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Urine proteomic profiling of pediatric nephrotic syndrome

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Abstract

The prognosis of pediatric nephrotic syndrome (NS) correlates with the responsiveness to glucocorticoid therapy. Steroid-resistant NS (SRNS) patients progress to end-stage renal disease, while steroid-sensitive NS (SSNS) and steroid-dependent (SDNS) patients do not. We have performed proteomic profiling of urine samples from a cross section of pediatric and adolescent subjects with SSNS, SRNS, and orthostatic proteinuria (OP) to identify urinary biomarkers of steroid resistance. We performed surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF MS) on urine from 19 subjects with SSNS/SDNS in remission, 14 with SSNS/SDNS in relapse, 5 with SRNS in relapse, and 6 with OP. Genetic algorithm search of principal component space revealed a group of five peaks distinguishing SRNS subjects, with mass/charge (m/z) values of 3,917.07, 4,155.53, 6,329.68, 7,036.96, and 11,117.4. Our analyses identified the peak at m/z 11,117.4 with an accuracy of 95% for classifying SRNS. Multidimensional protein fractionation and mass spectrometric analysis of SRNS urine samples combined with immunodepletion identified the 11,117.4 protein as β_2 -microglobulin (B2M). Using an unbiased protein profiling approach, we have validated previously reported findings of B2M as a biomarker associated with SRNS. Prospective studies are warranted to establish additional biomarkers that would be predictive of SRNS.

Keywords

Focal segmental glomerulosclerosis; Nephrotic syndrome; Steroid resistance; Urine proteomics; β_2 -Microglobulin

Abbreviations

B2M: β_2 -Microglobulin; FSGS: Focal segmental glomerulosclerosis; GA: Genetic algorithm; HPRP: High-performance reverse phase; MALDI: Matrix-assisted laser desorption/ionization; MS: Mass spectrometry; m/z : Mass/charge; NS: Nephrotic syndrome; OP: Orthostatic proteinuria; PCA: Principal component analysis; SD: Steroid dependent; SDNS: Steroid-dependent nephrotic syndrome; SELDI: Surface-enhanced laser desorption/ionization; SR: Steroid resistant; SRNS:

Steroid-resistant nephrotic syndrome; SS: Steroid sensitive; SSNS: Steroid-sensitive nephrotic syndrome; TOF: Time of flight

Introduction

Idiopathic nephrotic syndrome (NS) is the most common glomerular disease in childhood, occurring with an annual incidence of 2–3 per 100,000 [1–4]. Prognosis of this disease correlates with responsiveness to glucocorticoid-based therapy. The majority of cases are steroid sensitive with a benign, protracted course [5–8]. In contrast, steroid-resistant (SR) NS poses a greater therapeutic challenge and is the most common acquired cause of end-stage renal disease in the pediatric population [9]. SRNS is associated with a 30–40% overall risk of post-transplant recurrence [10,11] with some centers reporting even higher rates [12]. Recent epidemiologic data suggest that the incidence of SRNS is increasing, particularly in the African-American population [13,14].

Steroid-sensitive (SS) NS and SRNS have similar clinical presentations, and there are no specific laboratory parameters to distinguish these two clinical entities. Pathological evaluation of renal cortical tissue, by means of a renal biopsy, has traditionally been used to establish a distinction between SSNS and SRNS. The pathological correlates to SSNS and SRNS are minimal change disease and focal segmental glomerulosclerosis (FSGS), respectively. However, these histological diagnoses do not always parallel patients' clinical response to treatment [15]. Identification of noninvasive biomarkers that accurately distinguish SSNS from SRNS would be most beneficial to steroid-resistant patients, preventing their exposure to high-dose, yet ineffective steroid courses.

Proteomics is a technology with high-throughput capabilities that permits the evaluation of complex protein mixtures from biological samples. Proteomic technologies have been leveraged to identify sensitive and specific prognostic biomarkers in a wide range of disease states, particularly in the field of cancer research [16–19]. Accurate classification of 5/6 nephrectomy-induced glomerulosclerosis in rats is achievable by proteomic profiling [20]. Urine protein profiling [21,22] has been evaluated in the setting of renal allograft rejection [23], acute renal injury [24], and IgA nephropathy [25].

