Mining the human urine proteome for monitoring renal transplant injury

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The human urinary proteome provides an assessment of kidney injury with specific biomarkers for different kidney injury phenotypes. In an effort to fully map and decipher changes in the urine proteome and peptidome after kidney transplantation, renal allograft biopsy matched urine samples were collected from 396 kidney transplant recipients. Centralized and blinded histology data from paired graft biopsies was used to classify urine samples into diagnostic categories of acute rejection, chronic allograft nephropathy, BK virus nephritis, and stable graft. A total of 245 urine samples were analyzed by liquid chromatography–mass spectrometry using isobaric Tags for Relative and Absolute Quantitation (iTRAQ) reagents. From a group of over 900 proteins identified in transplant injury, a set of 131 peptides were assessed by selected reaction monitoring for their significance in accurately segregating organ injury causation and pathology in an independent cohort of 151 urine samples. Ultimately, a minimal set of 35 proteins were identified for their ability to segregate the 3 major transplant injury clinical groups, comprising the final panel of 11 urinary peptides for acute rejection (93% area under the curve [AUC]), 12 urinary peptides for chronic allograft nephropathy (99% AUC), and 12 urinary peptides for BK virus nephritis (83% AUC). Thus, urinary proteome discovery and targeted validation can identify urine protein panels for rapid and noninvasive differentiation of different causes of kidney transplant injury, without the requirement of an invasive biopsy.

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Kidney transplantation is the optimal choice of treatment for end-stage kidney disease.[1] Despite improved short-term outcomes,[2] long-term outcomes and graft survival rates remain suboptimal.[2,3] Recent studies by our group and others have demonstrated that subclinical inflammation followed by tissue injury is an ongoing process in the transplanted kidney and is a primary cause of graft loss.[4–7] This injury cannot be identified by the currently used clinical biomarkers—serum creatinine and increased urine protein load, or proteinuria. Both markers reflect nonspecific and late organ injury and cannot distinguish between causes of graft dysfunction that may require diametrically opposed approaches to therapy, such as immunosuppression intensification for graft rejection and immunosuppression reduction for BK virus nephritis. Both entities, if untreated, result in chronic tubulointerstitial loss and fibrosis.[2,8]

In this study, we have continued our efforts[9–13] to fully map and decipher changes in the urine proteome and peptidome after kidney transplantation, and to understand the perturbations in specific proteomic panels in the urine during biopsy-confirmed injury to the organ, defined by acute rejection (AR),[2] BK virus nephritis (BKVN),[14] and chronic allograft nephropathy (CAN)[15] versus stable renal allograft (STA).[15] In addition, we have evaluated the biological processes that drive these specific injuries and assessed variances in perturbations of transcriptional and translational programs in graft and urine samples from the same patient.

RESULTS

Transplant injury-specific proteins segregate transplant injuries by proteomics using either iTRAQ-based or label-free LC-MS in 264 unique urine samples analyzed by different methodologies and in independent sample sets

Application of a 2-dimensional liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS) strategy using isobaric Tags for Relative and Absolute Quantitation (iTRAQ) reagents on 108 urine samples pooled into 6 pools/phenotype (5 independent phenotype-specific samples for AR, CAN, and STA and 3 independent urine samples for BKVN [due to limited number of available cases]), identified a total of 6379 unique peptides (false discovery rate <0.1%), spanning 958 unique human proteins (Supplementary Table S1).[13] Principal component analysis of this data (Figure 1a) demonstrated that urine proteins generally segregate samples

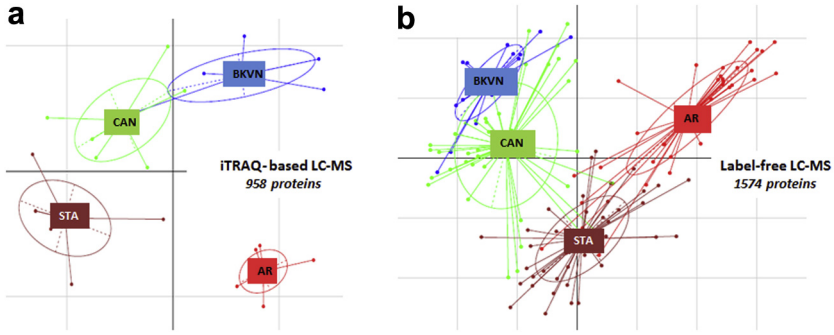

Figure 1 | Principal component analyses of proteomic data on 4 transplant phenotypes. Two different approaches were employed to identify potential biomarker urine proteins for kidney transplant injury. The first approach used quantitative proteomics using pooled samples that identified proteins specific to acute rejection (AR), BK virus nephritis (BKVN), and chronic allograft nephropathy (CAN) from stable graft (STA). (**a**) A principal component analysis plot demonstrates overall protein expression in-between injury phenotypes (AR, CAN, and BKVN) and no-injury phenotypes (STA) for all 958 proteins identified. The second approach used a shotgun proteomics (label-free) method analyzing individual samples from transplant injury phenotypes. (**b**) Principal component analysis plot demonstrates overall protein expression in-between injury phenotypes (AR, CAN, and BKVN) and no-injury phenotypes (STA) for all 1574 proteins identified. iTRAQ, isobaric Tags for Relative and Absolute Quantitation; LC-MS, liquid chromatography–mass spectrometry.

