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Shotgun Proteomics Identifies Proteins Specific for Acute Renal Transplant Rejection

Tara K. Sigdel¹, Amit Kaushal², Marina Gritsenko³, Angela D. Norbeck³, Wei-Jun Qian³, Wenzhong Xiao², David G. Camp II³, Richard D. Smith³, and Minnie M. Sarwal¹

¹ Department of Pediatrics, 300 Pasteur Dr, S378 Grant Bldg, Stanford University School of Medicine, Stanford, CA 94305

² Department of Biochemistry, 251 Campus Dr, Stanford University, Stanford, CA 94305

³ Battelle, Pacific Northwest National Laboratory, Richland, WA 99352, USA

Abstract

Acute rejection (AR) remains the primary risk factor for renal transplant outcome; development of non-invasive diagnostic biomarkers for AR is an unmet need. We used shotgun proteomics applying LC-MS/MS and ELISA to analyze a set of 92 urine samples, from patients with AR, stable grafts (STA), proteinuria (NS), and healthy controls (HC). A total of 1446 urinary proteins were identified along with a number of NS specific, renal transplantation specific and AR specific proteins. Relative abundance of identified urinary proteins was measured by protein-level spectral counts adopting a weighted fold-change statistic, assigning increased weight for more frequently observed proteins. We have identified alterations in a number of specific urinary proteins in AR, primarily relating to MHC antigens, the complement cascade and extra-cellular matrix proteins. A subset of proteins (UMOD, SERPINF1 and CD44), have been further cross-validated by ELISA in an independent set of urine samples, for significant differences in the abundance of these urinary proteins in AR. This label-free, semi-quantitative approach for sampling the urinary proteome in normal and disease states provides a robust and sensitive method for detection of urinary proteins for serial, non-invasive clinical monitoring for graft rejection after kidney transplantation.

Keywords

Renal transplantation; Acute rejection; Biomarkers; Urinary proteomics; ELISA

1. Introduction

Publication of human genome data has facilitated high throughput studies, including the large scale analysis of human proteins in different biofluids[1–2]. Biofluids such as blood, urine, nipple aspirate fluid, bronchoalveolar lavage and cerebrospinal fluid [3–6], have been analyzed by various proteomics techniques, and based on their relevance to the concerned disease; however, detection of low abundance proteins and the identification of the statistically significant proteins are still positioned as bottlenecks for proteomics analysis.

Recent studies [7–11], including those by Adachi *et al* [12] and Gonzales *et al* [13] have used gel-based as well as gel-free proteomics approaches to contribute to the atlas of urinary proteins in normal individuals. Using 1-D SDS-PAGE and a reversed phase HPLC protein separation

and fractionation method followed by application of LTQ-FT and LTQ-Orbitrap technology, Adachi *et al.* analyzed urine samples collected from healthy individuals and identified 1543 proteins [12]. Gonzales *et al.* analyzed urinary exosomes using LC-MS/MS and identified 1132 proteins [13]. In this study, we have undertaken a pilot study of 10 normal samples, 40 urinary samples from patients with nephrotic syndrome as well as renal transplant patients with stable graft function and biopsy proven AR. The purpose of the study was to study if phenotype specific differences could be identified in urinary samples from patients with different etiologies of native and transplant-associated renal injury.

The benefit of identifying rejection-specific proteome biomarkers in urine is very relevant. Renal transplantation is the ultimate treatment for patients with end stage kidney disease [14], but there is no current non-invasive means to monitor for acute graft rejection. The renal biopsy, the gold standard for diagnosing rejection, is an invasive procedure that suffers from sampling heterogeneity, has associated complications of pain, sedation, hematuria, arteriovenous fistulae, graft thrombosis and transfusion risk [15], and correlates poorly with treatment response and prognosis. Because of the ability of urine to reflect both local processes within the kidney as well as a reflection of changes within plasma, urine is particularly useful to diagnose kidney diseases and kidney transplant dysfunction [16]. Discovery of a urine biomarker for assessing the rejection status of patients following kidney transplant could significantly improve patient outcomes and decrease the cost of care.

To test the validity of the discovery of AR-specific protein biomarkers by our study approach, we performed ELISA assays on selected protein biomarkers using an independent set of 52 unique patient urines. ELISA results established that the approach taken in this study is a viable way to discover potential biomarkers. The report demonstrates how high-throughput, high-cost, labor-intensive MS-based discovery can eventually be developed into an economical, rapid turn-around, clinically applicable diagnostic assay for transplant patients.

2. Materials and Methods

Materials

The following reagents were used for the proteomics sample preparation: nanopure or Milli-Q quality water (~18 megohm-cm or better); Bicinchoninic acid (BCA) Assay Kit was purchased from Pierce (Rockford, IL); Amicon Ultra centrifugal filtration tubes were obtained from Millipore (Bedford, MA) ammonium bicarbonate, ammonium formate, and formic acid were obtained from Fluka (St.Louis, MO); Tris.HCl, urea, thiourea, dithiothreitol (DTT), iodoacetamide, calcium chloride, and trifluoroacetic acid (TFA), were obtained from Sigma-Aldrich (St.Louis, MO); HPLC-grade methanol (MeOH) and HPLC-grade acetonitrile (CH₃CN) were purchased from Fisher Scientific (Fair Lawn, NJ); 2,2,2-trifluoroethanol (TFE) was obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI); and sequencing grade modified trypsin was purchased from Promega (Madison, WI). PEDF(SERPINF1)ELISA kit was purchased from Bioproducts MD (Middletown, MD).

Samples

Forty individual and clinically annotated urine samples were included in the study. We used 10 renal transplant patients, each with biopsy proven acute rejection (AR), as diagnosed by the standardized Banff criteria [17], and 10 renal transplant patients with stable grafts (STA), classified by the absence of any acute rejection or significant chronic injury on a protocol biopsy. All urine samples were collected at the time of a clinically indicated or protocol biopsy, prior to any treatment intensification. Our controls included 10 non-specific proteinuria (NS) patients, from minimal change disease, not currently on any immunosuppression treatment, and 10 age matching healthy children as healthy controls(HC). As some element of proteinuria

from injury in AR can be seen, the NS patients provided controls for the presence or absence of proteinuria. Patient demographics were matched (Table 1). The samples were collected in between January 2005 and June 2007 and were obtained as part of an ongoing IRB approved study at Stanford University. Approval for the conduct of this research was obtained from the Institutional Review boards at Stanford University and Pacific Northwest National Laboratory (PNNL) in accordance with federal regulations.

Urine collection, initial processing and storage

Second morning void mid-stream urine samples (50–100 mL) were collected in sterile containers and were centrifuged at $2000 \times g$ for 20 min at room temperature within 1 h of collection. The supernatant was separated from the pellet containing any particulate matter including cells and cell debris. The pH of the supernatant was adjusted to 7.0 and stored at -80°C until further analysis. Urine osmolality was measured on all samples.

Recovering and quantification of urinary protein

Urinary proteins were isolated by removing small MW peptides and other pigments (<10 kDa) by filtering the supernatant through Amicon Ultra centrifugal filtration tubes (Millipore, Bedford, MA). The tubes were pre-equilibrated with 10 mL Milli-Q water and centrifuging at $3000 \times g$ for 10 min at 10°C using swinging bucket rotors. After equilibration, 10 mL of urine supernatant was centrifuged for 20 min at $3000 \times g$ at 10°C . The filtrate was recovered and saved for peptidomic analysis. Urine creatinine was measured using Quantichrom™ Creatinine Assay Kit (DICT-500) (BioAssay Systems, Hayward, CA). The manufacturer's manual was followed for the assay. The retentate was washed twice with 10 mL of 20 mM Tris-HCl (pH 7.5). The final volume of the retentate was brought to 400 μL with 20 mM Tris-HCl (pH 7.5) and was quantified by using bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). The mean protein to creatinine ratio for AR, STA, HC and NS were 0.22 ± 0.15 , 0.14 ± 0.06 , 0.05 ± 0.02 , and 0.67 ± 1.40 respectively. After the quantification of individual samples, 4 pooled samples for each AR, STA, NS and HC categories were prepared using 200 μg from each individual sample in each category.

