Original Article

Mass Spectrometric Identification of Urinary Biomarkers of Chronic Kidney Disease: A Proteomic-Related Preliminary Report

Abstract

Background: Chronic kidney disease (CKD) is a gradual loss of kidney function and has an increased prevalence rate worldwide. Our study was intended to identify potential biomarkers of progression using urine proteomics. **Materials and Methods:** This preliminary study consisted of 32 patients with stage V CKD. Urine samples were subjected to liquid chromatography–mass spectrometry (LCMS), and the network of protein interaction was analyzed using STRING. **Results:** A total of 135 proteins were identified, of which 35 were listed as candidates based on their clinical significance. Protein– protein interaction study provides novel insights into the functional constitution of the proteome, selecting urine as a source of biomarkers. **Conclusion:** The present study observed that the potential markers such as EndoG, HPX, APN, AnxA1, Mic60, LONP1, and HYOU1 correlate with renal damage and its progression to CKD stage V.

Keywords: *Biomarker, Chronic kidney disease, Protein-protein interaction, Urine proteome*

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Introduction

Chronic kidney disease (CKD)[1] has a global prevalence of 11–13%.[2] Progression of CKD depends on the primary disease and also other influencing factors such as smoking, diet, or coexisting metabolic diseases.[3] Inflammatory response associated with oxidative stress promotes renal damage by inducing necrosis, apoptosis, and fibrosis, which may play a role in the progression of CKD.[4] Several markers of various risk factors of CKD can be measured in the tissue or serum to monitor the disease and its progression.

Investigation of alternative biofluids such as urine as a potential non-invasive biomarker[5] would provide an easier means of disease monitoring. Urinary peptides identified through liquid chromatography–mass spectrometry (LCMS) could help in monitoring the disease progression and treatment response.

The study aims to identify a urinary proteome-based panel of novel protein biomarkers of CKD.

Materials and Methods

This preliminary study consisted of 32 patients with CKD stage V recruited at the Division of Nephrology, Department of General Medicine, Aarupadai Veedu Medical College and Hospital, Puducherry, India. The study was approved by the Institutional Human Ethics Committee of AVMC & H (IHEC No: AV/IEC/2021/010), and informed consent was obtained from each participant. The participants were asked to answer questionnaires regarding anthropometric measurements and recorded their laboratory and clinical examinations. The sample collection and preparation workflow are represented in Figure 1.

Sample collection and preparation

Fresh urine samples were collected in the early morning, clean catch specimen into a sterile urine container (15 mL falcon tube), and immediately transported to the ice to prevent proteolysis and microbe contamination. Samples were processed within 30 min to remove cellular components by centrifugation at ≈3000 relative centrifugal forces (RCF) for 20 min at 4°C.[6] The supernatant (5 mL each aliquot) was aliquoted and stored at −80°C.

Mass spectrometric analysis

The supernatants were concentrated and digested overnight with trypsin at 37°C. The precipitated protein pellets were re-

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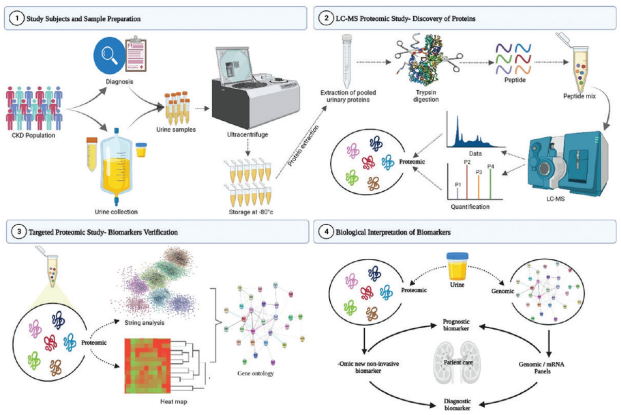

Figure 1: Urine sample collection and preparation work flow.

dissolved in 120 µL of the initial mobile phase consisting of 0.1% formic acid in acetonitrile. The resulting peptides were analyzed using nano-LC-MS/MS-LTQ-orbitrap discovery (Thermo Scientific). Database searches were performed using the proteome discover software, UniProt, and Mascot search engines for the human database.

Protein–protein interaction analysis

The network of protein interaction was analyzed the using STRING software, based on the STRING database and gene ontology. Verified candidate proteins that were found in urine samples of CKD stage V were subjected to further protein–protein network analysis.

