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Brief communication (Original)

Increased protein excretion, including albumin, by children of patients with urolithiasis

Thasinas Dissayabutra[a], Jakkapan Rattanapan[a], Nuttiya Kalpongnukul[a], Supawadee Mingmongkol[a], Wattanachai Ungchareonwattana[b], Chanchai Boonla[a], Piyaratana Tosukhowong[a]

[a]*Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand*

[b]*Division of Urology, Department of Surgery, Sunpasit Prasong Center Hospital, Ubon Ratchthani 34000, Thailand*

Background: Patients with urolithiasis usually have increased urinary protein excretion.

Objectives: To compare the urinary protein, including albumin, excretion by patients with urolithiasis and their children, and identify the urinary proteins that are excreted more than they are by the normal population.

Materials and Methods: We recruited 28 patients with urolithiasis after stone removal (G1) and their nonstone-forming children (G2), and 30 healthy volunteers who lived in the same region (G3) and their children (G4). Medical history and 24 h urine were analyzed. Total urinary proteins and albumin were measured, and the urine proteome analyzed by two-dimensional SDS–polyacrylamide gel electrophoresis and mass spectrophotometry.

Results: Age, sex, body mass index, and amount of smoking and alcohol drinking were not different between G1 and G3, or G2 and G4. G1 patients had more prevalent underlying diseases than participants in the other groups. Urinary protein and albumin levels were highest in G1 participants, and were higher in G2 compared with G4. Preliminary proteomics showed elevated urinary Tamm–Horsfall protein, albumin, κ- and λ-2 light chain immunoglobulin excretion.

Conclusions: Patients with urolithiasis and their children had elevated excretion of urinary protein, including albumin, compared with the normal population, even though the levels were not clinically important. Leakage of these proteins suggest a tubular cell reabsorption defect that might associate with the pathogenesis of stone formation.

Keywords: Albumin, family, urinary protein, urine proteome, urolithiasis

Urolithiasis is a common urologic problem worldwide, with a lifetime prevalence is 7.14% to 11.10% [1-3]. The prevalence of urolithiasis is up to 16.7% in the northeast region of Thailand [4]. Urinary stones form when the solute concentration in urine exceeds the saturation point, called a supersaturation state. In this state, the ionic lithogenic solutes, such as calcium, oxalate, phosphate, or uric acid spontaneously form a complex, and then insoluble complexes become crystals in the luminal part of nephrons. Tubular epithelial cells take up these crystals, which accumulate in the interstitium, inducing inflammation, bleeding, and oxidative stress. Patients with urolithiasis usually develop flank pain, hematuria, and frequently chronic renal failure.

Correspondence to: Thasinas Dissayabutra, Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand. E-mail: Thasinas.d@chula.ac.th

Although the pathophysiology of stone formation was well defined, its etiology has not yet been fully clarified. It is well recognized that the diabetes mellitus, hypertension, obesity, and metabolic bone disease increase the risk of urinary stone formation, as does a person who has a first-degree relative with urolithiasis. Until now, there is no evidence reported of any single genetic disease that causes urinary stone formation in Thai patients, and environmental hazards are considered only aggravating factors. However, close relatives of a patient with urolithiasis have higher chance of developing urolithiasis. Sritippayawan et al. reported that in Thai people from the northeast, relatives of patients with urolithiasis have a 3.18 times higher risk for stone formation [5].

Calcium oxalate is the most common stone found in humans, and its incidence is similar throughout the world. The most important risk factors for urolithiasis in Western populations are hypercalciuria and

hyperoxaluria (over excretion of calcium and oxalate in the urine). However, in Asian populations, such as in China, Taiwan, Malaysia, and Thailand, hypocitraturia (inadequate citrate excretion in urine) remains the most prominent risk factor, which is up to 69.6% in Thai patients, while hypercalciuria and hyperoxaluria were found in only 15.2% and 1.3% of Thais respectively [6]. The abnormal urinary characteristics are shared in family members. Children of patients with urolithiasis have a higher urinary oxalate and lower urinary citrate compared with a normal age- and sex-matched population. The validity of these risks for urolithiasis in family members is noted. However, the risks do not shed light on whether these abnormalities are derived from intrinsic or extrinsic causes.

