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Proteomic analysis of inhibitory protein profiles in the urine of children with nephrolithiasis: implication for disease prevention

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Abstract

Purpose In this study we aimed to screen for the presence of biomarkers that are downregulated in children with nephrolithiasis (RS) compared to healthy controls (HC) using a proteomic approach. We hypothesized that RS and HC would display unique inhibitory protein profiles that could be used for comparative pathway analysis.

Methods This is a prospective, controlled, pilot study of pooled urine from RS ($N=30$, 24 females, mean age 12.95 ± 4.03 years) versus age- and gender-matched HC, using liquid chromatography-mass spectrometry. The criteria for protein selection were: (1) patient/control abundance ratio of <0.5; and (2) ≤ 0.05 p-value for the Fisher's Exact Test. Results were confirmed by ELISA testing in individual samples.

Results 67 proteins were downregulated in RS group, and 17 of those were significantly different compared to controls. Of those seventeen proteins, five (two actins, annexin A5, keratin 6B, and serpin B4) were completely absent in the urine of stone patients but were found in controls. The remaining twelve proteins were significantly less abundant in the patient's urine compared to healthy controls. Protein–protein interaction modeling of significant proteins identified syndecan-1 as the key node, a protein associated with adhesion pathways. ELISA analysis by subgroups showed statistically significant difference in the urinary excretion of osteopontin (5.1 ± 3.22 ng/mg creatinine vs 14.1 ± 9.5 ng/mg creatinine, $p=0.046$) between stone patients with hypocitraturia and controls. Urinary osteopontin concentration was positively correlated with urinary citrate excretion ($r=0.417$, $p=0.03$).

Conclusions Children with RS have a different urinary inhibitory polypeptide profile compared to HC. Decreased urinary excretion of these proteins indicates their potential inhibitory role in renal stone formation, especially of the adhesion phase. Lower concentration of urinary osteopontin in children with nephrolithiasis and hypocitraturia suggests its potential involvement in the pathogenesis of this disease. Further characterization of these proteins in a larger sample is imperative.

Introduction

Urine supersaturation and abnormal balance between promoters (mainly calcium and oxalate) and inhibitors (mainly magnesium, citrate, and pyrophosphate) of urine crystallization are major contributing factors to stone formation. Various proteins participate in the crystal-crystal and crystal-cell interactions at the renal tubular epithelium either promoting or inhibiting the stone process [1, 2]. Proteins represent about 60% of the stone matrix [3], and a great number was detected in the urine of adult stone-formers [4], but little is known about their presence and function in pediatric population. The identification of the urinary inhibitory proteins in children is particularly important because of the potential role they could play in disease prevention. For this reason we performed a proteomic study aimed to screen for the presence of urinary biomarkers that are downregulated in children with nephrolithiasis (RS) compared to healthy controls (HC). We hypothesized that RS and HC would display unique inhibitory protein profiles that could be used for comparative pathway analysis.

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Methods

An initial screening of the significant proteins in the RS group compared to age- and gender-matched HC was performed in pooled urine samples using liquid chromatography-mass spectrometry (LC–MS/MS). Urine sample collection and preparation, as well as 2D LC–MS/MS and protein quantitation methods were described by our group in a previous paper [5]. In short, pooled samples consisted of the second morning mid-stream fresh urine obtained in sterile cups from patients and controls. The urine samples were prepared within 3 h of collection, centrifuged at 2500 rpm for 15 min, and stored until use as recommended by standardized proteomic protocols (developed by the Human Urine and Kidney Proteome Project, HUKPP, and the European Urine and Kidney Proteomics, EuroKUP Initiatives) [6]. Proteins in each sample were concentrated in a Centricon-type filter. Albumin and IgG were removed by anti-HAS/IgG resin (Sartorius). Protein concentration in each sample was measured by the BCA protein assay (Pierce), and 10 µg of protein per individual sample was used in each pool. Pooled samples were digested with trypsin and analyzed by two dimensional liquid chromatography—mass spectrometry (2D LC–MS/MS).

A detailed description of the 2D LC–MS/MS method can be found in the supplemental section

The criteria we used to define the urinary proteins as significantly different between patients and controls were: (1) patient/control abundance ratio of < 0.5 as a threshold to be well above observed technical variations in most label free proteomic analyses [7]; and (2) ≤ 0.05 p-value for the Fisher's Exact Test.

