iTRAQ-based comparative proteomics analysis reveals specific urinary biomarkers for various kidney diseases

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Background: Proteome studies for multiple renal diseases is bare. **Methodology & results:** Using isobaric tags for relative and absolute quantitation labeling, many differentially expressed proteins (DEPs) were identified in acute kidney injury (AKI), AKI + chronic kidney disease (CKD), diabetic CKD and nondiabetic CKD with or without IgA nephropathy (IgAN). Comparative analysis indicated that 34, 35, 17, 91 and 14 unique DEPs were found in AKI, AKI + CKD, CKD, diabetic CKD and nondiabetic CKD. Compared with nondiabetic CKD with IgAN, 47 unique DEPs were found in that without IgAN. Serum amyloid A1 (SAA1) and hepatocyte growth factor activator were unregulated in AKI and nondiabetic CKD without IgAN, respectively. Regenerating islet-derived protein 3- α (Reg3A) upregulation is associated with AKI and AKI + CKD patients. **Conclusion:** This research contributes to urinary biomarker discovery from multiple renal diseases.

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Kidney diseases, including acute kidney injury (AKI) and chronic kidney disease (CKD), often result in renal failure, especially among hospitalized patients. Causes of kidney disease are numerous and complex, including deposition of IgA antibodies in the glomerulus, termed IgA nephropathy (IgAN) [1], diabetes mellitus, which is termed as diabetic nephropathy (DN) [2], and high blood pressure-initiated hypertensive nephropathy [3]. To date, approximately 400 different rare renal diseases have been reported. Therefore, it is very important to identify reliable biomarkers that facilitate early diagnosis of kidney diseases and help distinguish one type of disease from another.

AKI involves an abrupt loss of kidney function that develops within 7 days and approximately 5–10% of AKI patients can progress into CKD and even end-stage kidney disease, depending on the conditioned stimulus [4,5]. The primary causes of AKI include ischemia, sepsis and nephrotoxicity [6]. Clinically, AKI is often diagnosed based on the elevation of blood urea nitrogen and creatinine or the amount of urine per 24-h period. Urinary proteomics approaches have been widely used to differentiate disease types and stages and to assist the discovery of diagnostic biomarkers, especially for kidney disease. Recently, numerous novel AKI biomarkers, such as neutrophil gelatinase-associated lipocalin [7], kidney injury molecule 1 (KIM-1) [8] IL-18 [9], L-FABP [10] and activin A [11], have been identified in urine and found to contribute to early detection, differential diagnosis and prognosis for AKI. Additionally, CKD, as a common type of renal disease, is characterized by a gradual loss of kidney function over a period of months or years and mainly includes DN, nondiabetic nephropathy and other types of nephropathy. AKI is closely linked with the increased risk of developing CKD, which presents a dependence on the severity of AKI [12]. In comparison with the CKD patients who do not experience superimposed AKI, CKD patients who sustain severe AKI have 30% higher long-term risk of death or end-stage kidney disease, implying that the AKI–CKD transition is a strong independent risk factor for CKD development [13]. Reduction in estimated glomerular filtration rate is

regarded as the best predictor of CKD, but this is only effective during the latter stages of renal disease [14]. Cystatin C and creatinine are the common protein biomarkers for CKD and cystatin C may improve estimated glomerular filtration rate in the progression of CKD [15]. Evidence suggests that urinary 8-hydroxyl-2'-deoxyguanosine (8-OHdG) [16], transferrin [17], type IV collagen [18], neutrophil gelatinase-associated lipocalin [19] and MCP-1 [20] can be used as biomarkers to predict patients with DN. However, challenges for the early diagnosis for AKI or progressive CKD derived from AKI are still to be addressed.

IgAN, characterized by deposition of IgA antibodies in the glomerulus, is the most common form of glomerulonephritis throughout the world [21]. Based on nano-liquid chromatography–tandem mass spectrometry (LC–MS/MS), 2D-electrophoresis matrix-assisted laser desorption ionisation-time-of-flight/time-of-flight (2DE-MALDI-TOF-TOF) mass spectrometry (MS) and ELISA analytical techniques, numerous studies have identified many noninvasive diagnostic markers in urinary excretions from relevant proteins in IgAN, including glycoprotein 2 (GP2), protocadherin [22], albumin fragments [23] and others. Although a growing number of urine protein markers for kidney disease have been identified using advanced biotechnological methods, only a few have been validated and used for clinical purposes. Additionally, case-crossover studies among multiple renal diseases can accurately distinguish specific urinary protein markers between different kidney diseases.