The supersaturation and crystal usually form at the loop of Henle region of nephron where the highest osmolarity of urine occurs. Urolithiasis is considered to induce tubulointerstitial injury, and elevated urinary protein found in patients with urolithiasis probably leaks from the tubulointerstitial lesions. Several proteins are considered to be upregulated in a calcium oxalate-mediated HK-2 cell model of urolithiasis, including FK506-binding protein 4, pyruvate kinase isozymes, and fascin [7]. Elevated urinary protein excretion, including albumin, comorbidities frequently found in patients with urolithiasis, but this is unable to be explained by tubulointerstitial damage, diabetes, or hypertension alone.

In the present study, we aimed to study the levels of urinary protein and albumin excretion in 24 h urine of patients with urolithiasis and their children, to validate these abnormalities for risk assessment, and to investigate further the urinary proteome and pathophysiology of urolithiasis.

Materials and methods

The present study, including assent forms for vulnerable participants (those aged 7–12 years, and 13–18 years old) was approved by Institutional Review Board of the Department for Development of Thai Traditional and Alternative Medicine as part of Ministry of Public Health, Thailand (approval No. RLC0029/55), and IRB of Sunpasit Prasong Hospital. We enrolled 116 participants in this study into 4 groups. Group 1 (G1) comprised 28 patients with calcium oxalate urolithiasis who underwent successfully surgical stone removal at Sunpasit Prasong Center Hospital, Ubon Ratchathani province, Thailand, with no visible stone larger than 0.4 cm seen in plain x-ray images after 1 month, while Group 2 (G2) comprised children of the urolithiasis patients (n = 28) who had no history or symptoms suggesting urinary stone disease. Group 3 (G3) were age- and sex-matched healthy volunteers living in the same region as Group 1, and Group 4 (G4) were children of the healthy volunteers. All participants were well-informed about the purpose and requirements of the study. Informed consent was freely obtained in writing from all participants before their enrollment in the study. Where the participant's age was between 7 and 18 years-old, written informed assent was obtained from the participant, and written informed consent was obtained from at least one of their parents.

Participants in Groups 2, 3, and 4 were assessed for urinary stone symptoms by taking a history and by a urine blood strip test. Medical records were reviewed, and a 24 h urine sample was collected. The total volume of the urine sample was measured, then tested using a urine strip, and preserved in a deep freezer with thymol until further analysis.

Total urinary protein was measured using the Bradford technique. In brief, 50 μL of urine was mixed with Coomassie Brilliant Blue G-250 dye in acidic solution. The mixture was filtered and then incubated for 5 min at room temperature before measuring its absorbance at 595 nm [8, 9].

Urinary albumin was measured using an electrochemiluminescence technique on a COBAS C6000 analyzer (Roche diagnostics, Basel, Switzerland), with the limit of detection (LOD) of 0.001 mg/dL for albumin [10, 11].

We randomly selected 10 samples from the 24 h urines of the G2 and G4 participants for proteomic study using two-dimensional (2D) SDS–polyacrylamide (PAGE) electrophoresis and proteins were identified using liquid chromatography followed by mass spectrometry (LCMS-2020, Shimadzu, Kyoto, Japan) and then compared with the protein UniProtKB and Swiss-Prot–ExPASy databases using Mascot software [12].

Data are expressed as mean ± SD. The differences of age, body mass index (BMI), urinary protein, and albumin were tested using a one-way analysis of variance followed by a least significant difference post hoc test, and sex by a χ^2 test. The differences in urinary protein and albumin levels between each family were assessed using a paired *t* test. Statistical analysis was performed using SPSS software, version 16 (Chicago, IL, USA), with $P < 0.05$ considered significant.

Results

Demographic data

The 28 calcium oxalate stone-forming patients who successfully had their stones removed and permitted their children to enroll in this study were allocated to the G1 group. These patients were predominantly male (64%) and their average age was 46.1 ± 9.7 years. Their children were predominantly female with an average age of 19.6 ± 8.7 years. Age- and sex-matched healthy volunteers were recruited from residents who lived in the same area as the G1 patients to minimize the effect of variability of climatic, cultural, and dietary patterns (G3). Most of the G3 participants were male (57%) with an average age of 45.6 ± 9.0 years old, which was not significantly different from G1 (P = 0.564 and P = 0.825, respectively). Children of the healthy volunteers were also recruited as the G4 group, and were predominantly female with an average age of 19.7 ± 7.3 years old. The sex and age of G4 participants was not significantly different from G2 participants (P = 0.773 and P = 0.955, respectively) (**Table 1**).

