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The Identification of Novel Potential Injury Mechanisms and Candidate Biomarkers in Renal Allograft Rejection by Quantitative Proteomics*⑤

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Early transplant dysfunction and failure because of immunological and nonimmunological factors still presents a significant clinical problem for transplant recipients. A critical unmet need is the noninvasive detection and prediction of immune injury such that acute injury can be reversed by proactive immunosuppression titration. In this study, we used iTRAQ -based proteomic discovery and targeted ELISA validation to discover and validate candidate urine protein biomarkers from 262 renal allograft recipients with biopsy-confirmed allograft injury. Urine samples were randomly split into a training set of 108 patients and an independent validation set of 154 patients, which comprised the clinical biopsy-confirmed phenotypes of acute rejection (AR) ($n = 74$), stable graft (STA) ($n = 74$), chronic allograft injury (CAI) ($n = 58$), BK virus nephritis (BKVN) ($n = 38$), nephrotic syndrome (NS) ($n = 8$), and healthy, normal control (HC) ($n = 10$). A total of 389 proteins were measured that displayed differential abundances across urine specimens of the injury types ($p < 0.05$) with a significant finding that SUMO2 (small ubiquitin-related modifier 2) was identified as a "hub" protein for graft injury irrespective of causation. Sixty-nine urine proteins had differences in abundance ($p < 0.01$) in AR compared with stable graft, of which 12 proteins were up-regulated in AR with a mean fold increase of

2.8. Nine urine proteins were highly specific for AR because of their significant differences ($p < 0.01$; fold increase >1.5) from all other transplant categories (HLA class II protein HLA-DRB1, KRT14, HIST1H4B, FGG, ACTB, FGB, FGA, KRT7, DPP4). Increased levels of three of these proteins, fibrinogen beta (FGB; $p = 0.04$), fibrinogen gamma (FGG; $p = 0.03$), and HLA DRB1 ($p = 0.003$) were validated by ELISA in AR using an independent sample set. The fibrinogen proteins further segregated AR from BK virus nephritis (FGB $p = 0.03$, FGG $p = 0.02$), a finding that supports the utility of monitoring these urinary proteins for the specific and sensitive noninvasive diagnosis of acute renal allograft rejection. *Molecular & Cellular Proteomics 13: 10.1074/mcp.M113.030577, 621–631, 2014.*

Although improvements in immunosuppressive drugs, organ procurement, and surgical methods have advanced, there remains the inability to noninvasively diagnose and predict acute allograft rejection in solid organ transplantation in the clinical setting (1, 2). Currently available strategies for monitoring transplanted organs are both inefficient and lacking in accuracy to assess the risks of drug toxicity, and acute or chronic rejection (3, 4). Rapid advances in genomics and transcriptomics technologies have facilitated their application toward the understanding of graft injury mechanisms and more recently, the evaluation of gene polymorphisms and the validation of blood-based gene-panels that can diagnose and predict allograft rejection (5–12). Proteomic measurements of urine, a noninvasive biofluid suitable for monitoring renal transplant recipients, have revealed promising candidate urine protein biomarkers that are highly correlative of the graft milieu with the added benefit that the identified proteins may directly reflect the underlying biology (13–17).

In this report, we have analyzed a highly annotated cohort of clinical samples from a large database of pediatric and young adult renal transplant recipients. Applying an unbiased

TABLE I

Demographic information for 262 independent samples used for proteomics discovery (n = 108) and ELISA validation (n = 154). The collection, storage and processing of urine samples in this study was within the guidelines proposed by HKUPP (http://www.hkupp.org/ Urine%20collectiion%20Documents.htm)

	Discovery			
Phenotype	AR	STA	CAN	BKVN
Number of patients	30	30	30	18
Steroid-free/steroid-based	17/13	15/15	17/13	6/12
Recipient gender (%male)	53.3	50.0	60.0	66.6
eGFR (ml/min/1.73 m^2)	65.4 ± 24.6	117 ± 28.1	58 ± 11.8	69 ± 20.0
Recipient age[a]	13.8 ± 4.7	15.9 ± 4.2	12.1 ± 5.6	15.7 ± 3.4
Donor age[a]	24.8 ± 11.5	30.6 ± 9.7	30.3 ± 9.3	29.1 ± 10.0
Donor gender (%male)	48.4	52.7	61.9	59.5
Donor source (% living donor)	63.1	75.4	71.1	72.6
post-txp (mo)	24.4 ± 29.4	26.6 ± 37.1	13.6 ± 17.0	8.6 ± 5.6
ESRD cause (%) 1,2,3,4,5,6,7,8	10, 10, 3, 17, 7, 10, 10, 33	0, 10, 0, 17, 3, 10, 23, 37	7, 13, 7, 7, 0, 16, 10, 40	17, 0, 0, 0, 22, 11, 11, 39

	Validation						
Phenotype	AR	STA	CAN	BKVN	CNIT	Nephrotic Syndrome	Healthy Controls
Number of patients	44	44	18	20	10	8	10
Steroid-free/steroid-based	27/17	20/24	10/8	8/12	5/5	NA	NA
Recipient gender (%male)	54.6	57.5	65%	84.6	68%	50%	50%
eGFR (ml/min/1.73 m^2)	91 ± 31.2	112.3 ± 26.2	107.2 ± 29.4	87.0 ± 19.1	105 ± 25.0	NA	NA
Recipient Age[a]	13.25 ± 5.57	15.03 ± 3.27	10.16 ± 6.53	14.86 ± 4.49	12 ± 5.4	11.5 ± 4	12.2 ± 6.4
Donor age[a]	30.10 ± 10.64	30.24 ± 10.44	26.12 ± 6.65	27.44 ± 11.59	25.6 ± 5.5	NA	NA
Donor gender (% male)	49.5	43.2	64.2	61.3	45.8	NA	NA
Donor source (% living donor)	60.1	69.3	58.9	52.6	57.9	NA	NA
post-txp (mo)	11.8 ± 7.7	33.4 ± 45.4	29.8 ± 32.0	11.1 ± 10.5	34.4 ± 22.6	NA	NA
ESRD cause (%) 1,2,3,4,5,6,7,8	9, 7, 2, 9, 2, 11, 9, 41	2, 9, 0, 14, 0, 11, 18, 36	11, 6, 0, 11, 0, 17, 28, 39	0, 10, 0, 10, 0, 20, 20, 40	30, 10, 0, 0, 20, 0, 0, 40	NA	NA