We are now reporting high-throughput profiling and identification of urinary protein biomarkers associated with steroid resistance in a cross section of children with idiopathic nephrotic syndrome.

Methods

Study eligibility

Children and adolescents ages 1–18 years with idiopathic NS and orthostatic proteinuria (OP) who were followed in the Renal Program at Children's Hospital Boston were eligible for study inclusion. The study was conducted in accordance with and was approved by the hospital's Institutional Review Board with respect to written informed consent and patient safety issues. NS was defined by the presence of nephrotic range proteinuria, hypoalbuminemia (serum albumin <2.5 g/dl), and edema. Nephrotic range proteinuria was characterized by a spot urinary protein-to-creatinine ratio ≥ 2 or by $\geq 2+$ proteinuria by urinary dipstick for 3 consecutive days. Orthostatic proteinuria was defined as a first morning urinary protein-to-creatinine ratio ≤ 0.2 and a midday urinary protein-to-creatinine ratio ≥ 0.2 . Those children and adolescents with known secondary causes of NS were excluded from the study.

Remission was defined as a negative to trace urine dipstick for protein for 3 consecutive days or a spot urinary protein-to-creatinine ratio <0.2 . Relapse was defined as persistent urine dipstick protein $\geq 2+$ for 3 consecutive days or a spot urinary protein-to-creatinine ratio >2 . Study participants were further categorized as steroid sensitive (SS), steroid dependent (SD), and steroid resistant (SR). Steroid sensitivity was defined as the ability to achieve remission in response to a standard minimum 8-week steroid course after initial diagnosis. Steroid resistance was characterized as a failure to achieve remission, despite receiving a standard steroid course, with a spot urinary protein-to-creatinine ratio >2 . Steroid dependence was defined by the presence of a relapse within 2 weeks of steroid discontinuation or upon steroid tapering [1].

Clinical assessment of each participant included a review of the medical records with respect to history, blood pressure measurements, height, weight, urinalyses, quantification of proteinuria, serum electrolytes, blood urea nitrogen, and serum creatinine.

Sample collection

A minimum of one and a maximum of four urine samples were obtained from each participant over a 1-year period following the date of consent. A minimum of 3 ml of urine was collected per sample. Upon collection, each urine sample was immediately centrifuged at 2,500 rpm for 5 min at room temperature. The supernatant was divided into 1-ml aliquots and placed on dry ice until storage at -80°C for subsequent proteomics applications [22].

Proteomics with SELDI-TOF MS

Samples were prepared for application on CM10 (cationic) and IMAC30 (immobilized metal affinity capture, anionic) ProteinChip (Ciphergen, Fremont, CA, USA) arrays and analyzed by surface-enhanced laser desorption/ionization (SELDI)-time of flight (TOF) mass spectrometry (MS), as previously described by our group [22].

SELDI-TOF MS was performed on the prepared chips (PBS-IIb, Ciphergen, Fremont, CA, USA). Each spot underwent SELDI-TOF MS with two laser intensities to assess proteins of low (2,000–20,000 dalton) and high (10,000–40,000 dalton) mass/charge (m/z) ratios as described [22]. The laser settings for the low protocol were as follows: IMAC30, sensitivity of 9, intensity of 224; CM10, sensitivity of 8, intensity of 220. The high protocol laser settings were as follows: IMAC30, sensitivity of 9, intensity of 246; CM10, sensitivity of 9, intensity of 239.