Results were confirmed by ELISA testing in individual urine samples. Statistical analyses were conducted with IBM SPSS® version 20. This study was IRB approved (IRB number: 075511MP4E).

Patient selection

Patient inclusion and exclusion criteria are presented in (Table 1). The criterion of two 24 h urine collections was chosen to increase the diagnostic yield since significant variation in the urinary parameters between urine collections was reported in the literature [8, 9]. At the time of urine collection, these children were asymptomatic (no flank or abdominal pain, no urinary symptoms), and were on no medication for renal stones. Hypercalciuria was defined as excretion of calcium greater than 4 mg/kg/day [10] and hypocitraturia was diagnosed when urinary citrate was less than 310 mg/1.73 m^2/day in girls and 365 mg/1.73 m^2/day in boys [11].

The control group consisted of age- and gender-matched healthy children seen in our clinic for bedwetting that resolved at the time of urine collection. Their inclusion and exclusion criteria are shown in (Table 1). The 24 h urine collection was not performed in these children due to technical reasons, and due to cost.

Results

The demographic characteristics of both groups are shown in (Table 2). Stone group consisted of 10 children with hypercalciuria, 10 with hypocitraturia, and 10 with normal metabolic work-up. Both groups had normal urinary dipstick at the time of urine collection.

Of the 1813 proteins identified by proteomic analysis, 1639 were found in children with nephrolithiasis, and 1396 were found in controls. Of those, 417 were seen only in patients and 174 only in controls. Using the above-mentioned criteria, 67 proteins were downregulated

Table 1 Inclusion and exclusion criteria used for the selection of control and study groups

Study group		Control group	
Inclusion criteria	Exclusion criteria	Inclusion criteria	Exclusion criteria
5–18 years of age; [a]History of proven renal stone; At least two satisfactory 24 h urine collections; [b]Absence of hematuria or pyuria; Normal renal function	Lack of toilet training; [c]Bladder and kidney diseases; Active urinary tract infection; Presence of blood in urine; Chronic kidney disease; [d]Any significant medical condition	Normal renal bladder ultrasound; Normal urine dipstick; Normal calcium-to-creatinine ratio in spot urine	Lack of toilet training; Bladder and/or kidney stones; Chronic medical conditions

[a]Typical renal colic and radiographically (ultrasound or CT) proven renal stone

[b]Urinary creatinine more than 15 mg/kg/day

[c]Bladder and kidney stones, nephrocalcinosis, neuropathic bladder, major congenital bladder abnormality, previous major reconstructive bladder surgery requiring catheterization

[d]Cardiac, pulmonary, gastro-intestinal, and neurological problems

Table 2 Demographic characteristics of the groups

	Study group ($N=30$)	Control group ($N=30$)	P-value
Gender (male/female)	8/22	9/21	1
Mean age ± SD (years) (Range)	12.96 ± 3.9 (5.5–18)	13.03 ± 3.86 (5.5–17.6)	0.96
Race/Ethnicity			
Caucasian	17 (56.7%)	14 (46.7%)	0.2
Africa America	5 (16.6%)	11 (36.7%)	
Other	8 (26.7%)	5 (16.6%)	

in RS group, and 17 of those were significantly different (Table 3). Five proteins (two actins, annexin A5, keratin 6B, and serpin B4) were completely absent in the urine of stone patients but were found in controls. The remaining twelve proteins were significantly less abundant in the patient's urine compared to healthy controls. Protein–protein interaction modeling of significant proteins identified syndecan-1 as the key node, a protein associated with adhesion pathways. ELISA analysis by subgroups showed statistically significant difference in the urinary excretion of osteopontin (OSP) (5.1 ± 3.22 ng/mg creatinine vs 14.1 ± 9.5 ng/mg creatinine, $p=0.046$) (Fig. 1) between stone patients with hypocitraturia and controls. Urinary OSP concentration was positively correlated with urinary citrate excretion ($r=0.417$, $p=0.03$).