Obesity is a risk factor for urolithiasis, the BMI of the participants in groups G1 and G3 was calculated and found not significantly different (24.0 ± 3.6 vs 24.5 ± 3.6 kg/m^2, P = 0.567). BMI was not calculated for participants in groups G2 and G4 because some participants in these groups were children or adolescents, to whom the normal range of BMI could not be applied.

Patients with urolithiasis appeared to have a higher rate of comorbidity than participants in the G3 group. Hypertension and diabetes mellitus were the most common noncommunicable diseases found in G1 patients, followed by dyslipidemia, cardiovascular disease, chronic kidney disease stage I or II (estimated glomerular filtration rate or eGFR was greater than 60 mL/min/1.73 m^2), while their children reported a lower frequency of diabetes, cardiovascular, and chronic liver diseases.

Urine analysis

Urine tested using a urine test strip showed that G1 still have abnormal urine after their stone was removed, hematuria, proteinuria, and glycosuria were frequently found in patients postoperatively. By contrast, glycosuria was found in 5 subjects, but no abnormality was found in the children of participants.

Table 1. Demographic data of the participants

	Patients with urolithiasis (G1) (n = 28)	**Children of patients with urolithiasis (G2) (n = 28)**	**Healthy volunteers (G3) (n = 30)**	**Children of healthy volunteers (G4) (n = 30)**
Sex (% male)	64.3%	42.8%[c]	56.7%	46.7%
Age (years)	46.1 ± 9.7	19.6 ± 8.7[c]	45.6 ± 9.0	19.7 ± 7.3[d]
(range, years)	(32–69)	(10–45)	(28–62)	(7–33)
BMI (kg/m^2)	24.0 ± 3.6	N/A	24.5 ± 3.6	N/A
Smoking (%)	28.6%	17.9%	26.7%	13.3%
Alcohol drinking (%)	35.7%	14.3%	46.7%	13.3%
Underlying disease (individuals)				
Diabetes mellitus	6	1	4	0
Hypertension	7	0	6	0
Dyslipidemia	3	0	1	0
Cardiovascular disease	2	0	0	2
Chronic liver disease	1	1	0	0
Chronic renal disease (eGFR >60 mL/min/1.73 m^2)	2	0	1	0

[a]P < 0.05 comparing G1 and G3 (parental groups), [b]P < 0.05 comparing G2 and G4 (descendant groups), [c]P < 0.05 comparing G1 and G2 (urolithiasis family groups), [d]P < 0.05 comparing G3 and G4 (healthy volunteer family groups), N/A data not available; eGFR = estimated glomerular filtration rate

G1 participants had the highest levels of urinary protein compared with other groups as shown in **Table 2**. Healthy volunteers (G3) had significantly lower levels of urinary protein than G1 participants ($P < 0.001$.) The children of G1 participants (G2) had elevated urinary protein compared with their counterparts (G4 participants) ($P < 0.001$). The level of urinary protein was not correlated with age, sex, or underlying diseases in any group (**Figure 1A**).

Like proteinuria, G1 participants had the highest levels of urinary albumin compared with other groups. Elevated urinary albumin excretion was also detected in G2 participants compared with their G4 counterparts ($P = 0.009$). We found no correlation between age, sex, or underlying disease with the level of urinary albumin in this study (**Figure 1B**).

Proteomic study

We performed a proteomic analysis of the 24 h urine samples from the children of patients with urolithiasis (G2). Four bands of proteins in G2 urine that were clearly elevated compared with G4 were chosen for sequencing. Four proteins, including Tamm–Horsfall protein or uromodulin, albumin, κ and λ-2 light chain immunoglobulin were identified (**Figure 2**).