Urinary proteome sample preparation

Samples were desalted using Micron Ultracel YM-3 centrifugal filters MWCO 3000 (Millipore, Billerica, MA) prior to the tryptic digestion following the manufacturer's protocol. Protein concentration was verified after buffer exchange using a BCA Protein Assay. A mixture of 3 standard proteins, purchased individually from Sigma-Aldrich (horse apomyoglobin, rabbit glyceraldehyde-3-phosphate dehydrogenase, and bovine ovalbumin), was added for quality control purposes. Proteins were denatured in 50 mM ammonium bicarbonate, pH 7.8, 8 M Urea for 1 h at 37°C and then reduced with 10 mM DTT at 37°C for 1 h. After this they were alkylated with 40 mM iodoacetamide at room temperature for 1 h in the absence of light. Samples were diluted 10 fold with 50 mM ammonium bicarbonate, pH 7.8 and sufficient amount of 1 M calcium chloride was added to the samples to obtain a concentration of 1 mM in the sample. Sequencing grade-modified trypsin was prepared by adding 20 μL of 50 mM ammonium bicarbonate, pH 7.8 to a vial containing 20 μg trypsin and after 10 min incubation at 37°C was used for digestion of the samples. Tryptic digestion was performed for 3 h at 37°C with 1:50 (w/w) trypsin-to-protein ratio. Rapid freezing of the samples in liquid nitrogen quenched the enzymatic digestion.

Digested samples were desalted by using a solid-phase extraction (SPE) C18 column (Discovery DSC-18, SUPELCO, Bellefonte, PA) conditioned with MeOH and rinsed with 0.1 % TFA, 1 mL, and washed with 4 mL of 0.1% TFA/5% CH_3CN . Peptides were eluted from the SPE column with 1 mL of 0.1% TFA/80% CH_3CN and concentrated in a Speed-Vac SC 250 Express (Thermo Savant, Holbrook, NY) to a volume of ~ 50 – 100 μL . The peptide

concentration was measured using the BCA Protein Assay. Digested samples were stored at -80°C until needed for analysis or further processing.

Strong cation exchange (SCX) fractionation

Digested samples (200.0–350.0 μg) were reconstituted with 900 μL of 10 mM ammonium formate, pH 3.0/25% CH_3CN and fractionated by SCX chromatography on a Polysulfoethyl A 2.1 mm $\times$ 200 mm, 5 μM , 300 $\text{\AA}$ column with 2.1 mm $\times$ 10 mm guard column (PolyLC, Inc., Columbia, MD) using an Agilent 1100 series HPLC system (Agilent, Palo Alto, CA). The flow rate was 200 $\mu\text{L}/\text{min}$, and mobile phases were 10 mM ammonium formate, pH 3.0/25% CH_3CN (A), and 500 mM ammonium formate, pH 6.8/25% CH_3CN (B). After loading 900 μL of sample onto the column, the mobile phase was maintained at 100% A for 10 min. Peptides were then separated using a gradient from 0 to 50% B over 40 min, followed by a gradient of 50–100% B the following 10 min. The mobile phase was held at 100% B for 10 min, followed by H_2O rinsing for the next 20 min and final re-conditioning with A for 10 min. A total of 60 fractions over 90 min separation were collected for each depleted sample, and each fraction was dried under vacuum in a Speed-Vac. The fractions were dissolved in 25 μL of 25 mM ammonium bicarbonate, pH 7.8 and combined into 32 fractions for LC-MS/MS analysis. The first 20 fractions were combined into one and were desalted by C18 SPE column (Discovery DSC-18, SUPELCO, Bellefonte, PA), the next 30 fractions were not pooled and each was analyzed separately, and 5.0 μL of each of the last 10 fractions were combined together into fraction number 32. A 5.0 μL aliquot of each fraction was analyzed by capillary LC-MS/MS.

Capillary LC-MS/MS analysis

The HPLC system consisted of a custom configuration of 100-mL Isco Model 100DM syringe pumps (Isco, Inc., Lincoln, NE), 2-position Valco valves (Valco Instruments Co., Houston, TX), and a PAL autosampler (Leap Technologies, Carrboro, NC), allowing for fully automated sample analysis across four separate HPLC columns [18]. Reversed phase capillary HPLC columns were manufactured in-house by slurry packing 3- μm Jupiter C₁₈ stationary phase (Phenomenex, Torrance, CA) into a 60-cm length of 360 μm o.d. $\times$ 75 μm i.d. fused silica capillary tubing (Polymicro Technologies Inc., Phoenix, AZ) that incorporated a 2.0- μm retaining screen in a 1/16" 75 μm i.d. union (Valco Instruments Co., Houston, TX). Mobile phase consisted of 0.2% acetic acid and 0.05% TFA in water (A) and 0.1% TFA in 90% $\text{CH}_3\text{CN}/10\%$ water (B). The mobile phase was degassed by using an in-line Degassex Model DG4400 vacuum degasser (Phenomenex, Torrance, CA). The HPLC system was equilibrated at 10k psi with 100% mobile phase A, and then a mobile phase selection valve was switched 20 min after injection, which created a near-exponential gradient as mobile phase B displaced A in a 2.5 mL active mixer. A 30-cm length of 360 μm o.d. $\times$ 15 μm i.d. fused silica tubing was used to split ~ 20 $\mu\text{L}/\text{min}$ of flow before it reached the injection valve (5 μL sample loop). The split flow controlled the gradient speed under conditions of constant pressure operation (10k psi). Flow rate through the capillary HPLC column was ~ 900 nL/min. A ThermoScientific LTQ linear ion trap mass spectrometer (ThermoScientific, San Jose, CA) was coupled with the LC-system using an in-house electrospray ionization (ESI) interface for all sample analysis. Home-made 150 μm o.d. $\times$ 20 μm i.d. chemically-etched electrospray emitters were used [19]. The heated capillary temperature and spray voltage were 200°C and 2.2 kV, respectively. Data was acquired for 90 min, beginning 30 min after sample injection (10 min into gradient). Full spectra (AGC setting: 3×10^4) were collected from 400–2000 m/z followed by data-dependent ion trap MS/MS spectra (AGC setting: 1×10^4) of the ten most abundant ions applying collision energy of 35%. A dynamic exclusion time of 60 s was applied.

Peptide and protein identification using MS/MS spectra

Peptides were identified from MS/MS spectra by matching them with predicted peptides from the protein FASTA file from the human International Protein Index (IPI -European Bioinformatics Institute) database (version 3.20, released at August 22, 2006) containing 61,225 protein entries using the SEQUEST™ algorithm [20]. A standard parameter file allowing for a dynamic addition of oxidation to the methionine residue and a static (non-variable) carboxamidomethylation modification to the cysteine residue, with a mass error window of 3 Da units for precursor mass and 1 Da unit for fragmentation mass was used. The searches were allowed for all possible peptide termini, i.e., not limited by tryptic-only termini. Not limiting by tryptic termini means that we did not require the peptide to end in a K, R, or be the termini of the protein. Peptide identifications were considered acceptable if they passed the thresholds determined acceptable for human plasma by Qian et al.[21] and passed an additional filter of a PeptideProphet score of at least 0.7 [22]. The PeptideProphet score is representative of the quality of the SEQUEST™ identification and is based on a combination of XCorr, delCn, Sp, and a parameter that measures the probability that the identification occurred by random chance. Peptide Prophet scores are normalized to a 0 to 1 scale, with 1 being the highest confidence value.