[a] Recipient Age, mean ± stdev; ESRD cause 1, Reflux Nephropathy;2, Focal Segmental Glomerulosclerosis; 3, Cystinosis; 4, Aplastic/ Hypoplastic/Dysplastic Kidneys; 5, Congenital Nephrotic Syndrome; 6, Obstructive Uropathy; 7, Unknown; 8, Other.

high-throughput proteomic approach, we identified candidate urine protein biomarkers for biopsy-confirmed acute rejection and then performed orthogonal, targeted validation by ELISA to select the most informative urinary proteins that would clearly define acute allograft rejection from all other confounding transplant phenotypes. Customized bioinformatics analysis of these datasets provided the additional benefit of revealing a common molecular network responsible for driving kidney transplant injury. The overall study is summarized in Fig 1.

MATERIALS AND METHODS

Study Population and Samples—A total of 262 urine samples were analyzed in this study: acute rejection (AR) (n = 74), stable graft (STA) (n = 74), chronic allograft injury (CAI) (n = 58 total samples; 48 samples had chronic allograft nephropathy based on the Banff criteria (18); 10 had evidence of chronic allograft injury because of calcineurin inhibitor drug nephrotoxicity based on the CNIT score (19)); BK (n = 38), patients with nonspecific proteinuria from native renal disease because of nephrotic syndrome (NS; to control for nonspecific renal

injury) (n = 8), and healthy normal control (HC) (n = 10). All samples were selected from a large and highly annotated urine sample biobank collected from pediatric and young adult recipients of kidney transplants in years 2000 to 2009 at Lucile Packard Children's Hospital at Stanford University. The biorepository consisted of ~2000 banked urine samples of which 770 were biopsy matched and collected before any treatment intensification for clinical graft dysfunction. From this subset of 770 patients, demographically matched clinical categories of different categories of stable graft function and allograft injury were selected for a total of 244 urine samples. The cause of ESRD is shown in Table I. Only first transplants from low risk patients with peak PRA <20% were included. Eight patients were on peritoneal or hemodialysis with little or no residual renal function before transplant and the majority of patients (n = 236) had received preemptive transplantation and had residual renal function with an eGFR (Schwartz) of ~15–20 ml/min/1.73 m². Eighteen additional samples were included for analysis from age-matched healthy controls (n = 10) and patients with nephrotic syndrome (n = 8). A summary of the patient demography selected for this study is presented in Table I. Urine samples were randomly split into (1) a training set of 108 patients, and (2) an independent validation set of 154 patients.

We balanced the study subjects in terms of their being either on steroid-based treatment or steroid-free treatment following transplantation. "*Allograft injury*" in this study was defined as a > 20% increase in serum creatinine from its previous steady-state baseline value and an associated biopsy that was pathological. The histological diagnoses spectra for the "allograft injury" cases (n = 170) were either acute rejection (n = 74), chronic injury (n = 58), or BK nephritis (n = 38). There were 74 samples collected at the time of stable graft function and normal histology on the paired biopsy and were called "stable." All biopsies were blindly, semiquantitatively scored by a single pa-

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[1] The abbreviations used are: AR, acute rejection; CAI, chronic allograft injury; BKVN, BK virus nephritis; NS, nephrotic syndrome; HC, healthy normal control; SUMO2, small ubiquitin-related modifier 2; FGB, fibrinogen beta; FGG, fibrinogen gamma; eGFR, estimated glomerular filtration rate; ESRD, end-stage renal disease; ESI, electrospray ionization; HCD, high-energy collisional dissociation; ROC, Receiver Operating Characteristic.

thologist using the most recent Banff criteria for both acute and chronic injury (18, 20–22). AR was defined at minimum, as per Banff Schema, a tubulitis score ≥1 accompanied with an interstitial inflammation score ≥1. Patients had varying grades of AR: Banff 1A, Banff 1B, Banff 2A, and Banff 2B; 2 patients had humoral AR as evidenced by C4d positivity in the allograft. Chronic allograft injury (CAI) was defined as patients with poor allograft function (see eGFR in Table I) (23) and on biopsy with a tubular atrophy score ≥1 accompanied by an interstitial fibrosis score ≥1 (18). BK nephritis (BKVN) was defined as acute (>20% increase in serum creatinine) or chronic graft dysfunction with demonstration of a positive blood BK viral load, graft inflammation and a positive immunohistochemical stain for the polyoma virus. Normal (STA) allografts were defined as patients with stable serum creatinine and on absence of significant injury on protocol biopsy. Given the very low incidence of acute tubular necrosis in our transplant program because of a bias toward living donation and a selection of deceased donors with low cold ischemia times, the category of acute tubular necrosis as a transplant injury subtype was not included for analysis because of low sample numbers. The patients included in this study were all on maintenance immunosuppression with tacrolimus, mycophenolate mofetil, and either on maintenance steroids or on a steroid avoidance protocol (24). The study was approved by the Ethics Committee of Stanford University Medical School and California Pacific Medical Center Research Institute (CPMCRI), and all patients/guardians provided informed consent to participate in the research, in full adherence to the *Declaration of Helsinki*. This study was approved by the Institutional Review Board for Human Subjects Research at Pacific Northwest National Laboratory in accordance with federal regulations.