Herein, based on the isobaric tag for relative and absolute quantitation (iTRAQ) labeling and LC-MS/MS, we identified differentially expressed proteins (DEPs) in urine from patients with AKI, AKI + CKD, diabetic CKD and nondiabetic CKD with or without IgAN. We subsequently performed gene ontology (GO), GO term and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analyses using the identified DEPs. In summary, we identified several novel urinary protein biomarkers that could distinguish and assist the classification of multiple kidney diseases.

Materials & methods

Subjects

A total of 300 urine samples were obtained for urinary proteomic profiling from healthy volunteers ($n = 50$) and patients with early AKI caused by acute ischemia-reperfusion injury ($n = 50$), AKI–CKD transition (AKI with chronic kidney disease, AKI + CKD; $n = 50$), diabetic CKD ($n = 50$), nondiabetic CKD with IgAN ($n = 50$) and nondiabetic CKD without IgAN ($n = 50$). Inclusion criteria for early AKI (stage I) was an increase in blood creatinine by $26.2 \mu\text{mol/l}$ or 1.5–1.9-fold in 48 h and when urine volume was less than 0.5 ml/kg/h for more than 6 h (less than 12 h). Inclusion criteria for early CKD was based on the glomerular filtration rate (GFR) which ranged from 45 to $59 \text{ ml/min/1.73 m}^2$. AKI + CKD patients were judged by clinical examination index as described above. Diabetic CKD, nondiabetic CKD with IgAN ($n = 50$) and nondiabetic CKD without IgAN patients were categorized by diabetic symptoms or IgAN from CKD patients. This study was approved by the Human Medical Ethics Committee of Zhejiang Provincial People's Hospital.

Urine collection, urinary protein extraction & digestion

In brief, void mid-stream morning urine samples (50 ml for kidney disease samples and 150 ml for the control group) were collected in sterile containers and divided into 10 ml/tubes; 1 ml protease inhibitor was added and samples were centrifuged at $2000 \times g$ for 20 min at 4°C . Supernatants from the same illness were removed, combined and stored at -80°C for the subsequent experiments. Urinary proteins were isolated using the trichloroacetic acid-acetone precipitation method and the concentration of total urinary protein was measured using a bicinchoninic acid protein assay kit (23225, Thermo Fisher Scientific, MA, USA). Urinary proteins were quantified and the quality was assessed using Bradford assays and SDS-PAGE. Based on the quantitation results, $100 \mu\text{g}$ urinary protein was diluted tenfold using 0.5 M tetraethylammonium bromide (TEAB) containing 0.1% SDS and digested with trypsin (50:1 protein/trypsin w/w; Promega, WI, USA) at 37°C for 3 h. Peptides were cleaned and redissolved in TEAB/water (1:1 v/v).

iTRAQ labeling

Peptide concentrations were measured using a bicinchoninic acid kit and sample volumes were adjusted to 15 μl containing 25 μg peptides using 0.5 M TEAB. Peptides were labelled using 8-plex iTRAQ reagent according to the manufacturer's protocol (4381663, Sigma, Darmstadt, Germany). Briefly, peptides were labeled with iTRAQ reagents for 1 h at room temperature and iTRAQ-113, 114, 115, 116, 117 and 118 were used to label peptide

Figure 1. Workflow diagram of the urinary proteomics approach. Approach employing isobaric tags for relative and absolute quantitation labeling combined with 2D LC-MS/MS analysis of samples from patients with acute kidney injury, acute kidney injury with chronic kidney disease, diabetic chronic kidney disease, nondiabetic chronic kidney disease with IgA nephropathy and nondiabetic chronic kidney disease without IgA nephropathy.

samples from patients with AKI, AKI + CKD, diabetic CKD, nondiabetic CKD with IgAN and nondiabetic CKD without IgAN, respectively. Labeled samples were mixed as a pool and dried in a vacuum concentrator.