Protein grouping

Due to the high redundancy of peptide-to-protein relationships inherent in the IPI database, two protein grouping programs were used to consolidate sequence identifications. ProteinProphet [23] uses the identified peptide sequences to weight the probability that the peptide originated from a particular protein. When parent protein distinctions cannot be determined, those proteins are grouped together and assigned an index value. The second method of grouping proteins involved aligning whole-protein sequences and assigning similarity scores based on the amino acid composition. This method utilized the BLAST algorithm to determine similarity of each protein to all other proteins in the input file. Then these similarities are clustered into groups of like similarities using a Perl script.

Differentially expressed proteins

Protein-level spectral counts were obtained by summing peptide-level spectral counts. To quantitatively compare relative protein abundances between different pools of samples, we considered either presence or absence of a particular protein in different phenotypes. For the proteins that were identified in multiple categories we used a cutoff criteria of ≥ 2 of fold change in log base(2) of spectral count with at least 5 spectral counts in one of the phenotypes being compared.

ELISA assays for Tamm-Horsfall protein (UMOD)

A total of 60 urine samples (20 AR, 20 STA and 20 HC) were included. Urine samples were diluted 200 fold in PBS buffer. Aliquots of diluted 100 μ L of urine was incubated in Reacti-Bind 96-Well Plates over night at 4 °C. The plate was washed 5 times with 1 $\times$ PBS buffer containing 0.05% Tween 20. The wells were then blocked by 100 μ L of 25% FCS in PBS to prevent non-specific binding of the antibody. The wells were then incubated with 1:3000 fold diluted anti-Tamm Horsfall Glycoprotein PAB at room temperature for 1 hr. The color was developed by using turbo-TMB (Pierce Inc, Rockford, IL) and stopped by the addition of 100 μ L of 2M H₂SO₄ and the plate was read by a SPECTRAMax 190 microplate reader (Molecular Devices, Sunnyvale, CA).

ELISA for Pigment Epithelium-Derived Factor-PEDF(SERPINF1), and CD44

Sandwich ELISA assays were performed to validate the observed elevated levels of PEDF (SERPINF1) and CD44 in urine collected from an independent set of patients and controls

which included AR (n=20), STA (n=20), NS [n=8 for PEDF(SERPINF1) and 6 for CD44], HC (n=6).

PEDF(SERPINF1)ELISA

An ELISA kit for Pigment Epithelium-Derived factor (PEDF) or SERPINF1 (BioProducts, MD) was used for the purpose and the reagents were prepared following the manufacturer's manual. Briefly, after an initial optimizing step for optimal dilution of urine, the urine samples were diluted (1:40) in Assay Diluent. The ELISA plate with 100 μ L of standards and the diluted urine specimens was incubated at 37 °C for 1 h. After the incubation the plates were washed 5 times with Plate Wash Buffer. The wells were incubated with 100 μ L PEDF (SERPINF1) detector antibody at 37 °C for 1 h and washed 5 times with the wash buffer. This step was followed by incubation of the wells with 100 μ L Streptavidin Peroxidase Working solution.

CD44 ELISA

An ELISA kit for CD44 (ABCam Inc, Cambridge, MA) was used for the purpose and the reagents were prepared following the manufacturer's manual. Briefly, after an initial optimizing step for the optimal dilution of urine, the urine samples were diluted (1:1) in Standard Diluent Buffer. The ELISA plate with 100 μ L of standards and the diluted urine specimens was incubated at room temperature for 1 h. After the incubation the plates were washed 5 times with washing solution. The plate was incubated for 30 min with 50 μ L of diluted biotinylated anti-CD44 in all wells. The plate was washed 5 times with the wash solution and was incubated with 100 μ L HRP solution in all the wells for 30 min. This step was followed by a wash step.

All the assays were developed by ready-to-use TMB substrate followed by addition of Stop Solution. All the plates were read by a SPECTRAMax 190 microplate reader (Molecular Devices, Sunnyvale, CA). Protein concentrations were determined from a standard curve generated from the standards obtained with the kit.

Correlation analysis between the spectral counts and the quantity observed from ELISA assay

We obtained quantitative data for UMOD, pigment epithelial derived factor (PEDF) (SERPINF1), and CD44 using ELISA assays on an independent set of patients. The quantitative data obtained from ELISA was compared with the spectral count data for each protein observed in the discovery phase using the LC-MS/MS platform. P Values and Pearson correlation coefficients were calculated using SAS® program (SAS Corporate Statistics, Cary, NC). The ROC was calculated by logistic regression model for 3 proteins based on the values of AR vs. no-AR that included STA, HC and NS.

Enrichment analysis and pathway impact analysis

The enrichment analysis for identified proteins was performed using Ingenuity Pathway Analysis (<http://www.ingenuity.com>). A list of all human genes was used as reference for computing significance, which was obtained from the Onto-Tools database [24]. The pathway analysis was also performed using Pathway-Express [25–26]. Pathway-Express performs a novel impact analysis on signaling pathways, which in addition to the number of proteins in IPA, considers important biological factors such as the topology of the pathway, position of the protein on the pathway, amount of change in protein expression, and the type of interaction between the protein in each pathway.

3. Results

Detection of novel urinary proteins expands the urinary proteome database

We identified 1446 urinary proteins by using LC-MS/MS-based shotgun proteomics on urine from renal patients and healthy individuals. A minimum of 2 unique, non-redundant peptides per protein to be identified was the criteria for positive protein identification. The false discovery rate (FDR) for protein identifications is ~0.1% based on target-decoy analysis using Sequest, while the FDR at the unique peptide level is ~3.0%. The number of total proteins identified in this study with a 1 peptide identified per protein criteria was 3296. The complete list of proteins is provided as supplemental data (Supplemental Table 1). We identified 1001, 1159, 1325, and 1340 proteins respectively in AR, NS, STA, and HC urine, respectively (Figure 1). We used Ingenuity Pathway Analysis -IPA (Ingenuity® Systems, Redwood City, CA-www.ingenuity.com) on predicted proteins based on the human genome database [27] and mapped the proteins identified with previously annotated urinary proteins and proteins of renal origin. A total of 756 urinary proteins from our 1446 protein list have been listed as urinary proteins (UP). This leaves 690 proteins in our list of urinary proteins as novel and were labeled as novel urinary proteins (NUP) (Supplemental Table 2). We compared the list of urinary proteins identified from healthy individuals in this study with 1543 identified by Adachi *et al.* [12] and 1160 by Gonzales *et al.* [13] (Figure 2A). This study has added 560 new proteins to the existing urinary proteome observed in healthy subjects.

Urinary proteins are enriched with extracellular proteins, complement and coagulation, glycan structures – degradation, cell adhesion, and ECM-receptor interaction

Gene ontological classification [28] sub-grouped the 1446 identified proteins into 5 major groups; 279 were cytoplasmic proteins, 325 were extracellular proteins, 28 were nuclear proteins, 304 were plasma membrane, and 108 had as yet unknown sub-cellular localization. We found that extracellular and plasma membrane proteins were enriched, and nuclear proteins were relatively underrepresented in the urine proteome when compared with the predicted human proteome from the human genome database. This is in agreement with previously published data [27]. Hypergeometric analysis revealed that the enrichment of proteins of extracellular origin ($p < 1.00E-6$) and plasma membrane in urine ($p < 3.00E-6$) is highly significant compared to the human proteome. The major representing pathways were complement and coagulation cascades ($P = 1.95E-12$), glycan structures – degradation ($P = 1.31E-11$), cell adhesion molecules (CAMs) ($P = 1.77E-11$), ECM-receptor interaction ($P = 1.87E-11$), cell communication ($P = 2.04E-11$), focal adhesion ($P = 2.62E-11$), axon guidance ($P = 2.86E-11$), regulation of actin cytoskeleton ($P = 4.97E-09$), cytokine-cytokine receptor interaction ($P = 3.26E-09$), and hematopoietic cell lineage ($P = 4.89E-08$).

