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Quantification of Urinary Protein Biomarkers of Autosomal Dominant Polycystic Kidney Disease by Parallel Reaction Monitoring

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Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Conflict of Interest

The authors have declared no conflict of interest.

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Abstract

Purpose: Autosomal dominant polycystic kidney disease (ADPKD) is a life-long disease in which the genes responsible are known, but the pathogenesis of cyst formation and cyst growth are not understood. Cyst growth ultimately leads to end-stage renal failure in most patients. Analysis of the urinary proteome offers the potential to identify proteins that indicate the presence of cysts (and thus provides diagnosis) as well as the rates of cyst growth (providing prognostic information).

Experimental design: A scheduled parallel reaction monitoring (sPRM) assay is performed on urine samples from 14 patients and 18 normal controls. For relative quantification, stable isotope-labeled synthetic peptides are spiked in the urinary protein digests prior to data collection. The data are subsequently normalized to creatinine and protein concentration in the respective urine samples to control for variations in water intake between individuals.

Results: Out of the 143 urinary proteins targeted for sPRM assay, 69 proteins are observed to be significantly dysregulated in ADPKD. The dysregulated proteins are used to cluster ADPKD patients into those who are more or less similar to normal controls.

Conclusions and clinical relevance: This study shows that sPRM is a promising approach to rapidly screen large numbers of proteins in urine in order to provide earlier diagnosis and potentially better understand the pathogenesis of ADPKD development and progression.

Keywords

autosomal dominant polycystic kidney disease; label-free quantification; parallel reaction monitoring; targeted mass spectrometry; urinary proteomics

1. Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is the fourth leading cause of end-stage kidney failure with a prevalence of clinical disease of about 4/10 000 in the population.^[1] ADPKD is a genetic disorder caused by mutations in either polycystin 1 or polycystin 2 encoded by *PKD1* and *PKD2*, which lead to the formation and growth of fluid-filled cysts in the kidneys, eventually progressing to kidney failure. Currently, there is no cure for ADPKD, and the available therapeutic options are limited. Tolvaptan, a vasopressin-2 receptor (VR2) antagonist, has been shown to slow the growth rate of cysts and preserve kidney function during clinical trials,^[2] but it is unclear how long the effect of tolvaptan persists or whether it will benefit patients with advanced disease.^[3]

Presently, ADPKD is diagnosed by the detection of fluid-filled cysts using radiological imaging.^[4] This approach can only detect cysts after significant, and possibly irreversible anatomical changes have occurred. Early diagnosis prior to macroscopic cyst formation might allow more effective treatment options which could delay or even prevent the development of clinically overt disease. One such approach is the analysis of the urine proteome by mass spectrometry. Since cysts initially develop as outpouchings of the tubule in communication with the urine, changes in the composition of urinary proteins can reflect the development and progression of cysts, and thus serve as the source of potential disease biomarkers. Monitoring the urine proteome therefore may provide a noninvasive early prognostic tool to diagnose ADPKD and monitor disease progression.

Several groups have used mass spectrometry-based methods to study the urine proteome in ADPKD. Kistler et al.^[5] were the first to use mass spectrometry analysis of the urine of ADPKD patients to gain insight into early disease progression. They used urine samples that were filtered to remove proteins higher than 20 kDa. Mass spectrometry results from ADPKD patients and age-matched healthy controls were compared to identify naturally occurring polypeptides that were differentially excreted.^[5] Pocsfalvi et al.^[6] analyzed the protein expression of urinary extracellular vesicles, and Hogan et al.^[7] analyzed the protein expression of exosome-like vesicles in urine to identify ADPKD-associated biomarkers. Bakun et al.^[8] have studied the higher molecular weight fractions of urinary proteome of ADPKD to characterize the patho-mechanism underlying the disease as well as establish a proteomic prognostic marker panel. However, most of these studies used discovery-based mass spectrometry method to identify and quantify urinary proteins.