Mass spectrometric analysis

Mixed peptides were fractionated by strong cation exchange (SCX) chromatography on a 20AD HPLC system using a polysulphoethyl column (2.1 × 100 mm, 5 mm, 200 Å; The Nest Group, MA, USA). Peptide samples were diluted tenfold with buffer A, consisting of 10 mmol/l KH_2PO_4 in 25% acetonitrile (CAN) at pH 3.0, eluted and loaded onto the column for separation with a gradient of 0–80% SCX buffer B (10 mmol/l KH_2PO_4 at pH 3.0 in 25% ACN containing 2 mol/l KCl) in SCX buffer A at a flow rate of 200 $\mu\text{l}/\text{min}$ for 60 min. After monitoring the absorbance at a wavelength of 214 and 280 nm, 20 SCX fractions were collected per min, pooled and dried in a vacuum concentrator. SCX fractions were dissolved in buffer A, consisting of 5% CAN and 0.1% formic acid (FA) and further separated using a QSTAR XL system (Applied Biosystems, Thermo Fisher Scientific) combined with a 20AD HPLC system (Shimadzu, Kyoto, Japan). Peptides were separated using a column (Zorbax 300SB-C18) at a flow rate of 0.3 ml/min for 70 min using a gradient of 5–35% buffer B (95% acetonitrile, 0.1% FA) in buffer A (5% acetonitrile, 0.1% FA). Survey scans such as MS/MS spectra were recorded at a resolution of 100 K with ranges of m/z 400–1800 and MS scans were obtained with m/z 100–2000 for MS/MS analysis. All experiments were repeated twice according to the workflow diagram shown in Figure 1 (differential abundance of specific proteins in various types of kidney disease).

Data analysis

MS data were searched against the UniProt human database using Proteome Discoverer software version 1.4 (Thermo Fisher Scientific). All peptides were validated with a false-discovery rate (FDR) <0.01%. Parameters for qualitative and quantitative analysis of urinary peptide samples were set according to general preferences. Trypsin was used with one missed cleavage allowed and carbamidomethyl was employed as a fixed modification. The tolerance of peptides and fragments were set to ± 20 ppm and 0.1 Da, respectively. The integration window tolerance was set as 20 ppm according to the most confident centroid method. Other parameters such as scan event

filters were set as follows: mass analyzer = Fourier transform ion cyclotron resonance mass analyzer (FTMS), MS order = MS2, activation type = higher-energy collisional dissociation (HCD). Protein ratios were calculated as the median of only unique peptides for each protein and normalized by the median protein ratio. A fold-change of >1.2 was applied as a cut-off for identifying DEPs from different kidney diseases.

Gene ontology enrichment & pathway analysis

Based on database searches, DEPs (>1.2 -fold-change and $p < 0.05$) were assigned biological functions using BLAST2GO annotation and annotated proteins were categorized based on biological process, cellular component and molecular function categories. Fisher's exact test was performed to evaluate enrichment and Benjamini–Hochberg adjustment was performed to assess multiple data. The KEGG database was employed for annotating pathways in combination with KOBAS software (version 2.0, KOBAS, London, UK). Identification of DEPs related to proliferation, survival, apoptosis, repair, inflammation and migration was based on published literature.

ELISA assay

Concentrations of urinary proteins serum amyloid A1 (SAA1), complement component 5 (C5), hepatocyte growth factor activator (HGFAC), since apolipoprotein C-I (APOC1) and regenerating islet-derived protein 3- α (Reg3A) were measured in samples from each of the five types of kidney disease ($n = 30$ per disease) according to the manufacturer's instructions. ELISA kits for SAA1 (DY3019-05), HGFAC (DY5940-05) and Reg3A (DY1514) were purchased from R&D (MN, US). APOC1 and C5 ELISA kits were obtained from (Abcam, Cambridge, UK) (ab108808) and (BD, NJ, USA) (557965), respectively.

Statistical analysis

All data are presented as mean $\pm$ standard error of the mean and were analyzed using SPSS (IL, USA) and GraphPad Prism software (CA, US). Statistical comparisons among groups were performed using one-way analysis of variance or Student's *t*-test and $p < 0.05$ was considered statistically significant.