There was no specific bias towards plasma and renal proteins in the urine of renal patients and depletion of ECM-receptors and integrins in renal patients

We identified the 1420 proteins that were detected in the urine of patients with normal renal function (HC and STA), and only 1206 proteins were found in patients with active renal dysfunction (AR and NS). There was no bias of the health status of the kidney in terms of known urinary, blood, and renal proteins when we used Ingenuity Pathway Analysis® based annotation. Among the total 1420 proteins identified in HC and STA; 578, 463, and 434 proteins were previously known urinary, blood and renal proteins; while in the total 1206 proteins identified in AR and NS; 504, 405, and 353 proteins were previously known urinary, blood, and renal proteins (Table 2).

67 proteins were uniquely identified only in healthy urine (HC) (Supplemental Table 3). EH-domain-containing protein 1 (EDH1) and creatinine kinase B-type (CKB) were the two most abundant proteins identified in this group. Among these proteins a significant number of

proteins are known to be involved in cell morphology (CEACAM6, CR1, CRYAB, ERK, GNA12, GNA13, GNAQ, KDR, NOS3, PAFAH1B1, PP1CB, PTPRF, RAB4A, RYR2), metabolic disease and lipid metabolism (ACO1, CD7, DDC, EHD1, EXTL2, FAM125A, FLRT3, LPHN3, MAN2A2, PPIC, RAB4B, RAB5B, SORD, VPS28, and VPS37D).

Correlation between data obtained based on spectral count and ELISA assays

The relative abundance of identified urinary proteins was measured by protein-level spectral counts adopting a weighted fold-change statistic, and assigning increased weight for the more frequently observed proteins. Observed abundances of highly abundant proteins are less likely to be biased in a particular direction due to random variations in the peptide detection from sample to sample. The higher the abundance of the proteins involved, the more confident we are that the observed fold-change difference is close to the actual fold-change difference. A complete dataset of identified proteins and observed spectral counts for each protein for all the phenotypes tested is provided in (Supplemental Table 4). UMOD, SERPINF1 (PEDF), and CD44 proteins were analyzed by ELISA assay to obtain concentrations for the proteins. The spectral counts for the proteins measured by LC-MS were compared and correlated to the concentration calculated from ELISA assays on an independent set of the urine samples from similar phenotypes that were used in the discovery phase (Table 2). We observed a good correlation in between the spectral counts and quantitative data measured from ELISA assays. An ELISA assay was run for urinary Tamm-Horsfall protein. It was performed on an independent validation set of samples with AR (n=20), STA (n=20), and HC (n=20). The mean UMOD concentration in AR urine (5.50 ± 0.85 $\mu\text{g/mL}$) stable graft urine (13.95 ± 2.94 $\mu\text{g/mL}$), and healthy normal control urine (19.80 ± 2.71 $\mu\text{g/mL}$) has been compared with the spectral counts for AR (122), STA (363), and healthy control (567) with a correlation coefficient of 0.99 and P-value 0.02. The mean PEDF(SERPINF1) concentration in AR urine (0.370 ± 0.350 ng/mL), STA urine (0.006 ± 0.009 ng/mL), and HC urine (0.01 ± 0.009 ng/mL) was compared with the spectral counts for AR (75), STA (54), and healthy control (15) with a correlation coefficient of 0.78.

When we assayed CD44 in an independent sample set of urine samples, we measured a mean CD44 concentration in AR urine (1.67 ± 1.17 ng/mL), STA urine (2.81 ± 1.10 ng/mL), and HC urine (2.54 ± 1.41 ng/mL). This was in good correlation with spectral counts observed from LC-MS experiments for AR urine (15), STA (42), and healthy controls (126) with a correlation coefficient of 0.59. When we combined total concentration measured from ELISA assay and compared to the spectral counts for corresponding samples, there was an excellent correlation ($R^2=0.84$) with a P-value <0.0012 (Table 2).

Urinary proteins specific to Nephrotic Syndrome (NS)

We analyzed the urine from patients with nonspecific proteinuria. Nine proteins were uniquely identified in the NS urine, which included proteins like keratin 5b (KRT78), Isoform DPI of desmoplakin (DESP), Protein-glutamine gamma-glutamyltransferase 4 (TGM4), secretogranin-3 (SCG3), Periplakin (PPL), collagen type V alpha 1 (COL5A1), and cell growth regulator with EF hand domain 1 (CGREF1) (Supplemental Table 5). We also identified 20 proteins present in both HC and NS that were altogether absent from the renal transplant patients (Supplemental Table 6). The proteins that were either only present in NS urine or up-regulated in NS urine were involved in the acute phase response signaling ($P=4.53\text{E-}21$), the coagulation system ($P=2.17\text{E-}11$), and the complement system ($P=1.63\text{E-}02$).

Differential expression of proteins in acute rejection (AR)

We analyzed the relative abundance of proteins which were identified in both renal transplant patients with AR episode and those with stable graft (STA). There were 9 proteins that were identified only in AR urine, but not in urine of HC, STA, and NS phenotypes. These included

HLA class II histocompatibility antigen, DP(W4) beta chain (HLA-DBP), HLA class II histocompatibility antigen, DRB1-8 beta chain (IgHM), C4b-binding protein alpha chain (C4BPA), MHC class II antigen (HLA-DR), Myosin light chain 1 (MYL6B), HLA class II histocompatibility antigen DQ(3) beta chain (HLA-DQB1) (Table 4A). In addition a total of 68 proteins that were absent in AR, but present in HC, STA, and NS categories, included Isoform 1 of Melanotransferrin (MFI2), Isoform 1 of FRAS1-related extracellular matrix protein 2 (FREM2), Isoform 2 of FRAS1-related extracellular matrix protein 2 (ROR1), Isoform 2 of Neural cell adhesion molecule L1-like protein (PLD3), Golgi apparatus protein 1 (CRYL1), and Thyrotropin-releasing hormone-degrading ectoenzyme (TRHDE) (Table 4B). For a more rigorous quantitative analysis of proteins that were identified in AR and STA, we used spectral counts observed for each protein. Protein-level spectral counts were obtained by summing up peptide-level spectral counts. A total of 284 proteins were observed to be either up-regulated or down-regulated in the AR urine when compared to the patients with stable graft with a cut-off criteria of ≥ 2 of fold change in terms of log base(2) of spectral counts (Supplemental Table 7). The list of 23 up-regulated proteins included ceruloplasmin (CP), complement C5 (C5), hemoglobin subunit alpha (HBA1), prostate-specific antigen (PSA), complement C2 (C2), etc. and are listed in Table 5A. The proteins in this group were associated mainly with acute phase response signaling ($P=3.77E-08$) and complement system ($P=5.46E-07$). The top 23 down-regulated proteins out of 261 included α -1-microglobulin (AMBP), cubulin (CUBN), Tamm-Horsfall protein (UMOD), amylase, alpha (AMY2A), fibrillin1 (FBN1), and Collagen type XII, alpha1 (COL12A1) proteins and are listed in Table 5B. From their spectral count evaluations all 9 identified collagens, COL5A3, COL4A2, COL1A2, COL27, COL1A1, COL15A1, COL6A1, COL12A1 were decreased in AR urine including type IV collagenase (MMP-9) and its inhibitor TIMP-1. A number of SERPIN family members SERPING, SERPINB12, SERPINB3, and SERPINB4 were decreased in AR urine; whereas, two members SERPINC1 and SERPINF1 (PEDF) were increased. The down-regulated proteins were found to be involved in ECM-receptor interaction, cell communication, and Glycan structure degradation (all with $P \leq 0.0005$). We used up-regulated proteins in AR to generate a heat map (Figure 3), which demonstrate some of the injury mechanisms in AR.