Table 2. Urinary sediment, protein, and albumin level

	Patients with urolithiasis (G1)	Children of patients with urolithiasis (G2)	Healthy volunteers (G3)	Children of healthy volunteers (G4)
Urine strip test (individuals)				
Blood ≥1+	4	0	0	0
Protein ≥1+	5	0	0	0
Glucose ≥1+	7	0	5	0
Ketone ≥1+	0	0	0	0
Urinary protein (mg/day)	436.6 ± 117.8	78.4 ± 8.6[c]	34.8 ± 7.7 [a]	23.2 ± 3.7[b]
Microalbuminuria (mg/day)	223.2 ± 73.0	6.3 ± 2.1[c]	7.7 ± 2.0[a]	0.4 ± 0.2[b]

[a]$P < 0.05$ comparing G1 and G3 participants, [b]$P < 0.05$ comparing G2 and G4 (children of G1 and G3 participants), [c]$P < 0.05$ comparing G1 and G2 (family groups including a urolithiasis proband), [d]$P < 0.05$ comparing G3 and G4 (healthy volunteer family groups)

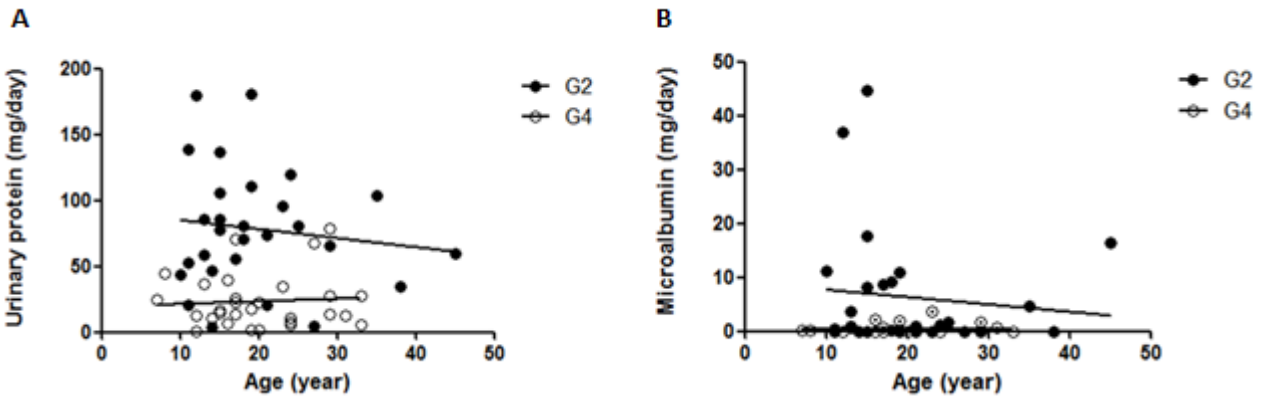

Figure 1. The level of urinary protein (**A**) and albumin (**B**) in the children of patients with urolithiasis (G2) compared with the children of healthy volunteers (G4). G2 participants had higher level of urinary protein and albumin than their G4 counterparts ($P < 0.001$ and $P = 0.009$, respectively)

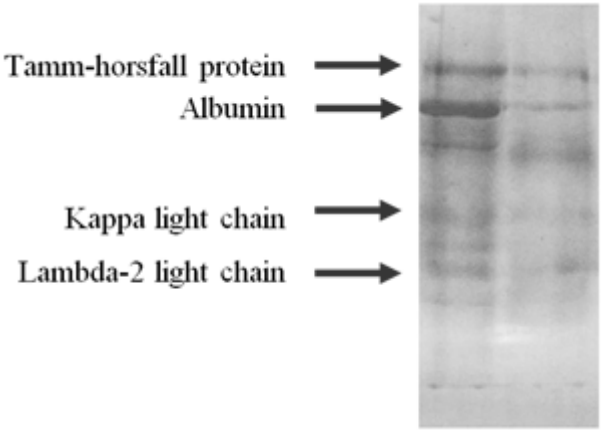

Figure 2. Two-dimensional SDS–polyacrylamide gel electrophoresis showing four bands of proteins, which were identified as Tamm–Horsfall protein, albumin, κ and λ-2 light chain immunoglobulin, respectively, were significantly increased in the 24 h urine samples of G2 participants (left lane) compared with their G4 counterparts (right lane).

Discussion

The present study aimed to elucidate the elevated urinary protein excretion, including albumin, in urolithiasis family members. We endeavored to normalize all possible confounders, including both intrinsic and extrinsic factors. To avoid possible confounders, such as genetic backgrounds, cultural beliefs, occupation, climatic weather, and dietary patterns that could not be completely matched, we recruited as the G3 participants healthy volunteers who lived in the same area as the G1 participants, and assumed that the confounding factors were not different between G1 and G3, or G2 and G4 participants. Metabolic syndrome, including diabetes mellitus, hypertension, hypertriglyceridemia, and obesity, is strongly associated with urinary stone disease. In this study, patients with urolithiasis tended to have a higher prevalence of metabolic syndrome than age- and sex-matched individuals, although no significant difference was seen because of the small number of participants. Excluding the underlying diseases, G1 participants shared similar characteristics with G3 participants in sex, age, BMI, and smoking and drinking habits.