Mass spectrometry-based protein quantification is achieved by acquiring data using either data-dependent acquisition (DDA) or targeted data acquisition (TDA) techniques. DDA is used in discovery-based studies and identifies thousands of proteins in an unbiased fashion. TDA is used in hypothesis-driven studies and monitors the expression of selected proteins across a large number of samples. The application of TDA in clinical research is gradually increasing and has been used for validating the expression level of urinary biomarkers in various kidney diseases, such as acute kidney injury,^[9] autosomal dominant tubulointerstitial kidney disease,^[10] ADPKD and chronic kidney diseases (CKD),^[8,11] and bladder cancer.^[12] Parallel reaction monitoring is a TDA method that is performed in a Quadrupole-Orbitrap-based mass spectrometer.^[13,14] In PRM, quantification is based on fragment ions observed in high resolution MS2 scans, making PRM a highly sensitive and selective method of quantification.^[15] PRM also possess multiplexing capability, that is, it can quantify hundreds of proteins/peptides in a single run.^[16]

In complex diseases, protein panels may provide higher levels of sensitivity and specificity than individual biomarkers, and ultimately high positive predictive values.^[17] Therefore, in this study, we used PRM quantification of a library of 268 unique peptides to assess a panel of 146 potential ADPKD biomarker proteins in urine. These 146 proteins were originally identified as differentially expressed during label-free quantification of pooled urine from three normal donors and three ADPKD patients. Individual urine samples from 14 ADPKD patients and 18 normal controls (NOR) were then analyzed by PRM using the library

peptides, resulting in identification of 69 distinct proteins as differentially expressed in the ADPKD patients.

2. Experimental Section

2.1. Sample Collection and Processing

Fourteen patients with ADPKD and 18 normal controls provided morning urine samples for analysis. All sample and data collection were performed after informed consent under a study protocol approved by the Yale Human Investigation Committee (HIC #1111009286). General characteristic and demographic features on these individuals including the method of urine collection are in Table S1, Supporting Information. After collection, urine samples were immediately refrigerated and then centrifuged at $5000 \times g$ for 10 min at $+4^{\circ}\text{C}$. Aliquots of 1 mL of supernatant were subsequently stored in cryovials at -80°C .

During processing, the samples were thawed and an aliquot was removed for determining urine creatinine concentrations, which was performed by Jaffe Method.^[18] The remaining urine samples were supplemented with protease inhibitors and dried under vacuum-centrifugation. Each sample was then resuspended in 150 μL water and subjected to methanol/chloroform precipitation. The protein pellets were reconstituted in denaturing buffer consisting of 8 M urea and 0.4 M ammonium bicarbonate, reduced with dithiothreitol, alkylated with iodoacetamide, and digested with trypsin (Promega Corporation, Madison, WI) for 8 h at 37°C . The samples were acidified to halt the trypsin activity, and desalted using C18 spin columns (The Nest Group, Inc., Southborough, MA). C18 eluates were dried under vacuum-centrifugation and dissolved in 0.1% trifluoroacetic acid. Protein concentration was determined by hydrolysis and amino acid analysis (AAA) of an aliquot of each C18 eluate. For scheduled parallel reaction monitoring (sPRM) assay, 200 ng of urinary protein digests were spiked with the stable isotope standard (SIS) peptide mixture and analyzed by LC-sPRM-MS/MS method.

2.2. Parallel Reaction Monitoring Setup

2.2.1. Selection of Peptides—A list of 268 unique peptides corresponding to 146 proteins selected for targeted quantification by PRM is provided in Table S2, Supporting Information. These proteins were observed as differentially expressed using label-free quantification (as previously described^[19]) to compare the proteome of pooled urine from normal subjects and ADPKD patients. The SIS version of each of these 268 peptides were purchased from JPT Peptide Technologies. The sequence of SIS peptides matches the sequence of the selected ADPKD peptides with the exception of the C-terminus being isotopically labeled with either heavy lysine ($[^{13}\text{C}_6, ^{15}\text{N}_2]$ -lysine) or heavy arginine ($[^{13}\text{C}_6, ^{15}\text{N}_4]$ -arginine). All cysteines in the SIS peptides carried a carbamidomethyl modification.

2.2.2. Development of sPRM Assay—The retention times of the 268 SIS peptides, obtained from LC-DDA-MS/MS run (details in Supporting Methods), were used for scheduling the sPRM assay. The DDA data of SIS peptides were processed using Proteome

Discoverer v2.1 (Thermo Scientific) software. The MS/MS spectra were searched against the Swiss-Prot human database (downloaded in November 2015) with precursor and fragment ion mass tolerance of 10 ppm and 0.02 Da (20 ppm), respectively. Additional parameters used for the database search included trypsin as digestion enzyme, carbamidomethylation (+57.03404 Da) of cysteine and heavy label (+10.008 Da and +8.014 Da) on arginine and lysine as a static modification.