Table 1: Clinical parameters of patients with chronic kidney disease stage V

Parameters	Mean ± SD (CKD stage-V [*n* = 32])
Age (years)	55.77 ± 8.04
Body mass index (BMI) (kg/m^2)	22.00 ± 4.0
Blood urea (mg/dL)	105.81 ± 23.75
Serum creatinine (mg/dL)	9.21 ± 2.78
Uric acid (mg/dL)	9.42 ± 1.47
24 h Creatinine clearance (mL/min)	165.10 ± 40.11
Creatinine clearance (Cockcroft–Gault equation) (mL/min)	15.63 ± 1.83
Proteinuria (g/24 h)	1.48 ± 0.28
Cystatin-C (mg/L)	2.55 ± 0.40
eGFR (mL/min/1.73 m^2)	8.93 ± 1.23
Albumin mg/dL	4.05 ± 0.64
Urine: ACR (mg/g)	271.59 ± 61.31
Ammonia (µmol/L)	53.16 ± 19.37
pH	7.31 ± 0.58
Specific gravity	1.020 ± 0.01
Serum electrolytes	
Calcium (mg/dl)	7.57 ± 1.48
Phosphorous (mg/dl)	6.69 ± 2.58
Sodium (mmol/L)	140.87 ± 4.92
Potassium (mmol/L)	4.73 ± 0.66

SD: Standard deviation, CKD: Chronic kidney disease, eGFR: estimated glomerular filtration rate, ACR: Albumin to creatinine ratio.

Results

The data on the baseline characteristics of the study subjects are presented in Table 1. We identified 135 proteins and segregated them based on gene ontology enrichment analysis, shown in Supplementary Data 1. Out of 135 proteins, 35 showed higher expression in urine [Table 2] and are involved in pathophysiological processes of kidneys such as protein sorting, ion and/or iron transport, metal binding, hemostasis, protease and antiprotease activity, transcriptional regulation, stress response, and apoptosis. We chose 10 candidates, namely, CD59, ANPEP, APOH, HPX, LMAN2, ANXA4, ANXA5, TPP1, ANXA11, and ATP1A1, having significance in the progression of CKD to end-stage renal disease.

A protein interaction network was constructed to evaluate any functional and/or physical interactions between the urinary protein candidates for 135 proteins using STRING and functional ontology (GO) enrichment analysis [Figure 2a-2b, Figure S1 and S2]. This analysis revealed that the majority of urinary protein candidates were membrane proteins associated with biological processes of heme homeostasis, protein trafficking and transport, and many of them were involved in the process of gene expression and cell signaling. Gene ontology analysis provides a comprehensive resource related to genes and gene products. The figure represents the list of proteins; proteins and peptides are interconnected in PPI networks, which can be altered in diseased conditions [Figure 2].

Glycoproteins (CD59, HPX, LMAN2, and APOH) showed increased expression in urine. SERPINA1 identified in urine samples of patients with CKD acts on SERPINE1, which is a potent fibrosis-promoting glycoprotein. Endonucleases are unique and may be assembled with a variety of enzymes typically associated with DNA damage and/or repair (e.g. XRCC3, FANCG, ATP23, FEN1, APTX, and SFN) [Supplementary Data 1]. Results showed the presence of the most active apoptotic endonucleases of the kidneys such as DNAse 1 and EndoG in urine. We identified F- and P-type ATPases in urine, which include ATP1A1, ATP2A3, ATP2B2, ATP7B, ATP2A1, and ATP5IF1; these are involved in sodium/potassium, calcium, and copper transport. ATP5IF1 acts as an inhibitor of endogenous F1F(o)-ATPase. The zinc finger proteins are involved in transcriptional regulation. These zinc finger proteins (e.g. SNAI3, ZNF621, ZNF511, ZNF205, ZNF331, ZNF787, and DNLZ) have a promising role in the development of podocyte damage or glomerular diseases.

Discussion

Proteomics provide good tools with clinically relevant diagnosis and prognosis models as translational research.