Urine Collection, Initial Processing and Storage—We followed the standards recommended by the Human Kidney and Urine Proteome Project (HKUPP) when collecting and processing samples from the kidney transplant clinic. Briefly, second morning, void mid-stream urine samples (50–100 ml) were collected in sterile containers, centrifuged at $2000 \times g$ for 20 min at room temperature within 1 h of collection, and the supernatant was separated from any pelleted particulate matter that included cells and cell debris. The pH of the supernatant was adjusted to 7.0 and stored at −80 °C until further analysis. In our previous report we established protocols allowing for stable urine collection from multicenter clinical studies (25), in which delays in storage and processing may occur. In our protocols, we determined that urine samples can be safely stored up to 1 h at room temperature and up to 12 h at 4 °C without significant protein degradation. Samples do not require the addition of protease inhibitors to maintain sample integrity, if stored at 4 °C or −80 °C within 72 h of collection. Centrifugal filtration is our optimal processing method of choice. Urine protein was quantified using the Bradford assay. Measured total protein concentrations in the urine of acute rejection, stable graft, chronic allograft injury, BK virus nephropathy, nephrotic syndrome, and healthy normal control were 1.8 ± 2.3, 2.4 ± 1.9, 2.6 ± 2.7, 1.2 ± 0.6, 15.9 ± 18.7, and 1.8 ± 1.7 mg/ml, respectively. To minimize impact of freeze thaw cycles, we aliquoted urine samples into 10 ml aliquots (5–10 tubes per sample) before freezing at −80 °C, to facilitate multiple assays without any freeze thaw cycles of the processed urine. Our assay uses a starting volume of 10 ml of urine so each aliquot only needs to be thawed once for each experiment.

Recovery, Quantification and Trypsin Digestion of Urinary Protein—Using a urine sample processing method that we developed, urinary proteins were isolated by filtering the supernatant through Amicon Ultra centrifugal filtration tubes (10K molecular weight cutoff, Millipore, Bedford, MA) to separate small MW peptides and other pigments (<10 kDa) from the larger proteins. The device was initially washed with 10 ml of 50 mM NH$_4$HCO$_3$ (pH 8.0) and discarded. Then a 10 ml aliquot of urine was loaded into the device and centrifuged for

20 min at $3000 \times g$ at 10 °C, the filtrate was recovered, and saved for a peptidomics analysis, and the retentate was used for proteomics studies. The final volume of the retentate was adjusted to 400 μl with 50 mM NH$_4$HCO$_3$ (pH 8.0). For iTRAQ-based quantitative analysis, the samples consisted of six unique pools for each phenotype with each pooled sample generated from: (1) 5 patients with acute rejection (AR); (2) 5 patients with chronic allograft nephropathy (CAN); (3) 3 patients with the BK virus (BK); or (4) 5 patients who are stable graft function (STA). All the samples were assayed with bicinchoninic acid (BCA) (Thermo Scientific, Rockford, IL) to determine the protein concentration. An equal mass of protein (200 μg) was collected from each sample and brought to a total volume of 100 μl using 0.5 M triethylammonium bicarbonate (TEAB) (all chemicals purchased from Sigma-Aldrich, St. Louis, MO, unless otherwise stated). Powdered urea was added to each tube to a final concentration of 8 M for protein denaturation and 500 mM dithiothreitol (DTT) was added to a concentration of 10 mM for reduction. The samples were briefly sonicated, and incubated at 37 °C for 1 h with constant shaking in a thermomixer (Eppendorf North America, Hauppauge, NY). Iodoacetamide was added to each sample to reach a concentration of 40 mM for alkylation and then incubated in the dark at 37 °C for 1 h with constant shaking. Samples were then diluted 10-fold using 0.5 M TEAB and digested with trypsin (50:1 protein/trypsin (w/w)) (Promega, Madison, WI) at 37 °C for 3 h. To clean the peptide preparations for analysis, C-18 solid phase extraction was performed using Supelco Discovery columns on a Gilson GX-274 ASPEC™ system (Gilson Inc., Middleton, WI). All samples were then concentrated in a speed-vac to 30 μl and measured with the BCA assay to determine the peptide concentration.

8-plex iTRAQ Labeling—The pH of each sample was measured and brought to ∼ pH 8 using 1 M TEAB. The sample concentrations were measured with the BCA assay and then vialed to contain 25 μg of peptides for each sample. Sample volumes were adjusted to 15 μl total volume either by adding 0.5 M TEAB or concentrated to 15 μl in a low-protein-binding 1.5 ml centrifuge tube. Each vial of 8-plex iTRAQ reagent (AB Sciex, Framingham, MA) was thawed and brought to room temperature. The reagents were pulse spun to ensure the contents were collected at the bottom of each tube and 60 of isoproponal was added to each reagent vial. The reagent vials were thoroughly vortexed, briefly centrifuged, and added to the appropriate sample. Each reagent vial was rinsed with an additional 10 μl of isopropanol and added to their respective samples.

The iTRAQ reagents with eight channels, ranging from 114 to 121, were used to label two AR, two BKV, two CAI, and two STA samples, respectively, for each eight-plex iTRAQ experiment. Three eight-plex experiments were performed for the 24 pools from the four phenotypes. For each labeling reaction, the pH was above 7.8 and the organic concentration was at least 60% (v/v). To mix the reagents, each sample was vortexed,d briefly centrifuged and incubated at room temperature for 2 h. The labeling reactions were stopped by the addition of 100 μl of nanopure water to hydrolyze any remaining iTRAQ reagent in each sample, and incubated for an additional 30 min. The labeled samples were partially concentrated in a speed vac to remove the organic solvent and then pooled together to obtain three samples with each containing all eight of the iTRAQ labels (two from the AR group (113, 114), two from the BK group (115, 116), two from the CAI group (117, 118) and two from the STA group (119, 121). The three mixed samples were concentrated to a volume of ∼100 μl in a speed vac.

High pH Reversed-phase C-18 Fractionation—Each of the three eight-plex iTRAQ-labeled peptide samples were fractionated into 24-fractions by high pH reversed-phase chromatography (26). Following dilution to a volume of 900 μl with 10 mM ammonium formate buffer (pH 10.0), each sample was resolved on a XBridge C18, 250×4.6

mm, 5-μm particle size, with 4.6 × 20 mm guard column (Waters, Milford, MA). Separations were performed at 0.5 ml/min using an Agilent 1100 series HPLC system (Agilent Technologies, Santa Clara, CA) with mobile phases (A) 10 mM Ammonium Formate, pH 10.0, and (B) 10 mM Ammonium Formate, pH 10.0/acetonitrile (10:90). The gradient was changed from at 100% A to 95% A over the first 10 min, 95% A to 65% A from 10 min to 70 min, 65% A to 30% A from 70 min to 85 min, maintained at 30% A from 85 min to 95 min, re-equilibrated with 100% A from 95 min to 105 min, and held at 100% A until 120 min had elapsed. Fractions were collected every 1.25 min (96 fractions over the entire gradient). The plate fractions were concentrated in a speed vac and then the 96 fractions in each plate were combined into a total of 24 final fractions samples (each with $n = 4$ fractions pooled) (*i.e.* each fraction combined with every 24th fraction). The pooled fractions were then completely dried down, 25 μl of 25 mM ammonium bicarbonate was added to each fraction, and plates with pooled fractions were stored at −20 °C until time for LC-MS/MS analysis.