Verification of AR associated proteins Tamm-Horsfall protein (UMOD), Pigment Epithelium-Derived Factor (SERPINF1), and CD44

We performed an ELISA assay on UMOD, PEDF(SERPINF1), and CD44 as AR specific novel urinary proteins for verification. We verified the decreased UMOD in AR patients. An ELISA assay was run for urinary UMOD, and was performed on an independent validation set of samples with AR ($n=20$), STA ($n=20$), and HC ($n=20$). The mean UMOD concentration in AR urine ($5.50 \pm 0.85 \mu\text{g/mL}$) was significantly lower than the stable graft urine ($13.95 \pm 2.94 \mu\text{g/mL}$) ($P < 0.01$) and the healthy normal control urine ($19.80 \pm 2.71 \mu\text{g/mL}$) ($P < 0.001$) (Figure 4A). In another experiment we observed an elevated concentration of PEDF(SERPINF1) in the AR urine compared to the urine collected from the urine from patients with stable graft function and other controls that included healthy normal control and non-specific proteinuric patients. The mean PEDF(SERPINF1) concentration in AR urine ($0.0396 \pm 0.0344 \mu\text{g/mL}$) was significantly higher than STA urine ($0.006 \pm 0.009 \mu\text{g/mL}$) ($P = 0.0001$), NS urine ($0.019 \pm 0.038 \mu\text{g/mL}$) ($P = 0.005$), and HC urine ($0.01 \pm 0.009 \mu\text{g/mL}$) ($P = 0.005$) (Figure 4B). When we assayed CD44 in an independent sample set of individual urine samples, we observed a decreased concentration of CD44 in the AR urine compared to the urine collected from stable graft function and other controls that included the healthy normals and non-specific proteinuric patients. The mean CD44 concentration in AR urine ($1.67 \pm 1.17 \text{ ng/mL}$) was significantly lower than STA urine ($2.81 \pm 1.10 \text{ ng/mL}$) ($P = 0.0001$), NS urine ($1.83 \pm 1.63 \text{ ng/mL}$) ($P = 0.005$), and the HC urine ($2.54 \pm 1.41 \text{ ng/mL}$) ($P = 0.005$) (Figure 4C). The ROC analysis was performed by the logistic regression model for the three proteins based on the values of AR vs. no-AR including STA, HC, and NS. The area under the curve for AR classification of CD44 is 97.3%

with $p=0.0058$, PEDF is 93.2% with $p=0.0205$ and UMOD is 84.6% with $p=0.0005$ (Supplemental Figure 1).

4. Discussion

This study describes the application of shotgun proteomics to expand the existing healthy normal urinary proteome database and its use in the identification and the verification of 3 potential biomarkers specific for AR in renal transplantation. Urine is the most relevant biofluid in biomarker discovery efforts for kidney diseases. Thus the analysis of its proteome is very relevant [29–30]. Mass spectrometry-based proteomics provides a fast and accurate means of obtaining protein identification from complex samples and allows for rapid screening for disease markers [31]. Renal transplantation has remained the optimal treatment for patients with end-stage kidney disease [14]. Even though improvement in the short term survival of grafts has been reported, AR of renal transplant still remains the primary risk factor for graft functional decline, chronic rejection and graft loss [32–35]. Therefore, AR specific biomarkers are important for patient and allograft surveillance. Herein, we used LC-MS based proteomics to investigate urine from kidney transplant patients. Using this approach, we have discovered the potential protein biomarker candidates that could provide a way to diagnose acute rejection of renal transplant effectively and non-invasively. For this discovery step, using pediatric and young adult patient samples, we applied a pooling approach to minimize individual and disease heterogeneity.

Different proteomics approaches have been applied to analyze the urinary proteome in the past, which has helped to build up the list of urinary proteins identified to date [7–10, 12–13]. Early studies used gel-based techniques to identify a relatively smaller number of proteins; whereas use of gel-free LC-MS has proven to be an efficient way to identify a greater number of proteins. Adachi *et al.* identified 1492 proteins using the urine collected from the healthy individuals [12]. In a recent report Gonzales *et al.* have identified 1160 from human urinary exosomes [13] (summarized in Figure 2). In this report we have identified a new set of urinary proteins with a stringent criteria of a minimum of two unique, non-redundant peptides per protein with $\sim 0.1\%$ FDR for protein identification. As summarized in Figure 2 there is a significant overlap among the list of proteins identified by Adachi *et al.* [36] and Gonzales *et al.* [13]. Yet there are new proteins identified in each study, which will eventually help to build a comprehensive human urinary proteome database. Apart from contributing to the existing urinary protein database, we have analyzed urinary proteins identified from the healthy normal controls to those with nephrotic syndrome and renal transplantation which yielded specific proteins related to renal injury associated with nephrotic syndrome as well as renal transplantation that included AR and stable graft function.

One of the challenges of translational research is that there is such a wide range (approximately as high as 10 orders of magnitude) of protein concentrations present in the bio-specimen, especially in blood and urine. The experimental design applied in this study has provided us with protein identifications for high abundance proteins such as UMOD with a concentration measured at 5 orders of magnitude (~ 0.07 mg/mL). This is more than the concentration measured for protein S100 calcium binding A4 protein (~ 2 ng/mL) in urine. In this study we calculated spectral counts as a semi-quantitative means for comparison and a weighted fold-change was used to derive a list of potential biomarker proteins. We tested three proteins whose concentrations differed by 4 orders magnitude, whereas there was a nearly perfect correlation to a good correlation for the proteins ranging from mean spectral counts 9 to 360 ($r^2 = 0.59–0.99$). The data suggest that the label-free LC-MS/MS spectral count data provides a relatively good quantification for high abundance to moderate abundance proteins. If the spectral count is low, it has a poor correlation with the real concentration in the sample and may require a more stringent labeling methods such as either iTRAQ [37] or the $^{18}\text{O}/^{16}\text{O}$ labeling method

[38]to achieve more accurate quantification. In this study, we used spectral counts as our measure of relative abundance to list potential AR specific proteins.

Given the scope of the study, we took three relevant protein candidates to verify their validity as being AR specific as discovered by the label-free approach using LC-MS/MS. Since the ELISA assay is known to be robust, sensitive for performing quantitative measurements of proteins in a simple setting unlike MRM. We performed the ELISA assay on THP (UMOD), PEDF(SERPINF1), and CD44 as AR specific novel urinary proteins. We have demonstrated that the reduced levels of THP (UMOD)and CD44 and the elevated level of PEDF(SERPINF1) in AR urine could be verified as both a highly specific and sensitive method to detect AR within the transplanted kidney, regardless of the confounding effect of proteinuria, immunosuppression, age or gender.