Table 2: List of urine proteome identified by LC-MS

Protein (Uniprot ID)	Synonym	pI	Molecular weight (kDa)	Gene	Anatomical entity	Theoretical expression	Function	Sequence
CD59 glycoprotein (P13987)	MIC11, MIN1, MIN2, MIN3, MSK21	6.02	14.17	CD59	Renal medulla	98.87	Transport, sorting, protein transport	TAVNCSSDFDACLITK
Aminopeptidase N (P15144)	CD13	5.31	109.4	ANPEP	Kidney	98.07	Protease activity, angiogenesis, differentiation, host-viral interaction	VVTVIAHELAHQWFGNLVTI
Annexin A1 (P04083)	ANX1, LPC1	6.57	38.71	ANXA1	Urethra	98.19	Inflammatory response, calcium/ phospholipid binding	QAWFIENEEQEYVQTVK
Progranulin (P28799)	GP88, PCDGF, PEP	6.43	63.54	GRN	Kidney	97.31	Integral membrane glycoprotein, immunity	DVECGEGHFCHDNQTCCR
Annexin A6 (P08133)	ANX6	5.41	75.87	ANXA6	Kidney	93.8	Regulate the release of Ca2+	EDAQVAAEILEIADTPSGDK
Sodium/potassium-transporting ATPase subunit alpha-1 (P05023)		5.33	112.8	ATP1A1	Kidney	99.54	Sodium/potassium transport	DAFQNAYLELGGLGER
Peroxiredoxin-1 (Q06830)	PAGA, PAGB, TDPX2	8.27	22.11	PRDX1	Kidney	99.29	Antioxidant	YVVFFFYPLDFTFVCPTEII AFSDR
Tripeptidyl-peptidase 1 (O14773)	CLN2	6.01	61.24	TPP1	Renal glomerulus	97.09	Tripeptidyl-peptidase I activity, metal binding	FLSSSPHLPPSSYFNASGR
Beta-2-glycoprotein 1 (P02749)	B2G1	8.34	38.29	APOH	Renal glomerulus	86.47	Inhibitor of serine proteases, acute phase, blood coagulation, hemostasis	TFYEPGEEITYSCKPGYVSR
Vesicular integral-membrane protein VIP36 (Q12907)	C5orf8	6.46	40.22	LMAN2	Kidney	93.99	Heparin binding	LFQLMVEHTPDEESIDWTK
Hemopexin (P02790)		6.55	51.67	HPX	Kidney	60.29	Heme binding, transport	DGWHSWPIAHQWPQGPSAVDAAFSWEEK
Annexin A11 (P50995)	ANX11	7.53	54.38	ANXA11	Kidney	97.17	Cell division	GFGTDEQAIIDCLGSR
Annexin A5 (P08758)	ANX5, ENX2, PP4	4.93	35.93	ANXA5	Urethra	99.19	Anticoagulant protein, hemostasis	DPDAGIDEAQVEQDAQALFQAGELK
Annexin A4 (P09525)	ANX4	5.83	35.88	ANXA4	Urethra	97.72	Exocytosis. calcium/ phospholipid binding	AASGFNAMEDAQTLR
Glutaminase kidney isoform (O94925)	GLS1, KIAA0838	7.85	73.46	GLS	Kidney	97.1	Acid-base homeostasis	ILQEYQVQYTPQGDSDNGK
MICOS complex subunit MIC60 (Q16891)	HMP, MIC60, MINOS2	6.08	83.67	IMMT	Renal medulla	96.32	Mitochondrial cristae morphology, host-viral interaction	LSQEQVDNFTLDINTAYAR
Lon protease homolog (P36776)	PRSS15	6.01	106.4	LONP1	Kidney epithelium	85.64	ATP-binding	EIFDIAFPDEQAEALAVER
Hypoxia-inducible factor 3-alpha (Q9Y2N7	BHLHE17, MOP7, PASD7	5.67	72.43	HIF3A	Kidney	73.12	Transcriptional regulator, angiogenesis, stress response, apoptosis, transcription regulation	DTEAVETDLDIAQDADALDL

(*Continued*)

Table 2: (Continued)