Results

Identification of DEPs in urine from kidney disease patients

In order to identify DEPs in urine from patients with multiple kidney diseases, the urinary proteomes of patients with AKI, AKI + CKD, diabetic CKD, nondiabetic CKD with IgAN, nondiabetic CKD without IgAN and healthy volunteers were labeled using iTRAQ reagents (Figure 2, cluster and volcano plot analyses of urinary DEPs identified in the five renal diseases). Cluster analysis of AKI and controls revealed that apolipoprotein L1 (APOL1), protein S and rheumatoid factor were increased threefold, while histone H4, desmoyokin (neuroblast differentiation-associated protein AHNAK) and calcitonin were upregulated by more than fourfold (Figure 2A & Supplementary Table 1, first image). Among urinary DEPs in AKI+CKD patients, nicotinamide adenine dinucleotide reduced (NADH) dehydrogenase (ubiquinone) 1- α subcomplex subunit 5 and follistatin-related protein 3 were increased threefold, while pancreatic alpha-amylase, polymeric immunoglobulin receptor and Golgi integral membrane protein 4 were diminished by more than fourfold (Supplementary Figure 1A & Supplementary Table 2, second image). In diabetic CKD, urinary proteins α -adducin and chromogranin-A were markedly upregulated, as were ephrin type-A receptor 4 and profilin-2 in nondiabetic CKD with IgAN patients, as well as α -1 globin and apolipoprotein C-I in nondiabetic CKD without IgAN samples. Meanwhile, tubulin-specific chaperone A and pro-epidermal growth factor were significantly downregulated in diabetic CKD patients, as were secreted and transmembrane protein 1 and tumor-associated calcium signal transducer 2 in nondiabetic CKD with IgAN patients, as well as histone H2B type 1-K and Nectin-4 in nondiabetic CKD without IgAN samples (Supplementary Figures 2A, 3A & S4A & Supplementary Tables 3–5, images 3–5). Based on the analysis of DEPs using volcano plots, 31, 22, 73, 29 and 45 DEPs were significantly increased by over 20% in the five kidney disease groups, while 125, 134, 213, 158 and 139 DEPs were downregulated by over 20% (Figure 2B, Supplementary Figure 1B, 2B & 4B).

GO annotation of targeted DEPs

Urinary DEPs identified in different renal diseases were subjected to BLAST2GO annotation. Urinary proteins were classified based on biological process (BP), cellular component (CC) and molecular function (MF) categories according to GO assignment. In the BP category, the majority of DEPs in the five kidney disease groups were mainly implicated in cellular process, stimulus subcategories, single-organismal process, biological regulation, metabolic

Figure 2. Cluster and volcano plot analyses of urinary differentially expressed proteins identified in acute kidney injury diseases. **(A)** Cluster analysis of filtered urinary DEPs in AKI diseases compared with healthy volunteers. **(B)** Volcano plot of all urinary DEPs in AKI and in healthy controls.
AKI: Acute kidney injury; DEP: Differentially expressed protein.

process and multicellular organismal processing. At the CC level, the identified DEPs were mainly associated with cell, organelle, membrane and extracellular region subcategories for all five renal diseases. MF analysis indicated that binding, catalytic activity and receptor activity were the primary functions of DEPs in these renal diseases (Figure 3A–E, GO annotation of urinary DEPs identified among various renal diseases). Unlike urinary DEPs in other diseases, electron carrier activity was an exclusive MF category in AKI + CKD patients (Figure 3B). In comparison with the AKI group, DEPs mediating MF did not include chemoattractant activity in AKI + CKD, nondiabetic CKD with IgAN, or nondiabetic CKD without IgAN patients (Figure 3A, B, D & E). Additionally, there were unique MF subcategories in diabetic CKD patients, including protein binding and nucleic acid-binding transcription factor activities (Figure 3C).

Figure 3. Gene ontology annotation of urinary differentially expressed proteins identified among the five renal diseases. (A–E) GO annotation of DEPs in AKI, AKI + CKD, diabetic CKD, diabetic CKD with IgAN and diabetic CKD without IgAN compared with healthy controls.

AKI: Acute kidney injury; AKI + CKD: Acute kidney injury with chronic kidney disease; CKD: Chronic kidney disease; DEP: Differentially expressed protein; GO: Gene ontology; IgAN: IgA nephropathy.

GO term enrichment analysis of DEPs in different renal diseases

GO term enrichment analysis revealed that all DEPs were linked to a variety of biological functions. The top ten most significant biological functions in each group were shown in Figure 4 (GO enrichment analysis of DEPs identified in different kidney diseases). As indicated in Figure 4A, single-organism process, protein binding, multicellular organismal process and developmental process were the main biological functions related to urinary DEPs in AKI patients (Figure 4A). Meanwhile, DEPs in AKI + CKD played an important role in developmental processes, system development and biological adhesion (Figure 4B). Among DEPs mediating biological functions, regulation of response to external stimuli, enzyme inhibitor activity and endopeptidase regulator/inhibitor activity were the most obvious in diabetic CKD patients compared with the control group (Figure 4C). Multicellular organismal process, developmental process and tissue development were predominant in nondiabetic CKD with IgAN, as well as single-multicellular organism process, multicellular organismal development and metal ion binding were the main biological processes in nondiabetic CKD without IgAN patients (Figure 4D & E). These results suggest that the biological functions regulated by DEPs in these renal diseases are diverse and complex.

