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URINARY PROTEOMICS IN DIAGNOSIS OF KIDNEY DISEASES

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Summary

Urinary proteomics is a technique to identify specific proteins and polypeptides in urine in different renal diseases. Much progress has been made in this technology during last 5 years. Until recently the principal proteins measured in urine are albumin, total protein, and immunoglobulin. Current available tests measure either total level of urine protein or single protein species. They are urine dipstick, 24 hr urinary total protein, urine protein- creatinine or albumin –creatinine ratio and immunoelectrophoresis. But urinary proteomics is the method of identifying other novel proteins in urine by depleting urine of albumin and immunoglobulin. It analyses multiple urinary proteins simultaneously. By comparing in healthy individual this technique can discover biological markers of disease as well as can identify pathogenesis. Henceforth this technique became disease diagnostic tool, provides prognostic marker and target sites for therapeutic decision. Urine is readily available, cheap bodily fluid and urinary proteomics is the phenotypic expression of genetic variation of disease. Urinary proteomics is non-invasive diagnostic and prognostic tool as well as it monitors therapeutic response. This urinary proteomics technology depends on mass spectrometry (MS). It has reached its validation phase by successful passing of development phase. It is safe, accessible, user friendly and serial sampling is possible. It may replace renal biopsy even in resource limited country like Bangladesh if technology support is available.

Key words

Proteomics; mass spectrometry; biomarkers

Introduction

Proteomics is the study of protein expression in a tissue or biological fluid. Urinary proteomics is urine analysis for various protein constituents. It is a novel, noninvasive diagnostic test which also provides therapeutic guidance and prognostic information.

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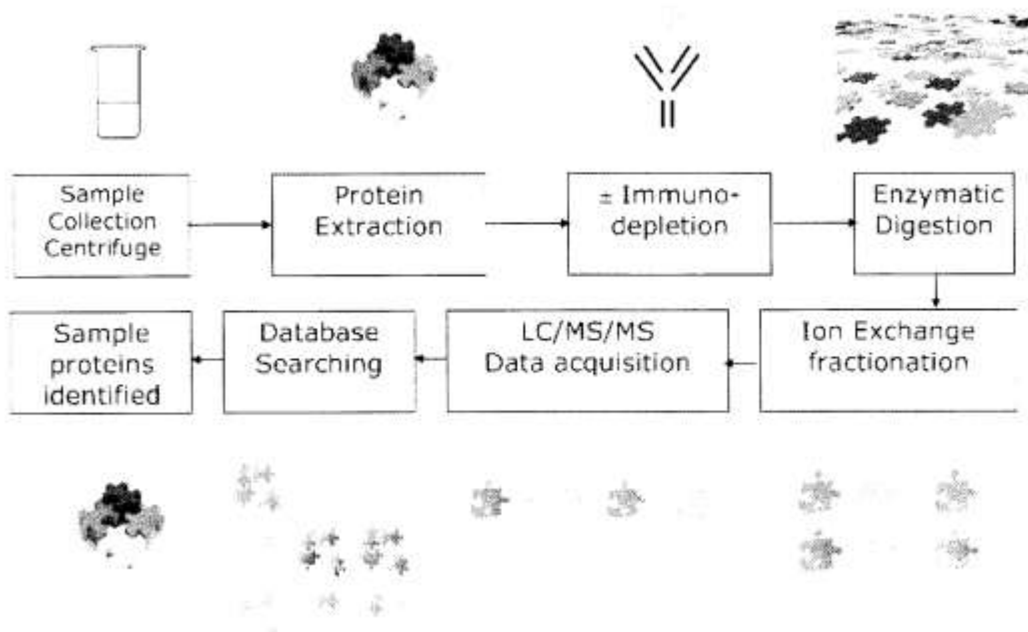
By comparing protein expression in urine of healthy individual with that of patient it discovers biological markers of disease (biomarkers) and suggests pathogenesis. The currently available tests for urine proteins measure either 24hr total urinary protein (or protein creatinine ratio) or the presence of a single protein species like albumin (glomerular disease), immunoglobulin (plasma cell dyscrasias), human chorionic gonadotropin (pregnancy), β_2 microglobulin (tubular disorder), myoglobin (rhabdomyolysis), Haemoglobin (Intravascular Haemolysis) and lysozyme (Myelomonocytic leukaemia). The emerging proteomic technologies allow simultaneous examination of the patterns of multiple urinary proteins[1]. Urinary total protein >150mg/d is taken as abnormal, and low level albuminuria or microalbuminuria [albumin (30-300mg/d)] is also used to detect early renal disease[2]. Normally glomerular filtration , tubular secretion, urinary sediment proteins (sloughed epithelial cells) and urine exosomes contributes 30%, 19%, 48%and 3% of total urine proteins respectively[1,3]. The filtered proteins (like albumin and β_2 microglobulin) and tubule-derived proteins (*N*-Acetyl-ß-D-glucosaminidase) are currently measured, but they offer no specificity in terms of the underlying renal insult, and their ability to predict early renal injury is uncertain. Their current clinical utility is therefore limited[4]. So far more than 1500 different proteins have been characterized in normal healthy individuals' urine[5]. Separation, differentiation and quantitation of constituent proteins can be achieved by proteomics. In this review the technology for analysis of urine proteomics and role of urinary proteomics in clinical settings will be discussed.