Selection of differentially expressed peaks

We performed two parallel analyses. First, we developed a noise reduction algorithm to reduce each intensity value into a binary value of “peak” or “no peak” [22]. Briefly, the peak-defining threshold of each spectrum was defined as the nadir of the bimodal distribution of peak intensities for that spectrum, such that each spectrum (i.e., each spot on a protein chip) had a data-driven specific threshold. The noise reduction algorithm eliminated the low-level background noise while preserving the observable peaks (Fig. 1). In addition to subtracting peaks present in OP samples, we also subtracted peaks present in urine from NS subjects in remission. The rationale for this maneuver is that we hypothesized that proteins present in OP urine and in urine from NS subjects in remission are benign, nonpathogenic proteins that are passively filtered into the urine. Therefore, every m/z value that demonstrated a peak in either OP samples or remission samples was “removed” from the population of peaks submitted to the genetic algorithm, and all peaks shown in Fig. 2 were absent in OP and remission samples. We then used a genetic algorithm (GA) [26] which was given the task of taking the entire list of proteins, minus the peaks present in OP and remission samples, and was charged with searching for the protein or small group (≤ 10) of proteins that most accurately distinguishes

relapsed SRNS from relapsed SS/SD NS. The genetic algorithm is essentially a type of artificial intelligence method for efficient searching (i.e., rather than comparing all possible combinations of proteins, which is computationally intractable). The GA was permitted to run until the top-performing model's accuracy was $\geq 95\%$, or for 2,000 generations, whichever came first.

Given that any method that transforms continuous data into discrete values (such as our noise reduction algorithm) risks eliminating more subtle information within the data, we performed principal component analysis (PCA) of the normalized, nondiscrete data set. For this approach, we again utilized our GA to search for the group of mass peaks that yield the maximum separation of samples in the top three dimensions of principal component space. Noise reduction, GA, and PCA were performed within the R programming environment [27].

Purification of the peak at m/z 11,117.4 dalton

A 100- μ l volume of urine obtained from a study subject with high expression of the 11,117.4 dalton protein peak was mixed with 500 μ l of 50 mM Tris pH 9 and incubated with Q Ceramic HyperD F beads loaded with copper sulfate (Biosepra, Pall, East Hills, NY, USA) rocking at room temperature for 2 h. After centrifugation through a spin column, the protein bound to the beads was washed with 50 mM Tris pH 9 and eluted with Tris buffer of decreasing pH followed by 75% acetonitrile and 0.5% trifluoroacetic acid (TFA). The acetonitrile fractions were analyzed by SELDI-TOF MS for the presence of the 11,117.4 peak. The fractions that contained the peak of interest were pooled and injected in the second dimension of a ProteomeLab PF2D protein fractionation system (Beckman Coulter, Fullerton, CA, USA) with a high-performance reverse phase (HPRP) column (Beckman Coulter, Fullerton, CA, USA). The proteins were eluted with a gradient of acetonitrile and fractions were collected. The 11,117.4 peak was present in several consecutive fractions that were then pooled. This pooled sample was passed through a membrane of molecular weight cutoff of 50 kDa (Microcon YM50, Millipore, Billerica, MA, USA). B2M is only 12 kDa and easily passes through the 50-kDa filter. The 50-kDa cutoff filter was used for both eliminating the high molecular weight protein (via the cutoff structure) and separating proteins according to their hydrophobicity with the gradient of acetonitrile. The membrane was washed with increasing concentrations of acetonitrile and the flow-throughs were collected. The 11,117.4 peak was found in the 30 and 50% acetonitrile fractions that were then concentrated by SpeedVac (Thermo Sarvant, Holbrook, NY, USA), reconstituted in 50 mM Tris pH 9, and incubated with Q Ceramic HyperD F beads (Pall, East Hills, NY, USA). The proteins eluted in the organic fraction were loaded for a second run of reversed phase separation on the PF2D column. The fraction containing the protein of interest was dried for subsequent trypsin digestion and protein identification.

Protein identification

The samples were dried and digested by trypsin (Promega, Madison, WI, USA) in 100 mM NH_4HCO_3 overnight, spotted on a stainless steel matrix-assisted laser desorption/ionization (MALDI) target with a C18 ZipTip (Millipore, Billerica, MA, USA) and analyzed on an Applied Biosystems MALDI-TOF/TOF 4700 mass spectrometer (Framingham, MA, USA) with a Mascot (Matrix Science, London, UK) algorithm analysis.