The Tamm-Horsfall Protein (also known as uromodulin -UMOD) is localized in the epithelial cells of the thick ascending limbs of Henle's loop and the most proximal part of the distal convoluted tubule[39]. This protein is suggested to be involved in the constitutive inhibition of calcium crystallization [40]. Mutation of the UMOD gene has been linked to familial juvenile hyperuricemic nephropathy (FJHN) as well as autosomal-dominant medullary cystic kidney disease (MCKD2) in children[41]. It has also been reported to be involved in prevention of urinary tract infection[42]. This protein has intrigued nephrologists for a long time because of its high abundance in healthy urine with no obvious role [39]. Kaden *et al.* observed reduced urinary UMOD delayed onset of transplanted function and increased urinary UMOD with the recovery of kidney health[43]. However, the use of UMOD as a diagnostic parameter was not recommended. Sejdieu *et al.* have recently related decreased UMOD in urine to the development of renal failure and cardiovascular death within 20 years in type1, but not in type 2 diabetes [44]. Our observation of the reduced level of Tamm-Horsfall protein in AR does agree with the pattern of low urinary UMOD in naturally occurring peptides that is seen in acute rejection and chronic allograft rejection and may need to be further validated with a larger cohort of patient samples[30,45].

Pigment epithelium-derived factor precursor (PEDF) is also known as serpin peptidase inhibitor. Clade F (SERPINF1) is a member of serine protease inhibitors and is known to be a potent inhibitor of angiogenesis in the eye [46]. PEDF(SERPINF1)was detected as one of the proteins whose level was elevated in the AR urine. PEDF(SERPINF1)is one of the major inhibitors of angiogenesis and is involved in physiological activities including wound healing, ischemia reperfusion injury and cancer metastasis to name a few examples. No direct correlation has been established for PEDF(SERPINF1)in renal injury. In a recent report Matsuyama *et al.* observed an increased PEDF(SERPINF1)level in the serum of diabetic patients with both diabetic retinopathy and nephropathy and suggested this could be a reflection of microvascular damage[47]. Our observation of the increased level of PEDF(SERPINF1)in AR urine could provide a new way to monitor the health status of renal transplant. It would take further investigation to understand the underlying mechanism related to its involvement in AR.

CD44 is a cell-surface glycoprotein known to be involved in cell-cell interactions, cell adhesion and migration [48]. It acts as a receptor for hyaluronic acid (HA), osteopontin, collagens, and matrix metalloproteinases (MMPs) [49]. A number of activities for this protein have been reported. These include lymphocyte activation, recirculation and homing, hematopoiesis, and tumor metastasis. Transcripts for this gene undergo a complex alternative splicing that results in many functionally distinct isoforms; however, the full length nature of some of these variants has not yet been determined.

High throughput genomics or proteomics studies generate not only a list of disease specific genes/proteins, but also help in understanding the underlying molecular pathways and events. Their biological activity and their association to different pathways provides a better understanding of the acute rejection event which is generally known to be mediated by T cell responses to antigens from the donor organs which are different than the ones in the recipient. This study has provided a broad view of underlying events in the kidney at the time of acute rejection. We observed up-regulation of MHC proteins which are involved in the presentation of foreign antigens to T cells. This observation is along the same trend from the observed role of increased abundance of MHC class II antibodies in previous reports [50–51]. By impact analysis on signaling pathways, we identified a number of AR specific urinary proteins that are part of the acute phase response, complement and coagulation cascades. On the other hand, there is a significant down-regulation of proteins involved with ECM, cytoarchitecture in AR urine when compared to STA and healthy controls. This suggests a significant turnover of extracellular matrix during an AR episode.

Conclusion

In summary, for the first time, we have demonstrated that shotgun proteomics is a viable way to discover potential biomarkers in transplantation. The outcome of this study demonstrates that comparative analysis strategy using pooled samples is a simple and effective way to achieve a list of potential biomarkers that can track with normal and disease states. Cross-validation of selected results from these studies, by an economically viable and convenient ELISA assay with an independent set of urine samples, demonstrates the feasibility of the translation of this approach to clinical practice. In conclusion, this label-free, semi-quantitative approach to analyze the urinary proteome in normal and disease states provides a robust and sensitive method for detection of urinary proteins for serial, non-invasive clinical monitoring for graft rejection after kidney transplantation. Since this study included pediatric and young adult patients as study subjects, a similar study on adult renal transplant patients will contribute in finding biomarkers that are more effective. In order to further validate the effectiveness of this approach in identifying AR specific marker proteins, a longitudinal response analysis on prospectively collected urine samples from a larger independent cohort of patients is warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

AR	Acute rejection
STA	Stable graft function
NS	Nonspecific proteinuria
HC	healthy normal control
BCA	Bicinchoninic acid

PEDF Pigment Epithelium-Derived factor

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Figure 1. Urinary proteins were identified from urine collected from healthy as well as renal patients with or without kidney transplant

Number of proteins identified in urine collected from renal transplant patients with biopsy proven acute rejection (AR), renal transplant patients with stable graft function (STA), healthy control (HC), and renal patients with nephrotic syndrome (NS).

Figure 2.

Urinary proteins identified from different patients groups including the healthy controls (HC) were compared. **[A]** A Venn diagram to compare urinary proteins from healthy normal individuals identified in this study to the proteins identified by Adachi *et al*¹² and urinary proteins identified by³⁸. **[B]** A comparison of proteins identified in healthy urine (HC) and urine of nephrotic syndrome (NS). **[C]** A comparison of proteins identified in healthy urine (HC) and urine of renal transplant patients both stable graft (STA) and acute rejection (AR) combined. **[D]** A comparison of proteins identified in urine from stable graft (STA) to urine of acute rejection (AR).

Figure 3.
A heat map demonstrating level of elevated proteins in AR compared to STA when compared to healthy urine and NS.

Figure 4. Verification of discovery of potential biomarker candidates by ELISA assay

Urinary protein level of three candidate proteins, THP, PEDF (SERPIN1), and CD44 were measured by ELISA using an independent set of samples from different phenotypes. **(A)** A decreased level of THP was observed in AR urine (n=20, mean concentration 5.50 μg/mL) when compared to STA urine (n=20, mean concentration 13.95 μg/mL) with $P < 0.01$ and healthy control urine (n=20, mean concentration 19.80 μg/mL) with $P < 0.001$. **(B)** An increased level of PEDF protein was observed in AR urine (n=20, mean concentration 0.40 μg/mL) when compared to STA urine (n=20, mean concentration 0.01 μg/mL) with $P = 0.0001$, with healthy control urine (n=8, mean concentration 0.01 μg/mL) with $P = 0.02$, and with nephrotic syndrome urine (n=6, mean concentration 0.02 μg/mL) with $P = 0.005$. **(C)** A decreased level of CD44 protein was observed in AR urine (n=20, mean concentration 1.67 ng/mL) when compared to STA urine (n=20, mean concentration 12.57 ng/mL) with $P < 0.00001$, with healthy control urine (n=6, mean concentration 11.76 ng/mL) with $P < 0.02$, and with nephrotic syndrome urine (n=6, mean concentration 8.54 ng/mL) with $P < 0.0002$. The boxes in the box plots are bounded by 75th and 25th percentiles of the data and the whiskers extend to the minimum and maximum values. As there are no statistically significant differences in urine osmolality between the samples groups (data not shown), the differences in concentration between groups are unlikely to be in part due to differences in water excretion, variably diluting or concentrating the markers.

Table 1
Patient demographic information

Patient information on 60 renal transplant patients (30 AR, 30 STA).