KEGG pathway analysis of identified DEPs in different kidney diseases

Further analysis of biological pathways using the KEGG database revealed 6, 13, 12, 10 and 14 signalling pathways that were enriched among the five kidney diseases (Figure 5, multiple signalling pathways involved in different kidney diseases). KEGG pathways in the AKI group included complement and coagulation cascades, microRNAs in cancer, transcriptional mis-regulation in cancer, cysteine and methionine metabolism, starch and sucrose metabolism and apoptosis-multiple species (Figure 5A). As shown in Figure 5B, PI3K-Akt signalling, extracellular matrix (ECM)-receptor interaction and proteoglycans in cancer were the primary signalling networks mediated by DEPs in AKI + CKD patients, implying that urinary DEPs in AKI and AKI + CKD participated in different pathways, although starch and sucrose metabolism were common in both groups (Figure 5B vs A). In the diabetic CKD group, complement and coagulation cascades, PI3K-Akt signalling, lysosome and proteoglycans in cancer were the main DEPs-mediated networks, indicating similarities in signal transduction and cross-talk among diabetic CKD, AKI and AKI + CKD patients (Figure 5C). Meanwhile, tuberculosis, proteoglycans in cancer, Rap1 signalling and starch and sucrose metabolism pathways were significantly enriched in the nondiabetic CKD with IgAN group, while tuberculosis, alcoholism, *Staphylococcus aureus* infection and transcriptional misregulation in cancer were observed in nondiabetic CKD without IgAN patients (Figure 5D & E). Thus, bioinformatics analysis of KEGG pathways revealed commonalities as well as process-specific differences among these renal diseases. Importantly, these results imply that some unique signalling pathway can be targeted for the development of therapies for different renal diseases.

Biomarker discovery & verification of unique DEPs in different renal diseases

Next, we performed comparative analysis of all DEPs to detect specific protein markers in urine from each of the five diseases (Figure 6, identification and validation of DEPs specific to different renal diseases). Combined with the results of Venn diagrams, we identified 34 and 35 DEPs that were specifically expressed in AKI and AKI + CKD patients. Upon comparison of AKI and AKI + CKD groups, 17 specific DEPs were identified in diabetic CKD and nondiabetic CKD groups, including nondiabetic CKD with or without IgAN patients. In the CKD group, 91 unique and abnormally expressed proteins were identified, while 14 specific protein markers were identified in nondiabetic CKD patients. By comparison, we identified 38 and 47 unique urinary protein biomarkers in nondiabetic CKD with IgAN and nondiabetic CKD without IgAN groups, respectively (Figure 6A & Supplementary Table 6). Further biological function analysis indicated that 22 specific DEPs in AKI were closely related to proliferation, apoptosis, survival, inflammation, repair and migration. For example, SAA1 was associated with proliferation, inflammation and migration, while SHBG was involved in progression of proliferation, apoptosis and survival (Table 1). Using this method, 12 unique DEPs including IGHG1 and PAPP2 were identified in the AKI + CKD group (Table 2). In contrast to AKI and AKI+CKD groups, 12 urinary DEPs from CKD patients participated in the biological processes mentioned above (Supplementary Table 7). A total of 44 and six specific urinary protein markers were identified in diabetic CKD and nondiabetic CKD patients, respectively (Supplementary Table 8 & 9). Among nondiabetic CKD patients, we identified 17 specific DEPs in patients with nondiabetic CKD with IgAN and 27 in those with nondiabetic CKD without IgAN (Supplementary Table 10 & 11).

Figure 4. Gene ontology enrichment analysis of differentially expressed proteins identified in different kidney diseases. (A–E) GO classifications of urinary DEPs in AKI, AKI + CKD, diabetic CKD, diabetic CKD with IgAN and diabetic CKD without IgAN (top ten). AKI: Acute kidney injury; AKI + CKD: Acute kidney injury with chronic kidney disease; CKD: Chronic kidney disease; DEP: Differentially expressed protein; GO: Gene ontology; IgAN: IgA nephropathy.

Figure 5. Multiple signalling pathways involved in different kidney diseases. (A–E) The main signalling pathways related to urinary DEPs identified in patients with AKI, AKI + CKD, diabetic CKD, diabetic CKD with IgAN and diabetic CKD without IgAN. AKI: Acute kidney injury; AKI + CKD: Acute kidney injury with chronic kidney disease; CKD: Chronic kidney disease; DEP: Differentially expressed protein; IgAN: IgA nephropathy.