Review criteria

A literature search was performed in PubMed using the search term " Proteomics, urinary proteomics and biomarkers " . Reference sections of the identified articles were searched for additional relevant manuscripts. Human Kidney and Proteome Project (http://www.hkupp.org/) /World Human Proteome Organisation (http://www.hupo.org/) and European Kidney and Urinary Proteomics (http://www.eurokup.com) were also accessed.



Overview of current proteomic technique:
Figure 1 shows a general approach to urinary proteomic methods.

Fig 1. Overview of urinary proteomics: examples of simple workflow for protein identification. Proteins are extracted from urine, optionally immunodepleted of some abundant proteins including albumin and immunoglobulins, and enzymatically digested (usually with trypsin). The resulting peptides are fractionated by string ion exchange before liquid chromatography and tandem mass spectrometry (LC-MS/MS); precursor peptides and fragment ion masses are anlysed by complex search programmes to identify proteins from genomic databases [6].

Sample collection

To obviate diurnal variation urine specimens for proteomics is preferably collected after controlled bed rest[7]. First void urine tends to be more concentrated, contains all proteins found in 24-hrs-specimen, contains protein which is present only in morning specimen of female patient but may be contaminated by normal flora[8,9,10]. In some protocols random midstream sample is used as there is minimum variation at different times of day and least chance of contamination [11,12].

Sample storage

Urine is stored at -80 ^{0}C with prior centrifugation to reduce contamination by leaking proteins , cellular debris and bacteria. However protease inhibitor is not used as low amount of proteins which are identified by proteomics contain very little protease activity and protease inhibitor may interfere with mass spectrometry[13]. Besides in acute rejection urine pH becomes more acidic which allows protease to cleave beta-2 microglobulin so that it becomes abundant to be measured [14].

Sample preparation

Appropriate preparation of samples is strongly dependent on the proteomic techniques to be used and should be factored into the study design. Some of the more common approaches are discussed below.

1. Protein extraction and salt removal from urine samples: Isolating or concentrating urinary proteins may be essential in low concentration specimens, particularly for less sophisticated gel-based studies. Numerous methods have been compared including centrifugal filtration, lyophilization, Reverse phase extraction and ultra filtration but with varying results[15]. Different proteins appear to be lost with each of the preparative techniques and therefore a combination approach is most likely to give the complete proteome. These methods simultaneously remove sodium, potassium and urea from the sample, which otherwise could affect the efficiency of enzymatic proteolysis, for example trypsin activity [16].

2. Albumin removal

As in renal disease albumin and uromodulin are present in abundant amount and other isolating proteins are lowly abundant, albumin is to be removed [17]. But there is a chance of co-depletion of other proteins along with albumin as they are albumin bound, besides certain albumin are important biomarkers like repetitive fragmentation albumin in nephrotic syndrome and a specific fragment albumin in type-2 diabetic nephropathy before appearance of microalbuminuria [18,19,20]. The removal of albumin by immunoprecipitation, affinity capture or protein size fraction improves the identification of low abundance proteins[5,21].

3. Trypsin digestion

Mass spectrometry can detect peptides with positive charge. And trypsin is the endoprotease which cleaves proteins into peptides with a positively charged C-terminal arginine or lysine residue.

Protein/peptide separation

MS cannot analyze proteome comprehensively unless fractionation can be performed at the protein level or the peptide level [22]. Protein separation is commonly achieved using 1D-SDS–PAGE (1-Dimentional Sodium Dodecyl Sulphate — polyacrylamide gel electrophoresis), 2-DE (Dimensional electrophoresis), or capillary electrophoresis (CE); 1D-SDS–PAGE separates proteins according to molecular mass, CE by differences in isoelectric point and 2-DE by both molecular mass and isoelectric point. These methods separate many lower abundance proteins from higher abundance proteins . Peptide separation is typically performed using reverse phase-high performance liquid chromatography (RP-HPLC) and/or strong cation exchange (SCX), which separate peptides based on hydrophobicity and charge state, respectively. Both methods can be linked online to MS processing. Two-dimensional electrophoresis has been in use for several decades, requires isolation of protein spots and physical dissection before enzymatic digestion and entry into the MS for ionization. It is labour intensive and can analyse only 70–420 protein spots per gel, compared to 400–2000 polypeptides in a single run of CE–MS [23,24].