Discussion

The initial process of renal calculus formation involves several chemical and physical factors, the first of which are high urinary solute concentration (supersaturation) and low urinary volume. Further growth and aggregation of crystals is favored by ionic strength and urinary pH. Adherence to the renal tubular epithelial cells of the urinary tract is required to allow for crystal growth which prevents crystals from being washed out. In normal subjects, nucleated crystals, composed mainly of calcium oxalate, are excreted in the urine before they can adhere to tubular cells; this is due to the presence of various inhibitory macromolecules such as proteins, lipids, and glycosaminoglycans. Of these, several proteins appear to play a major role in the urinary tract's defense against the crystallization of calcium salts which prevents the formation of stones [12, 13]. These urinary proteins act by incorporating into crystals, causing decreased affinity for renal cells and facilitating intracellular destruction of crystals [14]. The protein's altered function and/or low concentration in the urine can result in stone formation. Therefore, the identification of these urinary proteins represents an important step for renal stone prevention, which is imperative in pediatric patients.

To our knowledge, this is the first proteomic study to investigate the urinary inhibitory protein profiles in pediatric nephrolithiasis. We found that children with RS have a different urinary inhibitory polypeptide profile compared to HC, as hypothesized. We isolated 17 urinary proteins that

Table 3 Urinary inhibitory proteins identified in children with nephrolithiasis with at least twofold decreased abundance relative to healthy controls

Name	Accession number	Assigned peptides [Patient-Control]	Ratio (Patient/Control)	Fisher's exact test (p-value)
Actin, alpha cardiac muscle 1	ACTC	0/43	0	< 0.00010
Actin, cytoplasmic 1	ACTB	0/66	0	< 0.00010
Annexin A5	ANXA5	0/5	0	0.033
Keratin, type II cytoskeletal 6B	K2C6B	0/21	0	< 0.00010
Serpin B4	SPB4	0/25	0	< 0.00010
Syndecan-1	SDC1	1/21	0.05	< 0.00010
Annexin A2	ANXA2	5/28	0.18	< 0.00010
Annexin A1	ANXA1	8/37	0.22	< 0.00010
Serpin B3	SPB3	8/33	0.24	< 0.00010
Osteopontin	OSTP	46/151	0.30	< 0.00010
Trefoil factor 2	TFF2	12/35	0.34	0.00070
Vasorin	VASN	23/64	0.36	< 0.00010
Collagen alpha-1(III) chain	CO3A1	17/46	0.37	0.00023
Cadherin-1	CADH1	10/27	0.37	0.0046
Granulins	GRN	81/204	0.40	< 0.00010
Actin, cytoplasmic 2	ACTG	23/56	0.41	0.00019
Cubilin	CUBN	83/182	0.46	< 0.00010

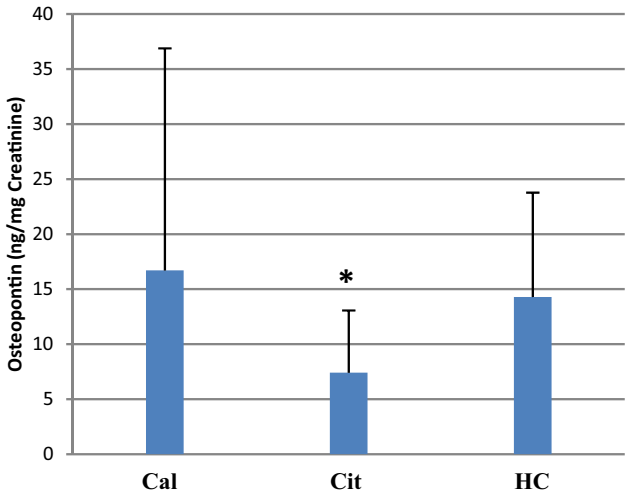

Fig. 1 Urinary osteopontin concentration in children with urolithiasis and hypercalciuria (Cal) and hypocitraturia (Cit) compared to healthy children (HC) assessed by ELISA. *$P = 0.046$

were downregulated in stone patients compared to healthy children. Decreased urinary excretion of these proteins indicates their potential inhibitory role in renal stone formation. Syndecan was identified as the key protein, suggesting a significant involvement of all these urinary proteins in the crystal's adhesion phase. Syndecan, also known as heparan sulfate, is a major glycosaminoglycan component of the matrix of calcium-oxalate stones and is a potent inhibitor of calcium oxalate crystallization [15, 16]. Indeed, several investigators have shown that syndecan facilitates nucleation, inhibits growth [17], and prevents aggregation of calcium oxalate crystals in undiluted, ultrafiltered urine [18]. By facilitating nucleation, syndecan promotes the precipitation of smaller crystals that will be excreted from the urinary tract much more easily. Additionally, syndecan creates a charge barrier against calcium oxalate crystal attachment on the renal epithelial Mardin-Darby canine cells [19].