LC-MS/MS Analysis—Peptide mixtures were analyzed on a high resolution, reversed-phase capillary LC system coupled with a Thermo Fisher Scientific LTQ-Orbitrap Velos MS (San Jose, CA). The automated LC system was custom built using two Agilent 1200 nanoflow pumps and one Agilent 1200 capillary pump (Agilent Technologies, Santa Clara, CA), and a PAL autosampler (Leap Technologies, Carrboro, NC). Full automation was made possible by custom software that allowed for parallel event coordination and therefore, ~100% of the MS duty cycle was used through the use of two trapping and two analytical capillary columns. Capillary reversed-phase columns were prepared in-house by slurry packing 3-μm Jupiter C18 (Phenomenex, Torrence, CA) into 35-cm x 360 μm o.d. x 75 μm i.d fused silica (Polymicro Technologies Inc., Phoenix, AZ). Trapping columns were prepared similarly, but using 3.6-μm Aeris Widepore XB-C18 packed into a 4-cm length of 150 μm i.d. fused silica. Mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid acetonitrile (B) operated at 300 nL/min constant flow with a gradient profile over the course of 100 min as follows (min:%B); 0:5, 2:8, 20:12, 75:35, 97:60, 100:85. Sample injections (5-μl) were trapped and washed on the trapping columns at 1.5-μl/min for 20 min before alignment with analytical columns. Two column operation also allowed for columns to be "washed" (shortened gradients) and regenerated off-line without any cost to duty cycle.

MS analysis was performed on a LTQ-Orbitrap Velos mass spectrometer outfitted with a custom electrospray ionization (ESI) interface. Electrospray emitters were custom made by chemically etching 150 μm o.d. × 20 μm i.d. fused silica (27). The heated capillary temperature and spray voltage were 350 °C and 2.2 kV, respectively. Full MS spectra were recorded at a resolution of 100K (for ions at *m/z* 400) over the range of *m/z* 400–2000 with an automated gain control (AGC) value of 1e6. MS/MS was performed in the data-dependent mode with an AGC target value of 3e4. The ten most abundant parent ions, excluding single charge states, were selected for MS/MS using high-energy collisional dissociation (HCD) with a normalized collision energy setting of 40%. A dynamic exclusion time of 45 s was used.

ELISA assays of FGB, FGG, and HLA DRB1—Commercially available ELISA assays for fibrinogen beta (FGB) (Cat#ABIN418172), fibrinogen gamma (FGG) (Cat#ABIN416913), HLA class II DRB11 (Cat#ABIN1137134), and SUMO2 (Cat# ABIN591073) were purchased from Antibodies-online (Atlanta, GA). After optimization for urine dilution, the manufacturer's protocol was followed.

Data Analysis—iTRAQ-labeled peptides were identified based on tandem MS/MS spectra. MS/MS spectra were converted to peak lists using DeconMSn (version 2.2.2.2, http://omics.pnl.gov/software/DeconMSn.php) (v1) using default parameters. The database used was SEQUEST v27. The protein database from Uniprot was downloaded (released May 5, 2010) with 20,776 total entries. Both fully and partially tryptic peptides were considered with two missed cleavages allowed. The mass tolerance for precursor ions was 50 ppm and fragmentation tolerance for HCD were 0.05 Da. All peptides were identified with <0.1% False Discovery Rate by using a MS-Generating Function Score (MS-GF) <1E-10 and a decoy database searching strategy (28). The reporter ion intensities for each peptide were summed for all identified spectra for each channel in each biological condition. Relative abundances at the peptide level were rolled-up to the protein level using the software tool DAnTE (29) with the abundances being log2 transformed and normalized by the central tendency approach. For each eight-plex iTRAQ experiment, a global mean (*i.e.* average abundance) was calculated for each protein across the eight channels in to serve as a baseline for normalization across the three iTRAQ experiments. All protein abundances (log2 transformed) in the three iTRAQ experiments were normalized against the global mean (*i.e.* subtracting by the global mean) obtained from their respective iTRAQ experiment to identify increased or decreased protein abundances in each phenotype. Differential expression analysis was conducted in AltAnalyze (30) version 2.0.8 with the Ensembl version 65 human database. Proteins were considered to have differential abundances with an empirical Bayes *t* test *p* value < 0.05 and fold change > 1.5. Pathway and gene set enrichment analysis and visualization were performed using the GO-Elite module of AltAnalyze. Protein-protein and protein-DNA interaction networks in AltAnalyze were built using annotated interactions from Wikipathways, KEGG, PAZAR and Amadeus using direct interactions only. For ELISA data, the urine protein levels were normalized to urine creatinine levels. GraphPad PRISM (GraphPad Software, Inc., La Jolla, CA) was used for statistical analysis, *p* values were calculated using an unpaired *t* test, and fold changes were used for significant increases or decreases of proteins in the urine. Nominal logistic regression model was performed on the ELISA data collected from the three proteins FGB, FGG, and HLA-DRB1. Receiver operating characteristic (ROC) analysis was conducted on a combined panel of 3 proteins to predict AR with fitted logistic regression models using log-transformed urine protein quantities. Given that different injury phenotypes can co-exist in the graft, we developed a multinomial regression model with an elastic-net penalty to estimate the risk score for each injury subtype. The computation was done in R using the package glmnet.

RESULTS

Peptide and protein identifications for kidney transplant urine—Application of the 2D-LC-MS/MS strategy for iTRAQ-labeled pooled samples led to the identification of a total of 6379 unique peptides with FDR <0.1% at the peptide level (supplemental Table S1), covering 958 human proteins (supplemental Table S2). The numbers of proteins quantified across the three eight-plex iTRAQ experiments were 824, 780, and 805, respectively. The normalized abundance profiles in log2 transformation format against a global mean are listed in supplemental Table S2.