	Acute Rejection (AR) (n=30)	Stable Graft Function (STA) (n=30)	P value
Mean Age	12±5	14±5	0.21
Age Range	3–19	6–21	
Immunosuppression, %SF [#]	66%	50%	0.19
Race [*]	63%,13%,0%,17%,7%	59%,7%,10%,17%,7%	0.45
Donor, % living donor	40%	53%	0.44
Mean GFR (mL/min/1.73m ²)	87.45±38.46	124±29.86	0.0001

[#] SF: Steroid-free immunosuppression treatment, consisting of daclizumab induction + mycophenolate mofetil + tacrolimus

^{*} Race: 1=Caucasian; 2= Hispanic; 3=Asian; 4=African American; 5=Other

Table 2

Quantitative measurement of THP, PEDF, and CD44

Protein concentration for these proteins were measured by ELISA and correlated the concentration obtained with the spectral count data observed from label-free LC MS.

Protein Name	Samples	Concentration measured by ELISA assays (ng/ μ L)				Spectral count
		Minimum	Maximum	Median	Mean	
THP*	AR (n=20)	216.00	13000.00	4150	5504.50	122
THP	STA (n=20)	374.00	56828.00	10248	13951.90	363
THP	HC (n=20)	7424.00	66622.00	17865	19798.10	567
PEDF**	AR (n=20)	10.00	1357.00	327	395.95	75
PEDF	STA (n=20)	0.00	40.00	0	6.00	54
PEDF	HC (n=8)	0.00	30.00	10	10.00	15
PEDF	NS (n=6)	0.00	96.00	5	19.33	124
CD44	AR (n=20)	0.34	3.96	1.27	1.67	15
CD44	STA (n=20)	3.42	19.87	13.2	12.57	42
CD44	HC (n=6)	4.06	19.87	11.1	11.76	126
CD44	NS (n=6)	1.99	17.97	6.51	8.54	20
Cumulative correlation among all the concentration and spectral counts for 3 proteins				Correlation	0.84	
				P value	<0.0012	

* THP: Tamm-Horsfall Protein (UMOD)

** PEDF: Pigment Epithelium Derived Factor (SERPINF1)

Table 3

No bias of plasma or renal proteins in renal patients.

	Proteins identified in AR and NS combined	Proteins identified in HC and STA combined
Proteins identified in this study	(1206)	(1420)
Previously known urinary proteins	504	578
Previously known plasma/serum proteins	405	463
Previously known kidney proteins	353	434

Table 4

Proteins specific to acute rejection.

Table 4A: List of proteins identified only in AR urine			
S. No.	IPI ID	Gene Symbol	Protein Name
1	IPI00103082.7	<i>HLA-DBP</i>	HLA class II histocompatibility antigen, DP(W4) beta chain
2	IPI00005180.2	<i>IgHM</i>	HLA class II histocompatibility antigen, DRB1-8 beta chain
3	IPI00021727.1	<i>C4BPA</i>	C4b-binding protein alpha chain
4	IPI00641889.1	<i>KIAA1522</i>	25 kDa protein
5	IPI00746396.1		302 kDa protein
6	IPI00760688.2	<i>HLA-DR</i>	MHC class II antigen (Fragment)
7	IPI00027255.1	<i>MYL6B</i>	Myosin light chain 1, slow-twitch muscle A isoform
8	IPI00783351.1	<i>SUMF2</i>	sulfatase modifying factor 2 isoform d
9	IPI00743218.1	<i>HLA-DQB1</i>	HLA class II histocompatibility antigen, DQ(3) beta chain

Table 4B. List of proteins identified in all other phenotypes (STA, HS, and NS) but were not identified in AR urine.			
S. No	IPI ID	Gene Symbol	Protein Name
1	IPI00029275.1	<i>MFI2</i>	Isoform 1 of Melanotransferrin
2	IPI00180707.8	<i>FREM2</i>	Isoform 1 of FRAS1-related extracellular matrix protein 2
3	IPI00644416.1	<i>ROR1</i>	Isoform 2 of FRAS1-related extracellular matrix protein 2
4	IPI00328243.1	<i>PLD3</i>	Isoform 2 of Neural cell adhesion molecule L1-like protein
5	IPI00645031.1	<i>CRYL1</i>	Golgi apparatus protein 1
6	IPI00007798.1	<i>TRHDE</i>	Thyrotropin-releasing hormone-degrading ectoenzyme
7	IPI00556267.1	<i>GPC1</i>	FAT tumor suppressor homolog 4
8	IPI00218795.1	<i>SELL</i>	Isoform 2 of Melanotransferrin
9	IPI00156171.2	<i>ENPP2</i>	Isoform 1 of Ectonucleotide pyrophosphatase/phosphodiesterase 2
10	IPI00641153.2	<i>GLG1</i>	Glypican 1 variant (Fragment)
11	IPI00007244.1	<i>MPO</i>	Isoform H17 of Myeloperoxidase
12	IPI00604773.1	<i>PODXL</i>	Target of Nesh-SH3 (Tarsh) (Nesh binding protein) (NeshBP)
13	IPI00010790.1	<i>BGN</i>	Biglycan
14	IPI00064262.1	<i>DCHS1</i>	Protocadherin-16
15	IPI00641672.1	<i>CD320</i>	Isoform 3 of Crumbs homolog 2
16	IPI00019907.1	<i>GPC3</i>	Glypican-3
17	IPI00025240.1	<i>CDH16</i>	Isoform 1 of Cadherin-16
18	IPI00182728.2	<i>VPS4B</i>	Vacuolar sorting protein 4b
19	IPI00413016.3	<i>CADM2</i>	CDNA FLJ35635 fis, clone SPLEN2011805
20	IPI00413781.3	<i>CXCL12</i>	Vacuolar sorting protein 4a
21	IPI00419215.3	<i>A2ML1</i>	Hypothetical protein DKFZp761G128
22	IPI00385751.3	<i>FUCA1</i>	Delta-notch-like EGF repeat-containing transmembrane
23	IPI00025512.2	<i>HSPB1</i>	Heat-shock protein beta-1
24	IPI00165360.4	<i>MPST</i>	3-mercaptopyruvate sulfurtransferase