MS/MS spectra of SIS peptides were used for building the spectral library using BiblioSpec algorithm in the Skyline v3.6,^[20] which is a freely available and open source Windows client application. The spectral library will be used for extracting the fragment ions corresponding to the targeted ADPKD peptides in sPRM data.

2.2.3. sPRM Assay for Urine Samples From 14 ADPKD Patients and 18 Normal Individuals

—We created the LC-sPRM-MS/MS assay based on 255 of the 268 SIS peptides that were identified by LC-DDA-MS/MS and provided the most robust quantitation (Table S3, Supporting Information). The LC-sPRM-MS/MS was performed on the nanoAC-QUITY UPLC and Orbitrap Fusion Tribrid mass spectrometer with the reverse phase gradient and duration similar to the LC-DDA-MS/MS that was used for analysis of the SIS peptide mixture. LC-sPRM-MS/MS acquisition combined two scan events: full scan (MS1) event followed by targeted MS/MS (tMS2) event of the doubly charged precursor ions of the 510 peptides (255 endogenous light peptides and their corresponding SIS heavy peptides). Full scan event consisted of quadrupole isolation of precursor ions of range of 350–1550 m/z and recorded for maximum fill time of 25 ms, AGC target of 2×10^5 , and an Orbitrap resolution of 120 000 (m/z 200). tMS2 event $\times$ consisted of quadrupole isolation of the targeted peptides with an isolation window of 1.6 m/z for a maximum fill time of 60 ms and AGC value of 5×10^4 . The targeted peptides were fragmented with HCD collision energy of 28%, and MS/MS spectra were recorded at an Orbitrap resolution of 30 000 (m/z 200). In all cases, single micro-scan MS1 and MS/MS acquisitions were recorded. Each sample was analyzed with three technical replicates ([14 ADPKD + 18 normal] $\times$ 3 = 96 total sPRM runs).

2.2.4. sPRM Data Analysis

—To identify differently expressed peptides in ADPKD patients, the sPRM data were imported into Skyline with the fragment ion resolution set to 30 000, fragment ions specified from ion m/z > precursor to the last ion, and five fragment ions designated for peak area extraction. The ion match tolerance was set to 0.5 m/z with peak areas restricted to only y-type fragment ions and precursor ions of only +2 charge. In Skyline, peak areas of the peptide fragment ions and precursor ions were extracted using information in the spectral library generated from the DDA data of the SIS peptides. Thus, each peptide is quantified by the sum of its fragment ion peak areas (referred to as total area fragment [TAF] in this manuscript), or sum of its first three isotopic precursor ions (referred to as total area MS1 [TAM] in this manuscript). The integrated peak areas, TAF and TAM, of the endogenous peptides and SIS peptides observed in the ADPKD and normal samples were exported in CSV format, which was then imported into R software (www.r-project.org) for normalization, quality control, and statistical analysis.

To control for run-to-run variations in the mass spectrometer, the peak areas of the light (endogenous) peptides were first normalized to their corresponding heavy-labeled partner. The normalized peak areas were then subsequently normalized to creatinine and protein concentration in the respective urine samples to control for variations in water intake between subjects.^[21] Normalization against creatinine and protein concentration adjusts the variations in urinary excretion rate and total urinary protein abundance across individuals (Equation (1)).

$$\text{Normalized PRM} = \frac{(\text{Raw abundance} / \text{Heavy isotope}) \times \text{Total protein Conc}}{\text{Creatinine Conc}} \quad (1)$$

To limit possible interference from co-eluting peptides, those peptides failing to meet any of the following criteria were removed from further analysis: 1) average mass error (ppm) < 10; 2) retention time variability of ± 2.5 min across samples; 3) isotope dot product (idotp) > 0.7, and 4) library dot product (dotp) > 0.7. Further quality control removed peptides detected in less than half of the samples from the ADPKD patients or normal donors.

After normalization and quality control, peak areas in the three technical replicates were averaged to enable ANOVA analysis. The peptide fold change was calculated as the ratio between the average of normalized peak areas in the ADPKD and normal samples. Differences in protein expression between ADPKD and normal samples were initially evaluated by a two-sample *t*-test. To account for multiple comparison testing, *p*-values obtained from *t*-test were corrected using the Benjamini–Hochberg method. Peptides with fold change ≥ 2 and Benjamini–Hochberg multiple testing correction *p*-value ≤ 0.05 were considered as differentially expressed in ADPKD patients. In addition to R, JMP Pro 11 software was also used for data processing and graphics.