In our study, further testing in individual urine samples showed significantly lower concentration of urinary OSP in children with nephrolithiasis and hypocitraturia compared to healthy controls. OSP (also known as uropontin) is a 40 kDa glycoprotein that is synthesized within the kidney (mainly in the thick limb of Henle), and is secreted into the urine by the renal tubular epithelial cells [20–22]. It is present in the matrix of calcium oxalate stones [23, 24] and in normal adult urine (> 100 nM) [25]. Few in vitro and in vivo studies demonstrated that OSP is an important modulator of stone formation [26, 27], but its precise role in the pathogenesis of nephrolithiasis remains controversial. Urinary OSP was reported as being downregulated in the adult stone former pools, but further testing in individual urine samples was not undertaken [28]. Using proteomic analysis, Cadieux identified OSP as an important urinary marker in 25 male patients with nephrolithiasis but found no significant difference in the urinary OSP levels measured by ELISA between stone group and controls [29, 30]. More recent reports showed that OSP inhibits calcium oxalate and calcium phosphate crystal nucleation, growth and aggregation by binding to calcium, and by disturbing the lattice of crystals during cell migration [31, 32]. In an osteopontin knockout mouse given ethylene glycol to generate oxalate, the formation of calcium oxalate crystal was exacerbated compared to the wild-type mouse [13].

Our finding of low urinary concentration of OSP in children with nephrolithiasis and hypocitraturia, in addition to the significant positive association between OSP and citrate suggests an important interplay between urinary OSP and citrate. This novel finding is supported by several known facts that apply to both OSP and citrate: (1) both represent important constituents of the normal human urine; (2) both were found to have a potent inhibitory effect on calcium oxalate crystallization, especially of the growth phase; and (3) both were reported to change the gross morphology of the calcium oxalate crystals. However, OSP and citrate act on different faces of calcium oxalate monohydrate crystals, suggesting their additive effects in changing the shape of calcium oxalate crystals [33]. Additionally, OSP facilitates calcium oxalate crystallization to the dehydrate phase, decreasing the adherence to the renal tubular cells compared to the calcium oxalate monohydrate form [32]. Based on all aforementioned accumulated knowledge by others and on our results it seems that both OSP and citrate may have simultaneous and cumulative inhibitory action on the crystal growth and adhesion to tubular epithelial cells, indicating their protective role in calcium nephrolithiasis. However, this remains speculative since we were not able to examine the causal relationship between OSP and citrate due to our study design. In spite of this limitation and of a small studied sample, our findings are significant because they provide additional insight in the pathogenesis of stone formation in hypocitraturia, a metabolic abnormality that is on the raise in the pediatric population [34].

Study limitations

The major limitation of this study is the inability to establish causal-effect relationship between these processes, due to the cross-sectional study design. Other limitations include (1) the small sample size, and (2) the initial use of pooled samples, which was intended to reduce the sample intra- and inter-variability, and the cost. However, this is an acceptable screening method in proteomics for the aforementioned reasons.

Conclusions

In conclusion, children with RS have a different urinary inhibitory polypeptide profile compared to HC. Decreased urinary excretion of these proteins suggests their potential involvement in the pathogenesis of nephrolithiasis.Lower concentration of urinary OSP in patients with nephrolithiasis and hypocitraturia compared to healthy children indicates its potential inhibitory role in renal stone formation, especially of the growth and adhesion phases. Therefore, further characterization of these inhibitory proteins in general and of urinary OSP in particular in a larger population of pediatric stone-formers obtained from a multi centric study is important because it will provide new insights in the mechanism of stone formation and will generate novel prophylactic and therapeutic measures in pediatric nephrolithiasis.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11255-022-03310-5.

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Declarations

Conflict of interest Author Larisa Kovacevic declares that she has no conflict of interest. Author Natalija Kovacevic declares that she has no conflict of interest. Author Yegappan Lakshmanan declares that he has no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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