Among this set, parametric analysis of all possible groups of pair-wise comparisons yielded a set of 389 proteins demonstrating a 50% difference in protein abundances among groups (moderated *t* test *p* < 0.05) (supplemental Table S3). Principal component analysis for the first three components indicated overall similarity among the distinct injury sample sets for these 389 proteins across the 24 pooled injury profiles (supplemental Fig. S1*A*). Unsupervised clustering of these

Increased in AR	Increased in BKVN	Increased in CAI (CAN and CNIT)
HLA-DRB1, FGB, FGA, FGG, KRT14, HIST1H4B, ACTB, KRT7, DPP4	KRT18, SUMO2, STMN1, CFHR2, KRT8, KRT19, RPL18, KRT75, FAM3C, HIST1H2BA	CALR, FAM151A, SERPINA2P, FAM3C, DAG1, KITLG, LUM, FABP4, AGT, LRG1

proteins using the HOPACH algorithm indicates largely distinct profile expression of AR, BKV, and CAI from STA (supplemental Fig. S1B and supplemental Table S3). SUMO2 (small ubiquitin-related modifier 2) was identified as a "hub" protein for graft injury irrespective of causation (see below).

AR Enriched Proteins—With criteria of >1.5 fold change and p value <0.05, there were 28 urine proteins increased and 125 urine proteins decreased in AR urine when compared with STA urine. The top 10 most significantly increased urine proteins in AR included ACTB, HIST1H4B, FGG, KRT18, FGB, HLA-DRB1, FGA, KRT14, KRT7, and DPP4 of which 9 were specific to AR ($p < 0.01$; fold increase >1.5) (Table II). The top 10 most significantly decreased proteins included COL1A1, BCAN, SHISA5, PGA4, HTRA1, RTN4RL2, VGF, SUSD2, HAVCR2, and GGT6.

BK Infection Enriched Proteins—Analysis of urine proteins collected from patients with BKV nephritis revealed 94 urine proteins increased and 26 urine proteins were decreased in BKV urine when compared with STA urine with the criteria of >1.5-fold change and p value <0.05. The top 10 most significant increased urine proteins in BKV are shown in Table II.

CAI Enriched Proteins (CAN and CNIT Samples)—Analysis of urine proteins collected from patients with CAI resulted in 48 urine proteins significantly increased with the criteria >1.5-fold change and p value <0.05. The top 10 most significant increased urine proteins in CAI are shown in Table II.

Comparison and Functional Analysis of Distinct Forms of Renal Transplant Injury—We compared urine proteins that are increased in different graft injury phenotypes (AR, BKV, and CAI) relative to STA. As illustrated by the Venn diagram in Fig. 2A, a set of eight proteins (CALR, CTSZ, DPP4, FABP4, FBXL19, FKBP1A, KITLG, and SUMO2) are increased in all the transplant injury phenotypes studied. Likewise, multiple overlapping sets were found when each individual group was compared. This core common set, was associated with T-cell activation (DPP4, FKBP1A) and ubiquitin protein ligase binding (CALR, SUMO2), with both direct and indirect predicted interactions among these proteins. Additionally, a group of proteins associated in a network that includes proteins that are known as extracellular matrix proteins and proteins involved in fibrosis (CD40, VIM, ACTB, CALR, CTSZ, KITLG, COL11A1, DPP4, and TGFBR1) (30–33)(Fig. 3B).

To properly evaluate those proteins with highly restricted injury profiles, we performed a marker discovery analysis in Alt-Analyze with the function MarkerFinder (supplemental Table S4).

Proteins most correlated with either AR, BKV, or CAI associated with a diverse and distinct set of biological processes (Fig. 2B). Proteins uniquely expressed in AR were characterized as belonging to pathways of blood clotting (FGA, FGB, and FGG) and keratin filaments (KRT1, KRT10, KRT14, KRT2, KRT5, KRT7, and KRT9). BKVN selective proteins were characterized as associating with contractile fiber (ACTA2, ALDOA, DES, ENO1, KRT19, MMP2, TPM1, TPM3, and TPM4), gene expression regulation (EEF1A1, HSPA8, RPL14, RPL18, SET, and SRP14), glycolysis (ALDOA, ENO1, PKM2, and TPI1), and response to virus (ACTA2, CFL1, ENO1, and STMN1). Proteins expressed specifically in CAI were predominantly annotated as sugar binding (ACAN, CLEC14A, GLB1, LGALS9B, LMAN2, MRC2, and SELL), regulation of caspase activity (CD27, DPEP1, and F2), and generally associated with immune system phenotypes (phenotype ontology). Although some of these "marker" proteins were up-regulated in more than one type of injury, they were still found to be more selective for the indicated injury group, such as KRT19 with BKV.

To better explore the functional relationships among differentially expressed proteins, we examined putative protein-protein and protein-DNA interactions using the analysis toolkit AltAnalyze. When viewed in the context of just direct interactions, proteins in both AR and BK formed a highly connected network of proteins (supplemental Fig. S2). In both sets, the common regulated protein SUMO2 formed a strong interaction hub to many up-regulated proteins.