3. Results and Discussion

To provide a list of candidate peptides for targeted analysis via PRM assay, we used the Yale Protein Expression Database (YPED)^[22] of peptide signatures for the 146 proteins identified as potentially differentially expressed based on label-free quantification^[19] of pooled urine from three normal subjects and three ADPKD patients. For 122 of the 146 proteins, we were able to identify two unique proteotypic peptides. Altogether, we identified 268 unique peptides that were surrogates of the selected ADPKD biomarker candidate proteins (Table S2, Supporting Information). The PRM experimental workflow is shown in Figure 1.

3.1. PRM Quantification

We developed the sPRM assay using retention times observed during LC-DDA-MS/MS of stable isotope-labeled synthetic (SIS) peptides corresponding to the 268 endogenous peptides to be quantified. DDA analysis of SIS peptides successfully identified retention times for 255 of the 268 peptides, covering 143 of the original 146 proteins (Table S3, Supporting Information). The sPRM assay was performed in the urine samples from 32 individuals: 14 ADPKD patients and 18 normal donors. The samples were analyzed using sPRM and the resulting spectral data normalized and filtered as described in Experimental

Section, resulting in the successful quantification of 241 peptides from 139 proteins (Table S4, Supporting Information).

Two-sample *t*-test was performed to identify the peptides that were significantly different in ADPKD as compared to normal samples. Considering a high biological variation among urine samples, we used a BH-corrected *p*-value 0.05 and relative fold change $2^{-1 \log_2 \text{ratio}}$ as a significance threshold for differentially expressed peptides. Of the 241 peptides successfully quantified, 109 peptides corresponding to 69 distinct proteins were differentially expressed in ADPKD compared to normal samples (Table 1). Among these 69 proteins, 58 were found to be upregulated and 11 were downregulated in the ADPKD urine samples. Forty proteins were quantified by both of their peptides targeted during sPRM assay. In all 40 cases, the direction and magnitude of the fold change was consistent for both of the quantified peptides from the same protein (Table 1). Consistent with previous studies, [23,24] angiotensinogen and fetuin-A were both quantified as significantly upregulated in ADPKD urine as compared to normal samples (Figure 2A,B). The expression level of the targeted peptides from all the proteins quantified by sPRM assay is shown in Figure S1, Supporting Information.

3.2. MS1-based Quantification of Targeted Peptides

During the sPRM assay, in addition to collecting fragment ion data of the peptides in targeted MS2 (tMS2) scans, we also collected precursor ion intensity data in MS1 survey scans. The MS1 scans were independent of the tMS2 scans, but the cycle was repeated throughout the duration of the LC-sPRM-MS/MS data acquisition. Quantification in MS1 is based on summed peak areas of the three precursor isotopes (*M*, *M*+1, *M*+2) of a peptide. We refer to the MS1-based quantification as total area MS1 (TAM) quantification. When we compared the fold change of the 241 peptides quantified using fragment ions in tMS2 mode (TAF method of analysis) and TAM method of analysis of precursor intensity in MS1, we observed a high Pearson correlation coefficient ($r = 0.85$, $p < 0.0001$) indicating an agreement in the expression level of peptides reported by TAF and TAM method of data analysis.

Of the 109 peptides that were detected as significantly differentially expressed using TAF, 82 peptides were also detected as significant by TAM analysis (Table S4, Supporting Information). The majority of the remaining peptides displayed a similar trend in fold change as that seen with TAF, but did not meet the *p*-value threshold of 0.05 using the TAM method of data analysis. Quantifying peptides based on MS1 precursor ion intensities may be compromised by a low signal-to-noise ratio; however, in certain cases MS1 signal can help identify MS2 interferences.^[25] Our results show that incorporating MS1 scans in PRM data acquisitions can be beneficial as it provides an independent means for validating changes in peptide expression level and hence can be used to support PRM quantification results.