Mass spectrometry

Each mass spectrometer consists of an ion source, mass analyzers 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 /ionization (MALDI) and its variant surface enhanced laser desorption/ionization (SELDI), which employ an organic acid matrix to sublimate and ionize analytes with laser pulses, and electron-spray ionization (ESI) which involves spraying voltage charged analyte solutions to desolvate and ionize the analytes. Different mass analysers include ion trap (IT), time-of-flight (TOF), quadrupole (Q) and Fourier transform ion cyclotron resonance (FT-ICR) devices. Sometimes mass analyzers are linked in series , it is called MS/MS or tandem mass spectrometry (e.g ,triple quadruple or QQQ, quadruple combined with time of flight or Q-TOF and dual time of flight or TOF/TOF) . Charged peptides are separated in the first MS. Selected peptide ion is then directed into a collision cell where it is fragmented by sequential removal of individual amino acids. Fragments are separated in second MS. The different mass analyser configurations can be alternately combined with the ionization sources although most commonly MALDI is coupled with TOF (MALDI-TOF and MALDI-TOF/TOF) and ESI with Q (ESI-QQQ and ESI-QTOF). More recent instruments include linear ion trap instruments which have expanded capability over traditional QQQ analysers, including MS/ MS/MS functionality and superior sensitivity. Additional coupling of the linear ion trap to the recent Orbitrap mass analyser further enables the simultaneous acquisition of selected ions with extremely high resolution and mass accuracy. The different MS platforms employed in proteomics have been comprehensively reviewed by others[23,24].

Urinary proteomics in clinical settings

Urinary proteomics are used for diagnosis, to understand Pathophysiology, to detect prognosis, to monitor treatment response. It is also used to diagnose some non-renal disease.

Diagnosis

Urinary Proteomic is a suitable non-invasive alternative to renal biopsy (Table I).

Table I. Advantages and disadvantages of urinary proteomics

Renal Biopsy	Urinary proteomics
Invasive but cheap	Non-invasive but expensive
Resampling difficult and inadequate sampling may happen	Resampling same day possible. Only 10 ml urine is required
Hypertension and coagulation disorder are contraindications	Immediately available, only in anuria it is impossible
Cannot guide treatment or predict prognosis	Response to treatment and prognostic information are available
Current gold standard	Sensitivity and specificity improving

The urinary proteomes of many glomerular and interstitial diseases have already been described. Lafitte et al. first compared 2-DE analysis of urine proteome of healthy controls with that of incipient diabetic nephropathy, minimal change disease, myelomatous kidney and proximal tubular acidosis[8]. later on 92.9% of patients with membranous nephropathy and 71.4% of patients with minimal change or FSGS were correctly classified by CE-MS analysis [25]. Further candidate biomarkers for lupus nephritis, IgA nephropathy were identified in the urine with near about 100% sensitivity and 90% specificity using by various techniques like SELDI ,CE-MS, MALDI-TOF-MS [26,27,28,29].

Lupus nephritis

Some techniques like SELDI-TOF may identify disease activity with both a sensitivity and specificity of 92% according to the International Society of Nephrology/Renal Pathology Society (ISN/RPS) class of lupus nephritis [30] . Using SELDI-TOF-MS sometimes even early stage of recovery or relapse or progression of one class to another class of lupus can be identified [31].

Allograft rejection

Renal biopsy is required to detect acute rejection and chronic allograft nephropathy. Spectra and virtual gels generated by surface-enhanced laser desorption and ionization (SLEDI), followed by time-of-flight mass spectrometry(TOF-MS) of urine samples from stable allograft and patient with acute rejection discriminated Mass-to-charge values for selected peptides and proteins [32].The proteins have subsequently been identified as cleaved forms of b-2 microglobulin [14] Subsequent identification of peaks detected a reduction in beta-defensin-1 and an increase in alpha-1 antichymotrypsin in patients with acute rejection [33] . MS in this case was found to have a sensitivity of 90.5–91.3% and a specificity of 77.2–83.3% in >90% of cases [34] .Urinary proteomics have also been able to discriminate between urinary tract infection and acute allograft rejection and between tubular and vascular rejection[35].

Quintana et al. have examined the urinary proteome of 39 patients with chronic allograft nephropathy (CAN) and 32 controls . Specific peptides derived from uromodulin and kininogen were more abundant in controls than patients and differential expression of two ions diagnosed CAN in virtually all cases [36] They propose that these biomarkers could form the basis of a biopsy-free urine test for the early diagnosis of CAN and will facilitate a more rapid introduction of targeted and personalized immunosuppressive regimes to improve long-term graft outcome.