with injuries from stable grafts and also clustered sample groups into different phenotypes. Within this cluster of proteins, fibrinogen β and fibrinogen ɣ have been previously confirmed by independent enzyme-linked immunosorbent assay validation to be significantly ($P < 0.05$) elevated in AR over the other injury phenotypes.[13] This approach allowed for interrogation of the dynamic range of protein abundance measurements in transplant dysfunction categories prior to proceeding with larger numbers of individual samples to be analyzed by label-free LC-MS and selected reaction monitoring (SRM).

The label-free LC-MS datasets from 137 individual samples identified 26,462 peptides mapped to 2291 proteins. Each peptide was evaluated to determine whether there was adequate data for an analysis of variance or a qualitative G-test.[16] Outlier and contaminant data were filtered using the $\log_2$ robust Mahalnobis distance.[17] Thus, at the end of filtering and outlier discovery, there were 133 samples associated with 16,218 human peptides from 1574 proteins. Principal component analysis demonstrated that these proteins cluster to separate injuries from stable grafts and also show scatter differences based on different injury phenotypes (Figure 1b).

Urine proteins uncover biological mechanisms of graft injury

A significant number ($n = 811$) of the total proteins ($n = 1719$) identified were impacted during kidney graft injury (Supplementary Tables S2 and S3). Major changes were noted for major molecular processes in transplant injury: the immune response ($n = 179$ proteins; $P = 4.01E-23$, response to external stimulus ($n = 224$, $P = 5.68E-24$), and extracellular matrix organization ($n = 118$, $P = 2.05E-51$) (Supplementary Table S2). A total of 517 urine proteins were significantly dysregulated in AR; 228 were increased and 289 were decreased in AR compared with other clinical categories ($P < 0.05$) (Supplementary Table S3) and were uniquely enriched for regulation of cell adhesion ($n = 51$, $P = 2.98E-17$), wound healing ($n = 48$, $P = 2.45E-12$), and regulation of body fluid levels ($n = 47$, $P = 1.68E-11$) (Supplementary Table S4). A set of 186 proteins were specific to CAN ($P < 0.05$; 99 increased and 87 decreased) when compared with AR, STA, and BKVN urine samples; these proteins were uniquely enriched for biological processes involving negative regulation of protein metabolic process ($n = 19$, $P = 1.25E-03$), innate immune response ($n = 20$, $P = 2.61E-3$), multiorganism process ($n = 33$, $P = 6.19E-03$), etc. (Supplementary Table S4). A set of 108 proteins specific to BKVN, showed overlapping biological processes with both AR and CAN, with unique enrichment of protein refolding ($n = 5$, $P = 1.54E-04$), regulation of response to stress ($n = 19$, $P = 1.15E-03$), xenobiotic catabolic process ($n = 3$, $P = 1.73E-02$), etc. (Supplementary Table S4). Significant and somewhat BKVN-specific urinary protein alterations were seen in lactotransferrin (TRFL), SUMO2 (SUMO2), granulins (GRNs), haptoglobin-related protein (HPTR), peptidase inhibitor 16 (PI16), alpha-1-antitrypsin (A1AT), and fibulin-1 (FBLN1).

Evaluation of transcriptomic data from matching urine and graft biopsies within patients shows specific overlapping and unique molecular processes in transplant injury

Of 811 proteins that were identified as significantly changed in the urine protein dataset (Supplementary Table S3), 26% ($n = 153$) were also dysregulated at the mRNA level in the matching biopsies. There was a relatively high level of agreement in the tissue genes and urine proteins from the same patient, showing dysregulation in each specific injury group: 50% in AR, 67% in CAN, and 42% in BKVN. These overlapping pathways, at both the gene level in tissue and the protein level in the effluent from the same tissue, were significantly involved in tissue development ($n = 29$, $P = 1.14E-07$), extracellular matrix organization ($n = 16$, $P = 2.85E-07$), and cell adhesion ($n = 36$, $P = 4.75E-07$). Enrichment analysis for cellular and