3.3. Hierarchical Clustering and Enrichment Analysis

To identify the protein regulation pattern in ADPKD, unsupervised hierarchical clustering was performed on differentially expressed peptides. First, the normalized abundances of

each regulated peptide were transformed to Z-scores. Then, unsupervised hierarchical clustering analysis was conducted on the Z-scores using the complete linkage method. Hierarchical clustering of 109 differentially expressed peptides representing 69 proteins show ADPKD samples group separately from the normal samples (Figure 3). As seen in the heatmap, even within ADPKD samples, sample number PRT11, PRT12, PRT17, and PRT25 formed a cluster, which in turn are clustered with sample number PRT13, PRT14, PRT16, and PRT24. The clustering shows strong correlation with the level of proteinuria. Patients PRT11, PRT12, PRT17, PRT24, and PRT25 have proteinuria, while remaining patients are negative (Table S1, Supporting Information). The large differences in protein levels within ADPKD samples suggest that biological differences exist between patients and may correlate with clinical status or progression.^[8]

An important clinical question remains regarding which of the 69 proteins identified as dysregulated in ADPKD urine samples represent specific tubular cell changes related to ADPKD pathogenesis, which are reactive changes related to inflammation and injury, and which are nonspecific due to late glomerulosclerosis. We used Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8, a high-throughput and integrated data-mining environment,^[26] and WebGestalt (WEB-based Gene SeT Analysis Toolkit)^[27] tools to gain biological insight into this question. DAVID analysis revealed statistically significant changes in broad range of biological processes, including serine-type endopeptidase inhibitor activity (adjusted p -value = $3.6E-07$), platelet degranulation (adjusted p -value = $1.6E-10$), negative regulation of endopeptidase activity (adjusted p -value = $1.4E-08$), proteolysis (adjusted p -value = $1.8E-07$), cellular iron ion homeostasis (adjusted p -value = $1.0E-04$), and acute-phase response (adjusted p -value = $1.5E-03$). WebGestalt mapping of the genes represented by the significant proteins to potential underlying diseases indicated linkage with diseases such as urological malignancies, blood pressure disorders, and cardiovascular disease (Figure S2A, Supporting Information), potentially reflecting the complex medical history of many adults with ADPKD (Figure S2B, Supporting Information). Therefore, the sensitivity and specificity of the 69 proteins in terms of predicting ADPKD must be tested in independent patient cohorts including those with kidney diseases other than ADPKD. Furthermore, validation of the 109 significant peptides in larger cohorts will improve the statistical power, and may reduce the number of peptides/proteins to a smaller number that can be used to most accurately diagnose ADPKD and predict rates of progression.

3.4. Comparison of PRM Results to Label-Free Quantification and to iTRAQ-Based Quantification by Bakun et al.^[8]

The initial discovery study to identify differentially expressed proteins in ADPKD was performed on pooled urine samples. Pooling urine from several individuals for analysis by mass spectrometry is cost-effective and reduces variability in protein expression levels, but also eliminates the underlying disease heterogeneity present in individual samples and thus may reduce the breadth of the analysis.^[28] In the current study, the use of pooled urines did provide valid peptide targets for subsequent PRM-based quantification in the individual samples, but may have prevented us from identifying additional peptides that would have proven equally valid in our PRM analysis.

To establish whether expression changes observed by PRM can also be observed by label-free quantification, we also performed DDA analysis on each of the ADPKD and normal urine samples. We compared the fold change of the 69 proteins that were significant in sPRM with the fold change observed by label-free quantification. Label-free quantification was performed using intensity-based absolute quantification (iBAQ) values, which is the sum of intensities of all tryptic peptides for each protein divided by the number of theoretically observable peptides. Out of the 69 proteins, 61 proteins demonstrated a fold change and direction of change consistent with the sPRM results, with 37 proteins having a *p*-value <0.05 (Figure S3, Supporting Information). Seven proteins were not detected using DDA while one protein (FCN2) showed an opposite trend in fold change.

Bakun et al.^[8] have also studied the urinary proteome of ADPKD to characterize the pathomechanism underlying the disease as well as establish a proteomic prognostic marker panel. They used iTRAQ, an isobaric-labeling-based quantification approach,^[29] for the quantitative analysis of the high molecular weight fraction (>10 kDa) of the urinary proteome in ADPKD as compared to a healthy control group. When we compare our significant proteins in sPRM results with the differentially expressed proteins reported by Bakun et al.,^[8] 22 proteins were found to be in common and showed a similar trend in fold change (Figure S4, Supporting Information).