Protein (Uniprot ID)	Synonym	pI	Molecular weight (kDa)	Gene	Anatomical entity	Theoretical expression	Function	Sequence
Peroxiredoxin-4 (Q13162)		5.86	30.53	PRDX4	Renal glomerulus	93.48	Antioxidant	YLVFFFYPLDFTFVCPTEII AFGDR
Hypoxia up-regulated protein 1 (Q9Y4L1)	GRP170, ORP150	5.16	111.3	HYOU1	Kidney	94.29	Cytoprotective, chaperone, stress response	LIPEMDQIFTEVEMTTLEK
Chloride intracellular channel protein 1 (O00299)	G6, NCC27	5.09	26.92	CLIC1	Kidney	98.89	Voltage-gated channel, chloride channel	FLDGNELTLADCNLLPK
Lysosome-associated membrane glycoprotein 1 (P11279)	CD107a	9	44.88	LAMP1	Renal glomerulus	99.92	Lysosome biogenesis, autophagy, cholesterol homeostasis, Host-viral interaction	YSVQLMSFVYNLSDTHLFPN ASSK
L-xylulose reductase (Q7Z4W1)	SDR20C1	8.33	25.91	DCXR	Kidney	98.65	Osmoregulation, carbohydrate metabolism, glucose metabolism, xylose metabolism	ALGSVGPVDLLVNNAAVALL QPFLEVTK
Radixin (P35241)		6.03	68.56	RDX	Urethra	94.32	Actin binding	VTTMDAELEFAIQPNTTGK
Myosin-9 (P35579)		5.5	226.5	MYH9	Kidney	95	Actin binding, calmodulin binding, cell adhesion	LQQLFNHTMFILEQEEYQR
EH domain-containing protein 4 (Q9H223)	HCA10, HCA11, PAST4	6.32	61.17	EHD4	Urethra	91.13	ATP- and membrane binding protein	LDGYELPSSLPPHLVPPSHR
Neutrophil gelatinase-associated lipocalin (P80188)	HNL, NGAL	9.02	22.58	LCN2	Kidney	75.41	Renal development, apoptosis, immunity, iron transport, ion transport	TFVPGCQPGEFTLGNIK
Annexin A3 (P12429)	ANX3	5.62	36.37	ANXA3	Kidney	80.25	Anticoagulant, phospholipase A2 inhibitor	GELSGHFEDLLLAIVNCVR
Serpin B6 (P35237)	PI6, PTI	5.18	42.59	SERPINB6	Kidney	96.52	Mitochondrial membrane ATP synthase (F1F0 ATP synthase of complex V), hydrogen ion transport	TYIGEIFTQILVLPYVGK
Calnexin (P27824)		4.46	67.56	CANX	Cortex of kidney	98.64	Calcium binding protein, chaperone	WKPPMIDNPSYQGIWKPR
Leukocyte elastase inhibitor (P30740)	ELANH2, MNEI, PI2	5.9	42.74	SERPINB1	Kidney	87.3	Transcriptional regulator	TYGADLASVDFQHASEDAR
Amyloid-beta precursor protein (P05067)	A4, AD1	4.73	86.94	APP	Kidney epithelium	98.63	Heparin-binding, apoptosis, cell adhesion, endocytosis	LALENYITALQAVPPRPR
Transmembrane emp24 domain-containing protein 9 (Q9BVK6)	GP25L2	7.81	27.27	TMED9	Renal glomerulus	98.34	Protein recruitment, mRNA transport, translocation, transport	VHLDIQVGEHANDYAEIAAK
Endoplasmic reticulum chaperone BiP (P11021)	HSPA5	5.07	72.33	GRP78	Renal medulla	99.08	Chaperone, apoptosis, cell proliferation	GINPDEAVAYGAAVQAGVLS
Stress-70 protein, mitochondrial (P38646)	GRP75, HSPA9B, mt-HSP70	5.87	73.68	HSPA9	Renal glomerulus	95.39	Chaperone	STNGDTFLGGEDFDQALLR

LC-MS: Liquid chromatography-mass spectrometry.

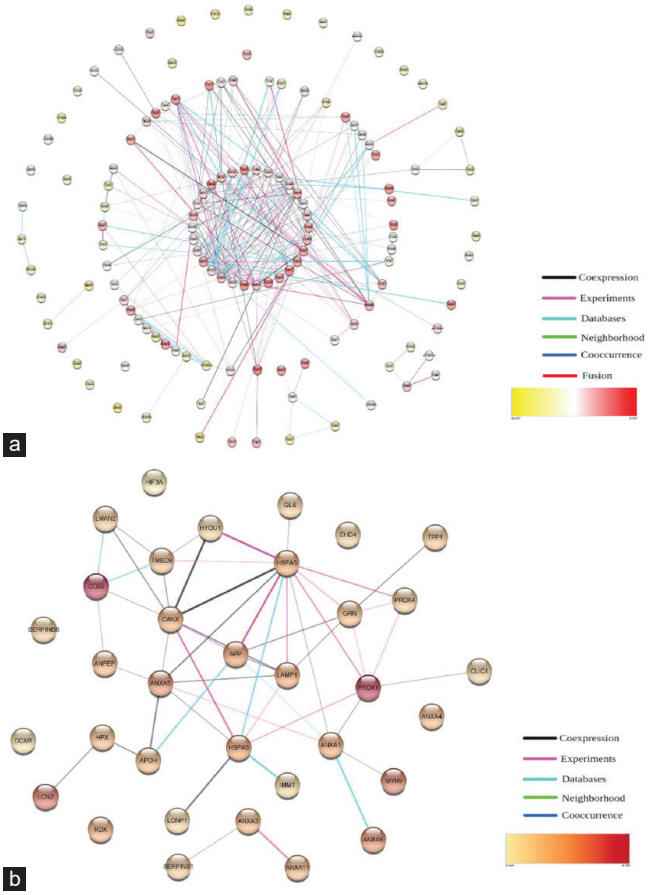

Figure 2: Protein interaction network analysis. (a) Protein interaction analysis of 135 proteins identified in urine. (b) Protein interaction analysis of 35 proteins identified in urine.