Immunodepletion

We performed immunodepletion studies to confirm protein identification. A volume of 100 μ l of urine was incubated for 2 h with 7 μ l of monoclonal antibody to β_2 -microglobulin (Abcam, Cambridge, MA, USA, catalog number 759) in 500 μ l of phosphate-buffered saline. As negative controls, aliquots of the same urine sample were incubated with normal goat immunoglobulin, water, or an antibody against NF- κ B p65. Protein A and G coated sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA) preincubated with bovine serum

albumin were added to the urine/antibodies and incubated, rocking, for 75 min. The samples were then spun down and the supernatant saved. These supernatants were spotted on a gold chip (CIPHERGEN, Fremont, CA, USA) with C18 ZipTip (MILLIPORE, Billerica, MA, USA) and analyzed by SELDI-TOF MS.

Results

Patient characteristics

A total of 44 patients were enrolled, including 19 with SS/SDNS in remission, 14 with SS/SDNS in relapse, 5 with SRNS in relapse, and 6 with OP. Table 1 shows the equal gender distributions across all groups. Table 2 displays the demographic and clinical characteristics of the study cohort of relapsed subjects. There were no significant clinical differences between the two groups of NS in relapse. Children with SSNS and SDNS do not routinely undergo renal biopsy. All children in the SS/SDNS category who did undergo renal biopsy had minimal change disease. All SRNS children had FSGS on biopsy.

Analysis 1: noise reduction and genetic algorithm

Excluding peaks observed in OP samples, CM10 profiles had 309 peaks, and IMAC30 profiles had 508 peaks. After filtering via our noise reduction algorithm, the GA found a single peak at m/z of 11,117.4 dalton that differentiated between SS/SDNS and SRNS with an accuracy of 95% (Fig. 2). No other peak or group of up to ten peaks demonstrated this degree of accuracy. This peak was traced back to the IMAC30 (anionic) ProteinChip. It was present in 4 of the 5 relapsed SRNS samples and in none of the 14 relapsed SS/SD samples.

Analysis 2: principal component analysis

We performed PCA of the normalized, nondiscrete (non-noise reduced) data set, and utilized a GA to search for the group of mass peaks that yield the maximum separation of samples in the top three dimensions of principal component space. After 2,000 generations, the GA selected a group of five m/z peaks, including 3,917.07, 4,155.53, 6,329.68, 7,036.96, and 11,117.4. Using these five peaks to project the duplicate samples into the top three components of principal component space reveals a clear line of separation between SRNS samples and relapsed SS/SD NS samples (Fig. 3). Figure 4 shows the box plots of the non-discretized intensity values for these peaks, demonstrating four peaks differentially expressed in SRNS and one peak differentially expressed in SS/SDNS.

Protein identification

The 11,117.4 peak was selected by both analyses. Therefore we focused on identifying this protein. In order to identify the protein encoding the peak clustered at 11,117.4, we fractionated by multidimensional chromatography the protein content of one of the urine samples highly expressing the peak as described in the “Methods” section. After each fractionation step, the protein profile of each fraction was determined by spotting an aliquot on a gold chip using ZipTip (MILLIPORE, Billerica, MA, USA) when needed and analyzing the mass spectrum via SELDI-TOF. After several fractionation steps the spectra showed only very few peaks in addition to the one of interest. This fractionated sample was digested and proteins identified by MALDI-TOF/TOF MS. The Mascot database search identified β_2 -microglobulin as the protein with the highest level of confidence and with a molecular weight closest to 11,117.4. We therefore proceeded to validate this candidate protein. We used a monoclonal antibody directed against β_2 -microglobulin to deplete the urine sample of β_2 -microglobulin, as described in the “Methods” section. This depleted sample was then analyzed by SELDI-TOF MS. We observed that the peak at 11,117.4 was dramatically reduced in the SELDI-TOF MS spectra

as compared with the samples treated with control antibodies while the other protein peaks remained unchanged (Fig. 5) confirming that the 11,117.4 peak is β_2 -microglobulin.