ELISA Validation of AR Specific Proteins—We chose four proteins to validate, fibrinogen beta (FGB), fibrinogen gamma (FGG), small ubiquitin-related modifier 2 (SUMO2), and MHC-II protein (HLA-DRB1), based on their statistical significance and biological relevance in AR. When measured by commercially available ELISA assays, the significance of three proteins, fibrinogen beta, fibrinogen gamma, and HLA-DRB1, were validated on an independent set of urine samples. The increased protein level of fibrinogen beta (FBB) in AR urine (27.8 ± 64.4 ng/mg urine creatinine) was significant when compared with STA urine (7.1 ± 8.7 ng/mg urine creatinine) ($p = 0.04$), CAI urine (8.1 ± 6.8 ng/mg urine creatinine) ($p = 0.05$), BKV urine (4.9 ± 3.9 ng/mg urine creatinine) ($p = 0.03$), NS urine (4.2 ± 2.2 ng/mg urine creatinine) ($p = 0.02$), HC urine (2.0 ± 1.1 ng/mg urine creatinine) ($p = 0.01$), and CNIT urine (6.6 ± 6.3 ng/mg urine creatinine)($p = 0.04$) (Fig. 3A). Similarly, the increased protein level of fibrinogen gamma (FBG) in AR urine (27.4 ± 60.1 ng/mg urine creatinine) was
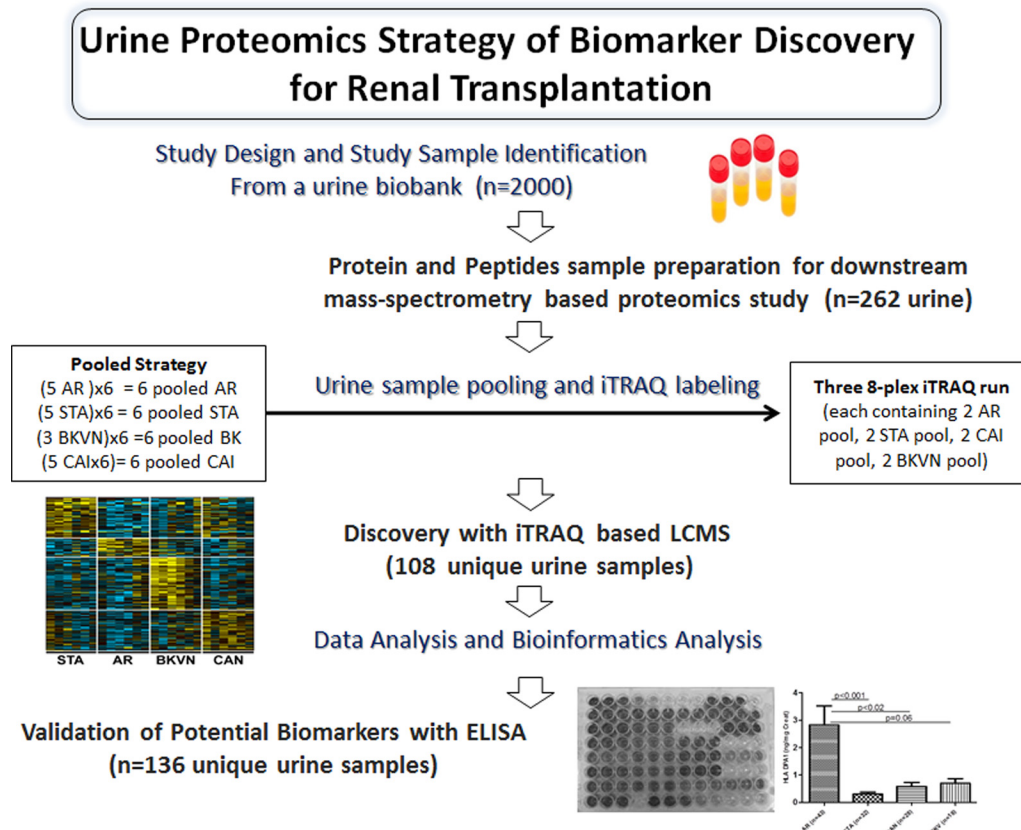
FIG. 1. **The study design used in this study for effective urine protein biomarker discovery for kidney transplantation.**

significant when compared with urine of STA urine (7.5 ± 8.5 ng/mg urine creatinine) ($p = 0.04$), CAI urine (8.5 ± 7.0 ng/mg urine creatinine) ($p = 0.05$), BKV urine (5.4 ± 4.3 ng/mg urine creatinine) ($p = 0.02$), NS urine (5.0 ± 2.4 ng/mg urine creatinine) ($p = 0.02$), HC urine (2.5 ± 1.5 ng/mg urine creatinine) ($p = 0.01$), and CNIT urine (6.6 ± 6.3 ng/mg urine creatinine)($p = 0.04$) (Fig. 3B). The protein level of HLA-DRB1 was significantly higher in AR urine (2.8 ± 4.6 ng/mg urine creatinine) when compared with its level in STA urine (0.4 ± 0.4 ng/mg urine creatinine) ($p = 0.001$), CAI urine (0.6 ± 0.8 ng/mg urine creatinine) ($p = 0.003$), BKV urine (1.2 ± 1.6 ng/mg urine creatinine) ($p = 0.04$) , NS urine (0.1 ± 0.1 ng/mg urine creatinine) ($p = 0.0003$), HC urine (0.1 ± 0.1 ng/mg urine creatinine) ($p = 0.0003$), and CNIT urine (0.2 ± 0.1 ng/mg urine creatinine)($p = 0.0005$) (Fig. 3C). The protein level of SUMO2 was significantly higher in AR urine ($n = 24$) and CAI urine ($n = 16$) combined (13.6 ± 18.1 ng/mg urine creatinine), when compared with STA urine (4.2 ± 5.8 ng/mg urine creatinine) ($p = 0.0005$) (Fig. 3D). SUMO2 level was significantly higher in AR urine (10.1 ± 5.6 ng/mg urine creatinine) when compared with STA urine (4.2 ± 5.9 ng/mg urine creatinine) ($p = 0.005$), BKVN urine (4.0 ± 5.1 ng/mg urine creatinine) ($p = 0.04$), NS urine (0.3 ± 0.2 ng/mg urine creatinine) ($p = 0.0003$), and HC urine (0.2 ± 0.4 ng/mg urine creatinine) ($p = 0.0003$). The SUMO2 protein level was also

significantly higher in CAI (18.9 ± 24.5 ng/mg urine creatinine) urine than in STA urine (4.2 ± 5.9 ng/mg urine creatinine) ($p = 0.009$), BKVN urine (4.0 ± 5.1 ng/mg urine creatinine) ($p = 0.03$), NS urine (0.3 ± 0.2 ng/mg urine creatinine) ($p = 0.008$), HC urine (0.2 ± 0.4 ng/mg urine creatinine) ($p = 0.008$), and CNIT urine (6.6 ± 6.3 ng/mg urine creatinine)($p = 0.03$) (Fig. 3E). ROC curve analysis performed to identify AR from the rest of the phenotypes (CAI, BK, and STA) on the data from three urine proteins yielded an AUC of 0.8.