3.5. Study Limitations

While our study demonstrates the potential breadth and depth of sPRM-based mass spectrometry proteomics for analysis of multiple urinary proteins in a single run, there are clear limitations to the data obtained. First, of the 268 peptides selected for sPRM assay, 11 methionine-containing peptides were included and 9 of them were quantified. Methionine can oxidize during sample preparation and storage. Stochastic oxidation can affect the reliability of quantification of methionine-containing peptides. Second, the urinary differences in protein amount that we identified are relative differences, rather than absolute concentrations. Absolute concentrations of the peptides of interest could be determined using sPRM, but it would require that we determine the absolute concentration of each SIS heavy peptide in the mix. Third, the concentration of proteins in the urine is highly variable even in normal persons due to the wide range of water ingestion and excretion among individuals. To account for this, we normalized the peptide results to urinary creatinine concentration for each sample since this is excreted at a nearly constant rate for any given individual, regardless of GFR, as long as they remain in steady state (as were the clinic patients and normal controls included in this study). Examination of those urinary creatinine values demonstrates the wide range of urinary concentration in both our normal subjects and our ADPKD patients (Table S1, Supporting Information). Fourth, all of our ADPKD patients had elevated serum creatinine values, and 5 out of 14 ADPKD subjects had overt proteinuria, as defined by a urinary protein concentration of $\geq 30 \text{ mg dL}^{-1}$ (Table S1, Supporting Information). This is likely attributable to chronic kidney disease (CKD) associated with their ADPKD, which is not present in any of the control subjects. The presence of CKD in our ADPKD cohort, along with their greater prevalence of coronary artery disease (11/14 in ADPKD vs 0/18 in controls), diabetes (3/14 vs 0/18), and hypertension (6/14 vs 0/18) (Table S1, Supporting Information), makes it clear that there are

disease processes in addition to ADPKD that could influence the differential expression of urinary proteins. Inclusion of patients with these chronic disease states as controls, as well as proteomic analysis of either the kidney itself or primary cells derived from ADPKD kidneys, would improve the ability of the PRM approach to identify dysregulated proteins that are specific to ADPKD.

4. Conclusion

ADPKD disease-specific protein signatures may be useful as both diagnostic and prognostic biomarkers, particularly if they are observed in samples that can be obtained noninvasively. Since pathologic changes in the kidney are reflected in the urine proteome, the characterization of urinary proteins in ADPKD is of great clinical interest and significance. In this study, we developed a sPRM assay to quantify and validate ADPKD biomarkers in urine. The inclusion of stable isotope-labeled synthetic (SIS) peptides enabled us to successfully quantify 241 peptides corresponding to 139 proteins, of which 109 peptides corresponding to 69 proteins were differentially expressed in ADPKD samples. Our study shows the potential of PRM assay to identify panels of biomarker proteins in urine samples for diagnosing ADPKD. A validation study will be required in a larger cohorts of patients in order to refine the biomarker panel and establish its use in ADPKD progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ADPKD	autosomal dominant polycystic kidney disease
DDA	data-dependent acquisition
iBAQ	intensity-based absolute quantitation
NOR	normal
PRM	parallel reaction monitoring
PRT	ADPKD
SIS	stable isotope standards
TAF	total area fragment
TAM	total area MS1
TDA	targeted data acquisition

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Clinical Relevance

Autosomal dominant polycystic kidney disease (ADPKD) is a life-long disease in which the genes responsible are known, but the pathogenesis of cyst formation and cyst growth are not understood. Cyst growth ultimately leads to end-stage renal failure in most patients. Currently, the only way to predict which patients have rapid rates of cyst growth is by repeated magnetic resonance imaging of the kidneys over multiple years with cyst volume calculations. Analysis of the urinary proteome offers the potential to identify proteins that indicate the presence of cysts (and thus provide diagnosis) as well as the rates of cyst growth (providing prognostic information). In the current study, we describe the use of targeted mass spectrometry with parallel reaction monitoring to identify significantly up and downregulated proteins in the urine of patients with ADPKD as compared to normals. We show that 69 of the targeted urinary proteins are significantly dysregulated in ADPKD, and can be used to cluster ADPKD patients into those who are more or less similar to normal controls. We propose that with further long-term validation, this approach could be used to provide urinary protein biomarkers of rates of cyst growth in ADPKD.

Figure 1.

Schematic illustration of the scheduled parallel reaction monitoring (sPRM) assay. For ADPKD biomarker discovery, 268 unique peptides corresponding to 146 distinct proteins were selected. Stable isotopelabeled synthetic (SIS) peptides were used to obtain retention time for scheduling and to generate spectral library for extracting fragment ion peak area.

