as its first mission. This network had a consensus to draft the HKUPP standard protocols and guidelines very soon. From our previous symposium and workshop, we have had a consensus to draft the HKUPP standard protocols and guidelines for urinary proteome analysis [33]. These standard protocols and guidelines will be generalized for all (or most) of techniques and balanced between perfection and practicality. Our initial standard protocols and guidelines will focus mainly on several important issues; e.g., study design, time of urine collection, addition of preservatives and protease inhibitors, removal of cells and debris, storage, removal of albumin and other high abundant proteins, normalization of the data, etc. We are planning to report the HKUPP standard protocols and guidelines very soon.

Another point-of-view on renal and urinary proteomics is that most of the previous studies applied expression proteomics, whereas only few performed functional analysis. The future of this field will move towards functional proteomics and protein bioinformatics will play a pivotal role in bridging the expression data to functional study (bioinformatics will be very useful to obtain additional information of the identified proteins to design further functional study). Also, proteomics is not a single perfect tool for renal research. It is just a piece of the jigsaw puzzle to fulfil the entire dynamic image of renal science and should be integrated with the other ‘omics’; e.g., genomics (genes), transcriptomics (transcripts), metabolomics (metabolites), lipomics (lipids), interactomics (interactions within or between genes, transcripts, proteins, etc.) to obtain as much information as possible. This is a concept of ‘integrative omics’ [34], in which many omics are linked together by bioinformatics. Finally, systems biology will be very useful for future renal research. It is not just integrative omics but also includes other aspects such as validation and modelling [35]. With this ideal approach, the ultimate goals of better therapeutic outcome and successful prevention of diseases, as well as personalized medicine, will be achieved. Of course, it will take time (more than a decade) before these ultimate goals can be reached.

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References


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