Figure 6. Identification and validation of differentially expressed proteins specific to different renal diseases. (A) Common and unique DEPs in the five kidney diseases displayed as a Venn diagram. **(B–F)** Verification of the specific DEPs SAA1, complement C5, HGFAC, since APOC1 and Reg3A using ELISA.

* $p < 0.05$; ** $p < 0.01$.

APOC1: Apolipoprotein C-I; C5: Component 5; DEP: Differentially expressed protein; HGFAC: Hepatocyte growth factor activator; Reg3A: Regenerating islet-derived protein 3- α ; SAA1: Serum amyloid A1.

Eventually, we chose five urinary protein markers SAA1, C5, HGFAC, APOC1 and Reg3A for validation to support our present work. In ELISA assays, an increase in SAA1 was only observed in urine from AKI patients, suggesting that SAA1 might be used as an indicator of AKI disease (Figure 6B). C5 upregulation was observed in all five kidney diseases, implying it might be a universal protein marker for all five diseases (Figure 6C). In samples from patients with nondiabetic CKD without IgAN, HGFAC level was significantly enhanced compared with other groups, which indicates that HGFAC was a potential biomarker for early diagnosis of nondiabetic CKD without IgAN (Figure 6D). Based on the results shown in Figure 6E, we speculated that detection of APOC1, which was upregulated in all renal diseases except for diabetic CKD, possibly could be used to distinguish diabetic CKD from other kidney diseases (Figure 6E). Enhancement in Reg3A abundance was observed in both AKI and AKI+CKD groups, which suggested that Reg3A differed significantly between AKI and CKD patients (Figure 6F). Importantly, these results were consistent with our MS data.

Table 1. Proliferation, apoptosis, survival, inflammation, repair and migration-associated urinary biomarkers in acute kidney injury.

Accession	Protein name	Proliferation	Apoptosis	Survival	Inflammation	Repair	Migration
Q14520	HABP2		Related		Related		
D3DQX7	SAA1	Related			Related		Related
A0A087WY13	FOLR3			Related			
P15169	CPN1		Related		Related		
Q7Z4G9	HBXBP	Related	Related	Related	Related	Related	Related
I3L145	SHBG	Related	Related	Related			
F5H3C5	SOD2	Related	Related	Related			
U5LGU2	APOL1	Related	Related	Related			
A2NB44	IGH@	Related					
P02774	GC	Related	Related	Related			
K7ENW7	DNMT1	Related	Related	Related			
A8K2Q6	PIN1	Related	Related	Related			
P22105	TNXB	Related	Related	Related			
Q66K79	CPZ	Related	Related				
D6W633	PTPRS	Related	Related	Related	Related		Related
E7EQB2	LTF	Related	Related		Related		
Q9NQ38	SPINK5	Related	Related				
Q14982	OPCML	Related	Related	Related			Related
Q4W4Y1	PDCD6IP	Related	Related				
I3L1M3	UMOD	Related	Related		Related		
Q5SZK8	FREM2		Related				
O96009	NAPSA	Related	Related				

Table 2. Proliferation, apoptosis, survival, inflammation, repair and migration-associated urinary biomarkers in acute kidney injury and chronic kidney disease.

Accession	Protein name	Proliferation	Apoptosis	Survival	Inflammation	Repair	Migration
A0A087WYC5	IGHG1	Related		Related			Related
Q9BXP8	PAPPA2	Related					Related
L8E853	vWF	Related					Related
Q06141	REG3A	Related				Related	
O95633	FSTL3				Related		
B4E2U0	6-phosphogluconate dehydrogenase	Related					
P30041	PRDX6		Related				
A0A052Z3Q0	HYAL1		Related				
P60953	CDC42	Related					
Q12860	CNTN1	Related					Related
K7EQ48	GPI	Related	Related		Related		
G3V5V4	COCH			Related			

Discussion

The urinary proteome consists of more than 1500 proteins that accurately reflect renal physiology and pathophysiology, as well as kidney dysfunction [24,25]. Given that urine collection is noninvasive and facile, urinary proteomics is growing in popularity for kidney disease diagnostics [26]. Currently, MS-based proteomic analysis is the dominant tool for urinary protein biomarker discovery in kidney disease [27]. However, at present there are no completely reliable indicators to specifically discriminate between diverse types of renal disease, due to the complexity of urine and renal pathology. In our present study, we discovered specific DEPs in patients with AKI, AKI + CKD, diabetic

CKD and nondiabetic CKD with or without IgAN (relative to healthy controls). Our results provide new evidence for biomarker discovery and may improve kidney disease diagnosis in clinical management.