Our study showed 35 urinary proteins with increased expression in urine in patients with CKD stage V. These proteins are involved in various physiological and pathophysiological activities of the kidneys including cell signaling, inflammation, oxidative stress, hypoxia, DNA damage/repair mechanism, cell cycle arrest, mitochondrial dysfunction, heme homeostasis, and chaperons. CD59, a glycosylphosphatidylinositol (GPI) is composed of a phosphatidylinositol group linked through carbohydrate groups acts as an anchored glycoprotein and is ubiquitously expressed on epithelial, endothelial and mesangial cells of glomeruli. A study used anti-CD59 to neutralize its activity observed reduced renal function with severe endothelial damage with increased platelet and also fibrin deposition in glomeruli.[7] Scavenging of free heme with hemopexin (HPX) is expected to mitigate heme-induced cell injury. Increased free heme concentration and HPX deficiency indicate that increased expression of CD59 could defend against complement-dependent renal injury.[8] Aminopeptidase N (APN) is identical to CD13, belongs to type I metalloproteases, and has a role in metastasis, tumor invasion, and angiogenesis. The potential use of ^{99m}Tc-probestin SPECT as a new technique is imaging kidney APN expression.[9] Increased urinary APN levels associated with microalbuminuria indicate tubular damage in diabetic nephropathy (DN).[10]

Annexins (Anx) can mediate a varied range of cellular processes including endo-, exocytosis, and membrane-cytoskeletal organization.[11] AnxA1 in renal tissue is negatively correlated with the degree of tubular damage.[12] AnxA1 levels correlated with normal kidney outcomes and showed higher expression during DN.[13] AnxA4 regulates adenylyl cyclase type 5 and affects β-adrenoceptor/cAMP-dependent signaling.[14] AnxA6 has a role in integrin recycling, aberrant Src, and FAK signaling, ECM cell surface delivery[15] and IL-2 homeostasis-related T-cell proliferation.[16] Progranulin (PGRN) is a cysteine-rich secretory protein used to predict the early signs of podocyte injury in DN[17] and also indicates an endotoxin-induced septic acute kidney injury.[18]

Sodium/potassium-transporting ATPase (Na/K-ATPase) is an important ion pump of P-type ATPase. Reduced Na/K-ATPase α1 causes deficiency in signaling protein Src and NFkB activation. A study identified sodium/potassium-transporting ATPase subunit gamma in urine using capillary electrophoresis-coupled mass spectrometry in IgA nephropathy patients.[19] Na/K-ATPase involved in CD40 receptor activation in the renal tubular epithelium during pathogenesis.[20] Tripeptidyl peptidase-I (TPP-I) is a lysosomal serine proteinase expressed in the kidneys. Urine TPPI was significantly increased in patients with CKD.[21] Peroxiredoxins (PRDXs) is an antioxidant enzyme key regulator of apoptosis and cell survival, it can intermediate during H_2O_2-induced activation of c-Abl/MST1/FOXO signaling.[22] Mic060 is an important factor involved in the regulation of cell apoptosis, mitochondrial biogenesis, and oxidative stress.[23] A study reported significantly decreased Mic60 levels in hemodialysis patients.[24]

Lon protease homolog, mitochondrial (LONP1) is a serine peptidase that protects the kidneys by inhibiting mitochondrial dysfunction and podocyte apoptosis.[25] Radixin is one of the membrane-cytoskeletal crosslinkers, and increased levels of radixin were observed in dialysis patients.[26] Limitations of this preliminary study are smaller sample size and that the proteins identified herein require further external validation.

Protein identification using LC-MS/MS allowed us to identify 135 proteins expressed in the urine of CKD patients. Possibly further studies on EndoG, HPX, APN, AnxA1, Mic60, LONP1, and HYOU1 potential markers will enable a better understanding of the mechanism of CKD and may provide novel opportunities for therapy. Sufficiently powered cohort studies are required to confirm the usefulness of these candidates to help improve understanding of CKD progression.

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