Controls for Clinical and Pathology Confounders—We checked the association between patients' phenotypes and their demographic information to ensure no demography related confounders were driving the data. Because height and weight of the recipients were highly correlative to their age, we looked into the age association with different phenotypes. Recipient age was significantly associated with patients' phenotypes with p value < 0.001. When examined for the association between the three potential biomarker (log-transformed) and recipients' age, there was no significant association found among them ($p = 0.187$ for FGB; $p = 0.260$ for FGG; $p = 0.633$ for HLA-DRB1). Based on this, we did not control for recipient age for the three potential biomarkers. There were significant associations between three potential biomarker proteins and initial infiltration amount at 95% confidence level. The correlations between three candidate bio-
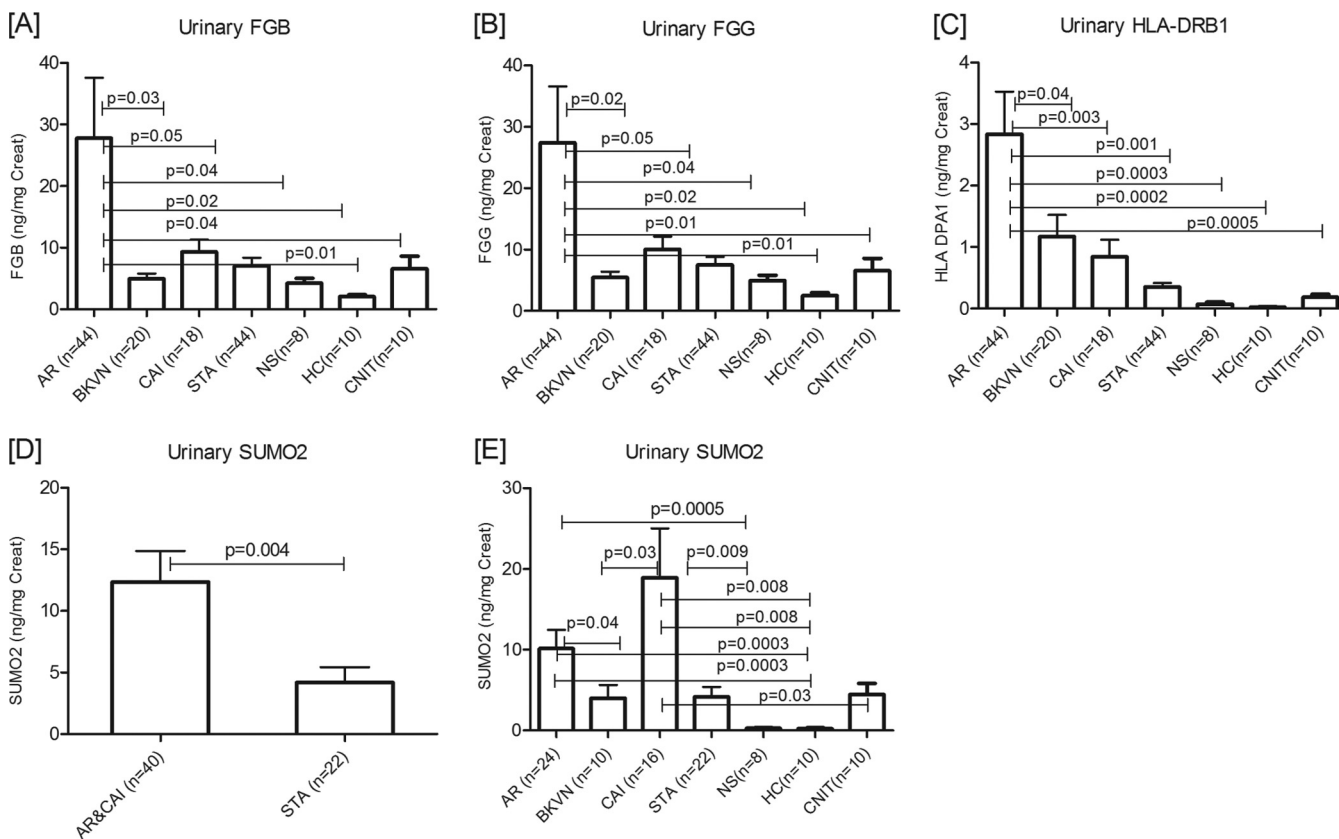
Fig. 2. **Distinct and common proteomic targets of allograft injury.** *A*, 133 unique up-regulated proteins overlapping among AR, BKV and CAI relative to STA. Protein names for overlapping up-regulated proteins are shown to the right of the Venn diagram. Predicted interactions (shortest path analysis) among core common injury proteins are shown on the right using predicted interactions from WikiPathways, KEGG, and BioGRID (undirected edges) (red = common injury proteins, gray = interaction partners not present among the 133 up-regulated proteins), with extracellular matrix fibrosis associated proteins indicated by blue text. *B*, Distinct marker profiles for the 133 uniquely increased injury proteins along with associated enriched Gene Ontology (GO), KEGG, Disease Ontology (CTD), Phenotype Ontology (MP), and WikiPathways (WP) terms from GO-Elite. Terms with a Fisher's Exact nonadjusted *p* value are reported. The top ranked marker proteins are shown in blue next to the heatmap.

markers and mononuclear cell interstitial inflammation (i-score) were positive (0.35 for FGB, 0.33 for GG, and 0.46 for HLA-DRB1). Urine HLA-DRB1 positively correlated with tubulitis score (t-score) with *p* value 0.003 and correlation coefficient 0.34.