Predictors of disease/prognosis

Acute kidney injury (AKI)

The current method of diagnosis of AKI is to monitor serum creatinine, but serum creatinine is raised when 50% of renal function is lost and also it varies in relation to sex, age, muscle mass and metabolism, drugs and hydration. So it may delay treatment. The goal for urinary proteomics is to define a panel of tests, which will allow early identification of patients at risk, in order to institute rapid and aggressive treatment. Nguyen et al. identified biomarkers with m/z of 6.4, 28.5, 43 and 66 kDa, using SELDI-TOF MS. A combination of these three markers predicted the development of AKI at 2 h in 100% of patients, despite serum creatinine not rising for 2–3 days after the procedure[37]. Using a rat model of sepsis-induced AKI and 2-DE/MALDI-TOF analysis, Molls et al. identified urinary peptides that were upregulated in AKI including albumin, aminopeptidase and neutrophil gelatinase associated lipocalin (NGAL) when compared with animals, which did not develop AKI[38]. Other studies utilizing microarrays and ELISAs have identified higher concentrations of Kidney injury molecule 1(KIM-1), IL-18, cystatin C, 1-microglobulin, in the urine of patients with incipient AKI [36,39,40].

Chronic kidney disease

The NGAL and livertype fatty acid-binding protein (L-FABP) are significantly higher in the urine of CKD patients with progressive disease than those with stable disease[6].

Diabetic nephropathy

Proteomic analysis of the urine of 112 patients with type 2 diabetes and healthy controls has identified the presence of the characteristic polypeptides (insulin-like peptide 3, uromodulinand an albumin fragment)[6]. This may indicate a population at risk of incipient nephropathy who require early intervention to prevent disease progression. MALDI-TOF analysis identified proteins including zinc alpha-2 glycoprotein, alpha-1 acid glycoprotein,alpha-1 microglobulin and IgG, and these could also be used as markers for the early detection of diabetic nephropathy [41]. Furthermore, Rossing et al. have recently described a panel of 40 biomarkers which identified patients with diabetes from healthy individuals in a large cohort with 89% sensitivity and 91% specificity. They also describe a profile, which accurately diagnosed nephropathy in patients with diabetes with 97% sensitivity and specificity and identified those with microalbuminuria who progressed to overt nephropathy over a 3 year period[42].

Prediction of response to treatment and disease monitoring

Proteomic analysis (SELDI-TOF-MS) identified ß2 microglobulin as having diagnostic accuracy in 95% of children with steroid-resistant disease, while reduced level of kininogen in urine in IgA nephropathy is related with nonresponsive to angiotensin-converting enzyme inhibitors (ACEi) or angiotensin II receptor blockers [6] The use of proteomics in this capacity would guide the early initiation of individualized treatment, which is not possible from information gained from the routine tissue biopsy.

Understanding renal Pathophysiology

Two hundred and ninety-five proteins in the exosomes in normal urine have been identified including many disease associated proteins; aquaporin-2, polycystin-1, podocyin, angiotensin-converting enzyme, thiazide-sensitive Na–Cl cotransporter, epithelial sodium channel[6,43]. Although many of the proteins are high abundanceplasma proteins and appearance in the urine is simply due to disruption of the glomerular basementmembrane, but certain fragments are characteristic of particular diseases e.g. albumin fragments specific for IgA nephropathy, diabetic nephropathy, minimal change disease, FSGS, membranous glomerulonephritis and autosomal dominant polycystic kidney disease reflecting tubular processing of these proteins. The lack of collagen fragments in diabetic nephropathy has led to the speculation that reduced protease activity per se contributes to disease pathology, resulting in excess collagen and extracellular matrix deposition. Indeed the abundance of certain specific collagen fragments has been shown to correlate with matrix metalloprotease activity[44].

Other applications for urinary proteomics in non-renal disease

markers for malignancies of the urinary tract (as Markers for prostate,bladder and renal cell tumours) have been identified in urine [45]. Other applications for urinary proteomics include diagnosis of interstital and bacterial cystitis, renal calculi, ureteropelvic junction obstruction,ovarian and lung cancer, graft-versus-host disease and coronary artery disease[6].

Conclusion

Urinary proteomics is the novel, noninvasive test for the diagno sis and monitoring of both renal and systemic diseases. But its high cost limits its generalized use.

To date, most biomarker studies have been conducted on small patient cohorts demanding its validation. Different MS techniques identifies different proteins and peptides in the same disease. It is due to confounding variables like age, sex, diet and immunosuppressive used as treatment. No well defined gold standard to compare each biomarker is available. Neither any single urine biomarker will have sufficient sensitivity and specificity for clinical use. So in every disease a biomarker panel using multiple biomarkers needs to be prepared . However Innovative urine proteomic studies are identifying increasing numbers of novel urine proteins that may prove useful for the diagnosis and monitoring of renal and systemic diseases in recent future which will obviate the need for renal biopsy

Disclosure

All the authors declared no competing interestes.

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