Discussion

A noninvasive biomarker such as a urine protein profile that accurately predicts responsiveness to steroid therapy in NS would be a valuable addition to the diagnostic armamentarium of nephrologists. Children, in particular, are adversely affected by long courses of high-dose daily steroid therapy, which inhibits growth. Children with new-onset NS are treated with an 8–12 week course of high-dose corticosteroids, which serves as both a diagnostic and therapeutic maneuver. Histopathologic findings on renal biopsy have been used by pediatric nephrologists to prognosticate long-term renal function, but the response to steroid therapy is the most important feature in the prognosis in NS [8,28,29]. Therefore, children with SRNS are exposed unnecessarily to the toxic effects of steroids without the therapeutic benefits. Access to a sensitive and specific noninvasive predictor of steroid responsiveness in NS would spare a substantial subgroup of patients from receiving high-dose, yet ineffective steroid therapy. Furthermore, steroid resistance in the setting of NS is prognostic of the leading acquired cause of end-stage renal disease in children [9].

We have shown that urine proteomics can be utilized to identify biomarkers associated with steroid resistance in NS. We identified one of the five protein peaks associated with steroid resistance as β_2 -microglobulin, a protein that has been previously linked to SRNS and renal tubular damage [30–40]. β_2 -Microglobulin is a small molecular weight protein that is an essential component of the class I major histocompatibility complex and is present on the surface of all nucleated cells. Its production is increased in patients with lymphoproliferative disorders and autoimmune diseases [41–43], suggesting increased levels may reflect immune activation. Because of its small size, β_2 -microglobulin is normally freely filtered by the glomerulus and almost completely reabsorbed in the proximal tubule [44–46]. Its reabsorption is via receptor-mediated endocytosis and is competitively inhibited by other proteins such as albumin [38]. Thus, one would expect that the massive albuminuria due to relapse of NS would lead to increased urinary β_2 -microglobulin, a marker of renal tubular damage [30–40]. While our identification of β_2 -microglobulin by mass spectrometric proteome analysis of urine is not novel, we are the first group to discover β_2 -microglobulin in SRNS by an unbiased high-throughput profiling approach, as opposed to the antibody-based targeted approaches previously described for β_2 -microglobulin. Furthermore, our analysis identified four additional protein biomarkers associated with SRNS that in conjunction with β_2 -microglobulin may enhance the diagnostic value as compared to a single biomarker.

In our study, we found a peak identified as β_2 -microglobulin (11,117.4 dalton) that is smaller than would be expected given the molecular weight of the intact β_2 -microglobulin protein (11,731 dalton). This difference in m/z value is beyond what could be explained as intact β_2 -microglobulin given the predicted resolution and mass accuracy of SELDI-TOF MS [47,48]. Schaub et al. have recently reported related findings of their experience with SELDI-TOF MS in the identification of urinary biomarkers of acute renal allograft rejection [21,49]. They identified a “rejection pattern” that was distinct from healthy controls, renal allograft recipients with stable graft function, post-transplant acute tubular necrosis, acute cystitis, and glomerulopathies [21]. Using tandem mass spectrometry, they subsequently identified their peaks as non-tryptic cleavage products of β_2 -microglobulin [49]. Based on their identification of the proteases responsible for the novel cleavages, they hypothesized that tubular injury causes shedding of lysosomal proteases whose activity is enhanced by the acidic urinary environment, leading to novel breakdown products of β_2 -microglobulin. Of the peaks identified in our study by PCA as distinguishing SRNS from steroid responders, 7,036.96 and 11,117.4 were similar in size to peak clusters identified by Schaub et al. as non-tryptic cleavage products

of β_2 -microglobulin, and 3,917.07 was comparable to the triple-charged form of intact β_2 -microglobulin [49], although we have no formal evidence to confirm the nature of these peaks as cleavage or triple-charged products of β_2 -microglobulin. We can only hypothesize that the relentless proteinuria present in our SRNS cohort may have precipitated significant tubular damage leading to a similar shedding of intracellular lysosomal proteases and generating novel β_2 -microglobulin breakdown products.