Figure 2.

A) Bar graph of normalized peak area of the fragment ions of the peptides SLDFTELDVAEEK and DPTFIPAPIQAK of angiotensinogen (ANGT) for each individual sample. B) Normalized peak area for the peptide EHAVEGDCDFQLLK of Fetuin-1 (FETUA). The results are one of the three technical replicates of sPRM assay. NOR = normal donor, PRT = ADPKD patient, QC = PASS indicate peptide qualified the stringent filtering criteria for quantification eligibility (see Experimental Section for details).

Figure 3.

Heatmap of 109 unique peptides representing 69 distinct proteins that were statistically changed in ADPKD urine samples (fold change ≥ 2 , BH-corrected p -value ≤ 0.05). Each column represents a single urine sample (NOR = normal sample, PRT = ADPKD sample), each row represents a single peptide. Red color and indicates upregulation and blue indicates downregulation.

Table 1.

List of differentially expressed 109 peptides representing 69 proteins quantified by sPRM assay.

Gene	Peptide #1	TAELog ₂ FC	TAEFDR	Peptide #2	TAELog ₂ FC	TAEFDR
A1BG	ATWSGAVLAGR	2.46	6.78E-03	HQFLLTGDTQGR	2.47	4.98E-03
ABHD14B	FSVLLHGHIR	1.61	1.90E-02			
AFM	LAPQLSTEEVLVSLGEK	3.82	8.72E-03	FLVNLVK	3.90	9.95E-03
AGT	DPTFPAPIQAK	3.55	5.77E-03	SLDFTELDVAEEK	3.79	6.13E-03
AHSG	EHAVEGDCDFQLLK	1.56	3.71E-02	CNLLAEK	3.01	9.63E-03
ALB	VPQVSTPTLVEVSR	3.49	8.46E-03	SLHTLFGDK	3.66	7.01E-03
AMB	ETLLQDFR	2.18	3.42E-03	AFIQLWAFDAVK	2.52	4.22E-02
APOA1	DYVSQFEGSALGK	3.79	3.35E-02			
APOA2	EQLTPLIK	3.21	3.16E-02			
APOA4	LAPLAEDVR	4.71	1.35E-02	ISASAEELR	4.78	1.10E-02
ARSF	ILDAIDDFGLR	-1.19	3.16E-02			
AZGP1	YSLTYIYTGLSK	1.99	2.21E-03	AYLEEECPATLR	2.90	2.09E-03
Clorf23	TIVEFEER	2.90	4.32E-02			
C3	SGIPVTSYQIHFTK	3.63	4.74E-02			
C9	LSPIYNLVPVK	3.38	1.34E-02	AIEDYINEFSVR	3.44	1.16E-02
CA1	ESISVSSEQLAQFR	3.95	4.32E-02			
CHGA	EAVEEPSSK	4.04	3.71E-02			
CP	GAYPLSIEPIGVR	2.65	7.01E-03	DLYSGLIGPLIVCR	2.70	7.75E-03
CTSC	NVHGINFVSPVR	1.98	1.64E-04			
CTSD	LSPEDYTLK	2.14	3.08E-06	VSTLPATLTK	2.17	5.68E-06
CTS2	VGDYGSLSGR	2.33	2.21E-03	NVDGVNYASITR	2.49	2.77E-03
DPEP1	GALADNLLR	-1.40	1.20E-03	AVGFGGDFDGVPR	-1.20	9.60E-04
DPP7	SLPFGAQTQR	1.34	3.65E-03	DLFLQGAYDTVR	1.44	1.20E-03
DSC2	GPGVDQEPK	-3.02	2.49E-02			
EGF	IIESSSLOGLGR	-2.05	9.37E-07	ITAVSLDVLDK	-1.74	9.37E-07
ENDOD1	ILEWNQIQDEER	1.24	2.21E-03			
FCN2	GYNYSYK	1.24	3.55E-02			
FTH1	MGAPESGLAEYLFDK	1.31	2.04E-02			