iTRAQ labelling combined with 2D LC-MS/MS is widely considered a powerful method for high-throughput protein identification and relative quantitation in various diseases. 408 N-linked glycoproteins showing significant variation and α -1-antitrypsin (SERPINA1) and ceruloplasmin identified by iTRAQ-based urinary glycoproteome analysis in DN patients may serve as indicators to distinguish microalbuminuria and normalalbuminuria, which facilitate the individualized care of patients with DN [28]. In comparison with healthy volunteers, 30 proteins including α -1-antitrypsin, apolipoprotein A-I and CD44 antigen are significantly up- or downregulated in IgAN patients [29]. However, whether there are common or specific urinary DEPs among the three disease types remains unknown. In the present research, among the DEPs in patients with AKI, AKI + CKD, diabetic CKD, or nondiabetic CKD with or without IgAN, compared with healthy individuals, a comparative analysis identified 34, 35 and 17 specific DEPs in AKI, AKI+CKD and CKD samples, respectively. Further investigation also identified 91 distinct DEPs in DN, compared with 14 unique DEPs in nondiabetic CKD. Numerous specific DEPs were also different between nondiabetic CKD with IgAN and nondiabetic CKD without IgAN. Thus, our results could potentially be applied to improve classification and diagnosis of renal disease. However, not all unique DEPs were verified in this study. The expression and biological functions of these specific top-ranking DEPs in the different group should be determined to decide whether they could be set the diagnostic biomarkers of certain renal disease.

Urinary proteomic studies commonly employ a long series of statistical filters to identify enrichment of biological functions and other processes during biomarker research. Previous studies suggest that cyclic helix B peptide-mediated protection in AKI is implicated in multiple molecular functions, such as protein-binding activity and oxygen transport and complicated signal transduction pathways, such as oxidative phosphorylation, alongside other diverse signalling and physiological pathways [30]. In the progression of DN, the decrease in some urinary proteins is presumably associated with impaired secretory function of other organs, such as a reduced excretion of pancreatic amylase and deoxyribonuclease I [31]. The DEPs identified in the IgAN group using iTRAQ appear to be involved in several molecular functions including responses to external stimuli and immune responses, as well as multiple signalling transduction pathways such as cytokine–cytokine receptor interaction and ubiquitin mediated proteolysis [32]. For the first time, we performed functional and KEGG pathway analysis of identified DEPs among patients with AKI, AKI + CKD, diabetic CKD and nondiabetic CKD with or without IgAN. GO analysis results revealed almost identical BP, CC MF categories for all five diseases. Protein annotation and KEGG analysis revealed some common molecular functions and signalling transduction pathways among the five diseases. Our findings could assist a possible strategy to better understand differences between the five kidney diseases by studying the specific molecular functions and signalling pathways involved in each disease.

SAA1, an inflammatory protein mainly produced by hepatocytes, is upregulated by factors that enhance inflammatory cytokines [33]. In blood circulation, it is closely related to high-density lipoprotein and SAA1 is of great importance in liver metabolism, infection and renal cell carcinoma [34–36]. HGFAC is a serine protease and also a key determinant in tissue injury and the progression of renal cell carcinoma [37,38]. Detailed analysis and filtering of synchronously altered DEPs identified SAA1 and HGFAC as highly expressed in patients with AKI and nondiabetic CKD without IgAN, respectively, implying that detection of urinary SAA1 and HGFAC might help to distinguish these two diseases in a clinical setting. The elevation of SAA1 and HGFAC in the two types of renal disease patients might be ascribed to the stimulation of inflammatory or metabolism-associated factors. Possibly, screening the unique risk factors in renal tissues from AKI and nondiabetic CKD without IgAN patients should contribute to the causal investigation of SAA1 and HGFAC elevation. C5, a member of the complement system, can moderate lipid metabolism and reduce fibrosis in DN [39]. C5 activation is also observed in patients with lupus nephritis and renal cell carcinoma, which suggests that activated C5 is widespread in kidney diseases [40,41]. Our results further confirmed that urinary C5 was notably increased in multiple kidney diseases, implying that it might act as a specific marker of multiple kidney diseases. Since APOCI, encoded by the *apoc1* gene, is expressed primarily in the liver, plasma APOCI is associated with an increased risk of developing DN and it acts as a key factor in the pathogenesis of glomerulosclerosis [42,43]. Nevertheless, in our study, we revealed that urinary APOCI was widely elevated in various kidney diseases except for diabetic CKD and the data indicated it might be used as a potential diagnostic marker to distinguish diabetic CKD from other renal diseases. Additionally, whether urinary APOCI participated in the progression of DN remains unclear. Reg3A is mainly increased during pancreatic inflammation and liver carcinogenesis and during mammalian urinary tract infection, both Reg3 gamma and HIP/PAP are induced, implying that RegA3 may be implicated in inflammation responses during renal disease [44]. Based on our