Previous efforts by our group at identification of markers of SRNS have included intrarenal gene expression of nuclear factor- κ B, angiotensinogen, and transforming growth factor- β [50]. The paucity of identified serum or urine markers may be due to the molecular heterogeneity associated with NS, which includes altered expression of cytokines, transcription factors, and podocyte structural proteins. Therefore it is likely that no single marker will prove useful in the prognostication of steroid responsiveness in NS. Rather, identification of groups of biomarkers or “profiles” will likely be necessary to distinguish subgroups of NS at the molecular level.

Our study has a number of limitations. We have identified biomarkers associated with, but not predictive of, steroid resistance. The cross-sectional design does not permit us to control for patient differences related to chronicity of disease or previous immunosuppressive therapy. In addition, unambiguous intact protein identification is not possible with SELDI and remains a limitation of this technology in contrast to the protein identification possible with MALDI or liquid chromatography tandem MS. However, in contrast to liquid chromatography tandem MS, which can only analyze peptides from one patient sample at a time, SELDI-TOF enables the parallel analysis of many patient samples at the protein level.

In conclusion, urine proteomics is a feasible technology to help distinguish SRNS from SSNS in children. Using a high-throughput profiling approach, we have validated previously reported findings of β_2 -microglobulin as a biomarker associated with steroid resistance in this disease. Prospective studies are warranted to establish biomarkers that would be predictive of SRNS.

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Fig. 1. Representative MS spectrum shown before (*top*) and after (*bottom*) noise reduction. For each spectrum, the algorithm determined the distribution of peak intensities, assumed a bimodal distribution, determined the intensity value at the nadir of the bimodal distribution, and removed all peaks from that spectrum that demonstrated intensity values below the nadir intensity value. The two spectra differ at the lower intensity levels

Fig. 2.

Representative MS spectra demonstrating peak found in 11,117.4 in SR subjects (*two spectra at the top*) and not found in SD (*two spectra in the middle*) and SS subjects (*two spectra at the bottom*). Spectra are shown from three subjects in duplicate

Fig. 3. Scatterplot of SRNS (*black*) and SS/SD NS (*gray*) samples in principal component space, projected using five mass peaks selected by the genetic algorithm

Fig. 4.

Box plots of the five mass peaks identified by the genetic algorithm within principal component space. These are the same five mass peaks that are used to project samples into principal component space in Fig. 3. IMAC30 refers to the anionic protein chip, m/z mass/charge ratio, *SS/SD* steroid sensitive/steroid dependent, *SR* steroid resistant

Fig. 5.

A urine sample containing the protein of interest was incubated with antibody against β_2 -microglobulin (a), water (b), normal goat immunoglobulin (c), or NF- κ B p65 (d). The samples were then immunoprecipitated and the supernatant was used to spot a gold chip for mass spectrometry analysis. The spectra shown are from the region of m/z 11,117.4. The major peak seen in this region is indicated by the arrow and is dramatically reduced with the B2M antibody and not with the controls used

Table 1

Gender distribution across all subjects enrolled

Diagnostic category	Female	Male
SS/SD in relapse	7	7
SR in relapse	3	2
Remission	9	10
OP	3	3

Table 2

Demographic and anthropomorphic data on study group^a

	SS/SD (n=14)	SR (n=5)
Age (years)	10.6 (3.3–20)	9.4 (3.6–17.9)
Weight (kg)	40.9 (20.4–151.1)	47.2 (25.2–93.8)
Height (cm)	129.6 (104.4–177.6)	144.8 (125.6–153.8)
Systolic blood pressure	111 (95–151)	131 (108–151)
Diastolic blood pressure	70 (48–85)	75 (58–86)
ACEI (mg/24 h)	0 (0–60)	10 (0–50)
ARB (mg/24 h)	0 (0–100)	0 (0–50)
Urine protein:creatinine ratio	1.8 (0.7–4.2)	5.7 (0–21.2)

^aNonparametric