Table 4B. List of proteins identified in all other phenotypes (STA, HS, and NS) but were not identified in AR urine.			
S. No	IPI ID	Gene Symbol	Protein Name
25	IPI00017672.4	<i>NP</i>	Hypothetical protein FLJ25678
26	IPI00103175.1	<i>CANT1</i>	Isoform 1 of Soluble calcium-activated nucleotidase 1
27	IPI00217253.2	<i>GCHFR</i>	GTP cyclohydrolase 1 feedback regulatory protein
28	IPI00645085.2	<i>LOC653163</i>	Intrinsic factor-vitamin B12 receptor
29	IPI00646907.1	<i>SLC12A3</i>	ROR1 protein
30	IPI00011564.1	<i>SDC4</i>	Syndecan-4
31	IPI00023014.1	<i>VWF</i>	von Willebrand factor
32	IPI00024012.4	<i>FZD7</i>	Frizzled-7
33	IPI00027769.1	<i>ELA2</i>	Leukocyte elastase
34	IPI00333140.7	<i>DNER</i>	Phospholipase D3, isoform 1
35	IPI00009294.1	<i>CRIM1</i>	Cysteine-rich motor neuron 1 protein
36	IPI00017160.3	<i>VTA1</i>	Protein C6orf55
37	IPI00027510.1	<i>IL2RB</i>	Interleukin-2 receptor beta chain
38	IPI00719786.1	<i>CD248</i>	Lambda-crystallin
39	IPI00759832.1	<i>YWHAB</i>	PREDICTED: similar to Von Ebners gland protein
40	IPI00157414.3	<i>ENPP6</i>	Ectonucleotide pyrophosphatase/phosphodiesterase 6
41	IPI00220253.1	<i>FGFR3</i>	Erythrocyte band 7 integral membrane protein
42	IPI00291737.1	<i>ITLN1</i>	Xaa-Pro dipeptidase
43	IPI00296922.3	<i>LAMB2</i>	Intelectin-1
44	IPI00004946.8	<i>CXCL16</i>	chemokine (C-X-C motif) ligand 16
45	IPI00024896.2	<i>PBLD</i>	Probable isomerase MAWBP
46	IPI00030887.1	<i>TYRO3</i>	Tyrosine-protein kinase receptor TYRO3
47	IPI00299059.5	<i>CHL1</i>	Tumor-associated calcium signal transducer 2
48	IPI00607804.1	<i>CRB2</i>	Thioredoxin
49	IPI00002441.1	<i>SDC1</i>	Syndecan-1
50	IPI00005517.1	<i>EFNA5</i>	Ephrin-A5
51	IPI00008554.1	<i>ANG</i>	Angiogenin
52	IPI00016027.3	<i>FZD2</i>	Frizzled-2
53	IPI00021812.1	<i>AHNAK</i>	Neuroblast differentiation-associated protein AHNAK (Fragment)
54	IPI00025252.1	<i>PDIA3</i>	Protein disulfide-isomerase A3
55	IPI00158145.4	<i>LILRA5</i>	leukocyte immunoglobulin-like receptor subfamily A member 5 isoform 1
56	IPI00297910.1	<i>TACSTD2</i>	Laminin beta-2 chain
57	IPI00411356.5	<i>VPS4A</i>	stomatin isoform b
58	IPI00440824.2	<i>ABI3BP</i>	chemokine(C-X-C motif) ligand 12 (stromal cell-derived factor 1) isoform gamma
59	IPI00552768.1	<i>TXN</i>	CDNA FLJ41598 fis, clone CTONG2025496
60	IPI00646217.2	<i>IL10RB</i>	24 kDa protein
61	IPI00000861.1	<i>LASP1</i>	Isoform 1 of LIM and SH3 domain protein 1
62	IPI00006601.5	<i>CHGB</i>	Secretogranin-1
63	IPI00021034.1	<i>COL4A1</i>	Collagen alpha-1(IV) chain

Table 4B. List of proteins identified in all other phenotypes (STA, HS, and NS) but were not identified in AR urine.			
S. No	IPI ID	Gene Symbol	Protein Name
64	IPI00219682.5	<i>STOM</i>	L-selectin
65	IPI00413641.6	<i>AKR1B1</i>	CDNA FLJ35595 fis, clone SPLEN2008007
66	IPI00257882.6	<i>PEPD</i>	Isoform 2 of Fibroblast growth factor receptor 3
67	IPI00419327.2	<i>FAT4</i>	Aldose reductase
68	IPI00641203.1	<i>CUBN</i>	podocalyxin-like isoform 1

Table 5

Table 5A. Quantitatively up-regulated urinary proteins in AR compared to STA						
S. No.	IP1 ID	Gene Symbol	Protein Name	AR Spectral Counts	STA Spectral Count	Fold change (LOG2)
1	IP100017601.1	CP	Ceruloplasmin	439	141	2
2	IP100032229.1	C5	Complement C5	26	8	2
3	IP100410714.4	HBA1	Hemoglobin subunit alpha	30	9	2
4	IP100010858.1	KLK3	Prostate-specific antigen	21	4	2
5	IP100303963.1	C2	Complement C2	12	4	2
6	IP100747314.1		13 kDa protein	15	4	2
7	IP100477804.2		Immunoglobulin heavy chain variable region	10	3	2
8	IP100464948.3	HLA-DRA	major histocompatibility complex, class II, DR alpha	10	1	3
9	IP100021304.1	KRT2	Keratin, type II cytoskeletal 2 epidermal	5	1	2
10	IP100741163.1	LOC65265	PREDICTED: similar to Ig heavy chain V-II region ARH-77	6	2	2
11	IP100783393.1		Immunoglobulin heavy chain variable region	10	2	2
12	IP100745363.1	LOC652113	PREDICTED: similar to Ig heavy chain V-III region VH26	6	2	2
13	IP100386142.1		Ig heavy chain V-II region ARH-77	12	2	3
14	IP100737304.1	LOC652141	PREDICTED: similar to Ig heavy chain V-III region VH26	6	1	3
15	IP100556442.1	IGFBP2	Insulin-like growth factor binding protein 2 variant	5	1	2
16	IP100736985.1	LOC441368	PREDICTED: similar to Ceruloplasmin	21	5	2
17	IP100477540.2		13 kDa protein	9	3	2
18	IP100382540.1		Ig heavy chain V-II region NEWM	11	2	2
19	IP100386135.1		Ig lambda chain V-VI region SUT	4	1	2
20	IP100554676.1	HBE1	Hemoglobin subunit gamma-2	4	1	2
21	IP100387119.1		Ig kappa chain V-III region POM	11	3	2
22	IP100419517.1	IGHV1-69	IGHV1-69 protein	6	2	2

Table 5B. Quantitatively down-regulated urinary proteins in AR compared to STA				
S. No	IP1 ID	Gene Symbol	Protein Name	Fold change (LOG2)
1	IP100022426.1	AMBP	AMBP protein	2
2	IP100160130.3	CUBN	Cubilin	2

Table 5B. Quantitatively down-regulated urinary proteins in AR compared to STA

S. No	IPI ID	Gene Symbol	Protein Name	AR Spectral Count	STA Spectral Count	Fold change (LOG2)
3	IP100012503.1	<i>PSAP</i>	Isoform Sapmu0 of Proactivator polypeptide	93	427	2
4	IP100640271.1	<i>UMOD</i>	Tamm-Horsfall Protein	122	363	2
5	IP100745705.1	<i>AMY2A</i>	Amylase, alpha 2A; pancreatic variant	89	264	2
6	IP100744362.1	<i>FN1</i>	Hypothetical protein DKFP686K08164	36	126	2
7	IP100021885.1	<i>FGA</i>	Isoform 1 of Fibrinogen alpha chain	60	176	2
8	IP100784458.1	<i>FBN1</i>	312 kDa protein	30	112	2
9	IP100000073.1	<i>EGF</i>	Proepidermal growth factor	37	140	2
10	IP100328113.2	<i>FBN1</i>	Fibrillin1	20	76	2
11	IP100744835.1	<i>PSAP</i>	Isoform Sapmu9 of Proactivator polypeptide	71	312	2
12	IP100641961.1	<i>COL12A1</i>	Collagen, type XII, alpha 1	39	128	2
13	IP100783446.1	<i>GAA</i>	Lysosomal alpha-glucosidase	29	120	2
14	IP100329573.8	<i>COL12A1</i>	Isoform Long of Collagen alpha1(XII) chain	32	117	2
15	IP100023673.1	<i>LGALS3BP</i>	Galectin3binding protein	46	134	2
16	IP100385896.1	<i>SPP1</i>	Isoform D of Osteopontin	27	109	2
17	IP100293088.4	<i>GAA</i>	106 kDa protein	28	114	2
18	IP100008787.3	<i>NAGLU</i>	AlphaNacetylglucosaminidase	27	96	2
19	IP100741768.1	<i>LOC64213</i>	PREDICTED: similar to Maltaseglucoamylase, intestinal	25	114	2
20	IP100003919.1	<i>QPCT</i>	Glutaminylpeptide cyclotransferase	30	87	2
21	IP100783792.1	<i>MGAM</i>	192 kDa protein	10	43	2
22	IP100220143.2	<i>MGAM</i>	Maltaseglucoamylase, intestinal	22	97	2
23	IP100240345.3	<i>CLEC14A</i>	Ctype lectin domain family 14 member A	5	29	3