findings, we concluded that RegA3 upregulation was only associated with AKI and AKI + CKD patients, but not CKD patients, regardless of diabetic CKD or nondiabetic CKD status and with or without IgAN. In our opinion, the reason for the increase of RegA3 in these two types of disease should be associated with the acute inflammation stimulation.

Conclusion

In summary, we performed urinary proteomics analysis of multiple kidney diseases for the first time and identified numerous urinary proteins that are common to five different types of disease, as well as several unique protein markers for each disease. Importantly, we also identified and validated SAA1, HGFAC, C5, APOC1 and RegA3 as common or specific biomarkers for different kidney diseases. This work provides fundamental biological information for multiple kidney diseases and can accelerate the development of this field in the future.

Future perspective

In the present study, we performed urinary proteomics analysis of multiple kidney diseases for the first time and identified SAA1, HGFAC, C5, APOC1 and RegA3 as common or specific biomarkers for different kidney diseases. Although urinary proteomics has been extensively applied to investigate renal diseases including AKI, CKD, IgAN and diabetic CKD. However, differential urinary proteome studies have not been reported for multiple diseases. The biomarkers observed in this study can be used as the diagnostic marker for multiple kidney diseases or specific types of kidney disease. Even so, whether these differentially expressed proteins have biological function during the progress of kidney disease remains unclear. This needs further clinical and biological experiments to verify its clinical application value. For instance, if the biological function of RegA3 on patients with AKI and AKI plus CKD were proved, the detection kit developing about RegA3 would contribute to distinguish patients with only CKD and patients with AKI or AKI plus CKD in the future. Collectively, this work provides fundamental biological information for multiple kidney diseases and could accelerate the development of this field in the future.

Summary points

In this article, we determined the differentially expressed urine protein among healthy controls, acute kidney injury (AKI), chronic kidney disease (CKD), IgA nephropathy (IgAN) and diabetic CKD, nondiabetic CKD with or without IgAN patients.

- Using 8-plex iTRAQ reagent, a total of 156, 156, 286, 187 and 184 differentially expressed proteins (DEPs) showing up- or downregulated were discovered in AKI, AKI + CKD, diabetic CKD, nondiabetic CKD with or without IgAN patients compared with healthy volunteers mass spectrometry using 2D LC-MS/MS.
- Among the differentially expressed urine proteins, 34, 35 and 17 unique DEPs were found out in AKI, AKI + CKD and CKD samples, respectively.
- In diabetic CKD, 91 specific DEPs were screened out, while 14 particular DEPs were discovered in nondiabetic CKD patients.
- In comparison with nondiabetic CKD with IgAN, which contains 38 specific DEPs. 47 unique urinary proteins were found in nondiabetic CKD without IgAN.
- Among these DEPs, 22, 12, 12, 27, 6, 44 and 17 specific DEPs were closely related with proliferation, survival, apoptosis, inflammation, repair and migration process.
- The majority of DEPs in AKI, AKI + CKD, nondiabetic CKD with IgAN and nondiabetic CKD without IgAN groups were mainly implicated in cellular process, response to stimulus, metabolic process and multicellular organismal processing.
- The biological functions regulated by DEPs in these renal diseases are diverse and complex.
- Bioinformatics analysis of Kyoto Encyclopaedia of Genes and Genomes pathways revealed commonalities as well as process-specific differences among these renal diseases.
- Urinary serum amyloid A1 and hepatocyte growth factor activator were employed as the indicators of AKI and nondiabetic CKD without IgAN, respectively. Component 5, apolipoprotein C-I and regenerating islet-derived protein 3- α could not be used for biomarker to distinguish one disease from the other.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/bmm-2019-0556

Author contributions

Q He conceived the idea; J Jin drafted the manuscript; J Jin and J Gong analyzed and interpreted the patient data and the screening results; L Zhao designed the figures and Y Wang collected the human samples. YG Wang revised the manuscript and provided the fund support. All authors read and approved the final manuscript.

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Ethical conduct of research

This study was approved by the Human Medical Ethics Committee of Zhejiang Provincial People's Hospital. The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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