DISCUSSION

Serial and noninvasive monitoring of transplanted kidney remains a critical unmet need to predict transplant injury and to distinguish among the different causes of transplant injury, such as AR, BKVN, and CAI. Noninvasive monitoring provides patient risk stratification for different immunosuppression loads and choices based on the nature of the injury. Special emphasis for the development of a noninvasive monitoring

assay needs to focus on distinguishing acute transplant injury because of AR (based on increased alloimmune reactivity) and BKVN (because of altered viral and innate immunity) as these conditions require diametrically opposite treatment approaches, with intensification of immunosuppression for AR and reduction or even discontinuation of immunosuppression for BKVN (1). When monitoring blood serum creatinine levels, a very redundant, minimally invasive, biomarker of nonspecific transplant injury, injury to the transplanted kidney gets diagnosed only when there is already established and often irreversible tissue damage. Kidney biopsy is invasive and cannot be used as a serial monitoring tool, though it is considered the gold standard for transplant injury detection, when implemented following an observed increase in blood serum

FIG. 3. **Validation of transplant injury specific proteins by ELISA on independent set of individual urine samples.** *A–C*, Potential AR specific protein biomarkers were validated on an independent set of urine samples on (*A*) Fibrinogen beta (FGB), (*B*) Fibrinogen gamma (FGG), and (*C*) HLA class II histocompatibility antigen (HLA-DRB1). [*D-E*] Small ubiquitin-related modifier 2 [SUMO2] was validated to be significantly increased in AR and CAI urine combined compared with no-injury phenotype including nontransplant control [D]. Urine level of SUMO2 was also found to be significantly increased in AR and CAI independently when compared with its level BKVN and STA urine and urine from nontransplant control. The error bars represent standard error of means (S.E.).

creatinine levels. For these reasons, identification and validation of clinically applicable noninvasive biomarkers remains an important and unmet need in the field of kidney transplantation.

Urine-based biomarkers provide a noninvasive monitoring and management alternative to assess renal diseases including kidney transplantation (34–36). With the publication of human genome data and ever improving molecular profiling assays our ability to accurately identify and quantify potential urine biomarker molecules has increased tremendously. Several reports on assessing mRNA level in urine sediments (12, 37, 38), urine protein (13, 35) and peptides (16) have highlighted the feasibility of identifying potential biomarker molecules in urine. Measurement of urine mRNA levels presents an attractive biomarker option, but susceptibility of mRNA in the urine in combination with low mRNA abundances requires more validation in the clinical setting (39). Unlike peak clustering for SELDI-TOF or a label-free shotgun MS/MS approach, the quantitative iTRAQ method provides an accurate assessment of protein levels in the urine samples analyzed in this study. In this report we applied a unique quantitative approach of identifying and profiling potential candidate

urine biomarker proteins that could provide surrogate noninvasive biomarkers to screen for transplant injury, provide a risk output of the type of transplant injury, and additionally, reveal underlying key molecules driving kidney transplant injury.

To accurately characterize urine protein profiles that are distinct to different injury phenotypes following kidney transplantation, we applied a quantitative proteomics approach that identified a unique set of proteins for each sub-type of transplant injury. Through this study we were able to further validate proteins that were observed as AR specific in our previously published report from a pilot study which was based on label-free shotgun proteomics (17). The associated molecular networks identified by AltAnalyze, an innovative bioinformatics suite, has helped to better characterize and understand the events that cause different injuries of kidney transplantation. Similar to our previously published observations of altered levels of PEDF and CD44 proteins in urine, in this study we observed a 1.7-fold increase in PEDF level in AR urine compared with STA urine and a 2.2-fold decrease in CD44 level in AR urine compared with STA urine; however, the candidate biomarker panel reported in this study had

greater statistical significance as biomarkers for renal allograft rejection.

Fibrin-fibrinogen and keratin degradation products have been previously shown to be present (decades ago) in urine at the time of renal allograft rejection using Western blotting (40). Fibrin deposition itself can be a key player in the evolution of chronic transplant glomerulopathy, a prime risk factor for acute rejection (41). Likewise, increases in excreted HLA peptides have been correlated with an increase in donor-directed HLA antibodies, which are important in humoral or antibody-mediated rejection (42). A number of other proteins identified in this study are markers of tubular epithelial injury, and many have been demonstrated to be deregulated in experimental models of ischemia reperfusion injury (43).

The antigenic protein DPP4, also known as CD26, was found to be increased in all injury models. This interesting molecule is a cell surface glycoprotein receptor involved in the costimulatory signal essential for T-cell receptor (TCR)-mediated T-cell activation. DPP4 acts as a positive regulator of T-cell coactivation and it is reported to be found in renal tubules (44).There are reports that DPP4 may play an unexpected role in modulating $Na^+/H+$ exchange mediated by NHE3 in proximal tubule cells (45). DPP4 is also a target for a class of drugs that are used to treat diabetes mellitus. Given the high incidence of glucose intolerance following chronic immunosuppression after organ transplantation, it would be tantalizing to hypothesize that DPP4 may be dysregulated during alloimmunity and help to drive an increased risk for glucose intolerance.

Among the eight core regulated injury set, a consistent twofold increase was observed with both CALR and FBXL19 proteins observed in all three injury types. The calreticulin (CALR) protein binds to misfolded proteins and has been previously reported to be associated with systemic lupus and other autoimmune disorders through its binding to autoantibodies (46, 47) and DNA (48). The F-box and leucine-rich repeat protein FBXL19 is associated with cellular inflammation (pulmonary) through its action as a ubiquitin ligase to target proteins for proteasomal degradation (49). Hence, these common injury-associated proteins may represent an autoimmune inflammatory end-point common to all forms of renal allograft injury. Among the common set of injury-associated proteins, SUMO2 was found to be the most tightly interconnected protein at the central hub of regulation in both acute injury settings (AR and BKV) and CAI (Fig. 2A). SUMO2 is a factor that regulates the functional activity of a broad range of proteins through a reversible covalent modification to those proteins known as SUMOylation. Although there is no clear identified functional role for SUMOylation in allograft injury, SUMO2's common regulation and predicted interactions with a large number of AR, CAI, and BKV proteins may suggest that its regulation is a central axis of allograft injury.

In conclusion, an unbiased, quantitative, high-throughput proteome characterization approach has been tested and optimized for biomarker discovery. Subsequent validation of proteomic discoveries with highly annotated clinical samples demonstrated that this method provides an effective strategy for biomarker discovery with the goal of monitoring transplant injury in kidney transplant patients. Because the study population consists of children and young adults with minimal incidence of acute tubular necrosis, these findings may not be generalizable to transplant populations that are not similarly highly selected for living donors and low cold ischemia times. Further application of these well-credentialed candidate protein biomarkers in clinical trials that utilize larger sample cohorts for prospective validation will forge the path toward clinical utility of these protein panels for predictive risk stratification of renal transplant patients.

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