Gene	Peptide #1	TAFlog ₂ FC	TAFDR	Peptide #2	TAFlog ₂ FC	TAFDR
FTL	LGGPEAGLGEYLFR	2.48	6.83E-03			
GAS6	GQSEVSAALQER	-1.79	2.43E-04	DLVQPINR	-1.59	4.44E-06
GC	VLEPTLK	3.49	1.20E-02	HLSLLTTLNRR	3.75	1.35E-02
GDF15	TDTGVSLSQTYDDLLAK	1.43	8.46E-03	ASLEDLGWADWVLSR	1.76	9.42E-03
GM2A	IESVLSSSGK	2.07	2.03E-03	VDLVLEK	2.10	1.65E-03
GP1BA	GLGELQELYLK	1.60	7.48E-03	LTQLNDR	1.69	8.46E-03
GPX3	QEPGENSEILPTLK	1.03	3.16E-02	FLVGPDPGIMR	1.73	1.76E-02
GSN	AGALNSNDAFVLK	1.60	5.75E-03	EPGLQIWR	1.94	4.12E-02
GUCA2A	DLQEPQEP	2.78	4.89E-02			
HP	VTSIQDWVQK	2.85	2.58E-02	VGYYVSGWGR	3.05	4.22E-02
HPX	NFPSPVDAFR	3.41	5.96E-03	GGYTLVSGYPK	3.52	7.46E-03
IGKC	SGTASVVCLNNFYPR	1.63	3.09E-03	DSTYSLSTLTLSK	2.01	2.03E-03
IGLV3-19	ITCQCDSL	2.62	3.27E-02			
KLK1	LTEPADTTTDAVK	-1.94	1.92E-03	VTDFMLCVGHLEGGK	-1.38	3.87E-02
LCN1	VTMLISGR	1.69	8.46E-03			
LRG1	DLLLPQDLR	2.41	6.39E-04			
LRRC15	LTLFGNSLK	-1.20	7.80E-03			
LTBP1	YTCICYECYR	1.17	3.84E-02			
LYVE1	NGVGVLJWK	1.46	1.01E-03	LLGLSLAGK	1.65	1.80E-04
MSLN	IQSFLGGAPTEDLK	2.47	6.83E-03	TDAVLPLTVAEVQK	2.52	3.06E-02
OMD	LLLGYNESK	-1.24	8.57E-03			
ORM2	EQLGEFYEAIDCLCIPR	2.83	3.09E-03	EHVAHLLFLR	2.86	2.24E-03
PAPPA2	YECKPCYYVAESAEGK	2.96	4.44E-06	VGISAVALR	3.27	9.37E-07
PI16	AQVSPTASDMLHMR	1.67	7.21E-03	WDEELAAFAK	2.12	6.80E-04
PIP	TYLISSIPLQGAFFNYK	-1.20	2.57E-02			
PLAU	MLCAADPQWK	-1.38	1.13E-02			
PLC	LSSPAVITDK	2.59	9.95E-03			
RBP4	YWGVASFLOK	4.40	3.79E-02	DPNGLPPEAQK	4.84	4.22E-02
RNASSET2	ELDLNSVLLK	1.75	3.77E-03			
SERPINA1	SVLGLQGITK	3.58	8.57E-03	LSITGTYDLK	3.69	9.63E-03
SERPINA3	ADLSGITGAR	2.44	7.29E-03	EQLSLDDR	2.48	9.74E-03

Gene	Peptide #1	TAFlog ₂ FC	TAFDR	Peptide #2	TAFlog ₂ FC	TAFDR
SERPINA6	WSAGLTSSQVDLYIPK	1.96	7.75E-03			
SERPINC1	EVPLNTIIFMGR	3.68	9.69E-03	VAEGTQVLELPFK	3.88	8.72E-03
SERPIND1	QFPILLDFK	2.75	4.22E-02	TLEAQLTPR	2.87	3.89E-02
SERPINF1	LQSLFDSPDFSK	4.22	2.39E-02	LAAAVSNFGYDLYR	4.58	3.16E-02
SOD1	CDGPVQGIINFEQK	1.76	1.73E-02			
SPINK5	DTCDEFK	3.12	2.86E-02			
TF	EGYYGYTGAFK	3.70	7.48E-03	SASDLTWDNLK	3.87	7.01E-03
TFF1	GCCHDDTVK	2.72	1.62E-04			
VCAM1	NTVISVNPSTK	1.75	1.20E-03	SLEVTFTPVIEDIGK	1.93	1.34E-03
XPNPEP2	VLCNIDLSK	-1.35	1.24E-03	AAVWTDNR	-1.23	9.11E-04