A urine strip test was used to screen for microscopic hematuria to exclude asymptomatic urinary stone disease in groups without urolithiasis in the present study. Even though the results showed that no hematuria was detected in the participants without urolithiasis, we could not definitively claim that all of these participants were stone-free. By contrast, glycosuria was the most prevalent abnormal urinalysis observed in parental (G1 and G3) groups, and most of these had diabetes mellitus. Approximately 14.3% of patients with urolithiasis had persistent hematuria after surgery for stone removal, suggesting a lack of complete recovery from the kidney injury or the retention of remnant nonvisible small stones.

Proteinuria is a frequent complication of urolithiasis and related to the renal progression and prognosis [13, 14]. Some proteins are considered to be stone promoters, because they facilitate crystal aggregation, stone-to-cell adherence or upregulate the inflammatory response. These proteins are likely be elevated before or during stone formation and are important contributors to lithogenesis. Extensive studies of stone enhancing proteins have included osteopontin, oxypurine, calgranulin (calB), and bone morphogenetic protein 2 [15-18]. Likewise, a number of proteins were increased during or following stone formation, and considered to be the result of stone-induced inflammation, damage and apoptosis, and the consequences of stone removal procedures. These proteins include neutrophil gelatinase-associated lipocalin, kidney injury molecule 1, *N*-acetyl glucosaminidase, tissue necrosis factor, and interleukin-6 [19-22]. In the present study, we aimed to investigate the urinary proteins that promote the stone formation, which are responsible for the increased risk of lithogenesis. Because the children of patients with urolithiasis are supposed to have higher risk for the disease, but not yet have stones, we studied their urine proteome. We found a higher risk in another study in which the G2 participants had higher urinary calcium

and lower urinary citrate excretion than G4 counterparts (data not published). A limitation of the present study is that the medical history and urinary blood test evidence to exclude stone formers from the group of participants without urolithiasis is presumptive. In the present study, we found that patients with urolithiasis had significant proteinuria, while the children of these patients with urolithiasis also had significantly elevated levels of protein excretion compared with the children of healthy volunteers.

Albumin is the most abundant protein in plasma, and passes through glomeruli, then is reabsorbed by proximal tubular epithelial cells (PTEC). Increased urinary albumin is observed in several nephrotic-type nephropathies, indicating glomerular dysfunction, and can be used as a biomarker for glomerular diseases. However, less commonly, albuminuria does not indicate glomerulopathies, but increased albumin leakage in urine caused by abnormal reabsorption from PTEC disease, such as in Fanconi's syndrome. Considering that urolithiasis is a tubulointerstitial disease, increased albumin excretion observed in the patients may indicate the tubular injury more than the glomerular dysfunction. This hypothesis is supported findings of Manoharan et al., who reported that the tubular reabsorption of protein was diminished in patients with urolithiasis [23], and Pourmand et al. who considered that Tamm–Horsfall protein was not significantly increased in the urine of stone formers, but albumin and transferrin were increased in the association of bacteriuria [24]. Patients with urolithiasis had significant albuminuria, which is caused by stone-induced complications, postoperative sequela, the course of lithogenesis, or the combination of these factors. However, the clinically insignificant elevated urinary albumin excretion in the children of patients with urolithiasis compared with their healthy counterparts suggested that the increased albumin excretion might also be associated with stone pathogenesis.

Proteomics is a useful tool by which to identify the crude function of proteins in any disease. Albumin, Tamm–Horsfall protein, osteopontin, and prothrombin fragment 1 (PF1) were increased in a model of calcium oxalate stones [25]. Intertrypsin inhibitor, PF1, CD59, and calB were elevated in patients with urolithiasis compared with their stone-free first-degree relatives [26]. At least 12 proteins, including the M1 isoform of pyruvate kinase isozyme, FK506-binding protein 4, isoform 1 of cytosol aminopeptidase, fascin, L-lactate dehydrogenase B chain, were observed in a proteomic study of calcium oxalate crystal formation performed in a cell culture model [7]. Proteomics has assisted the identification of stone-inhibiting proteins including human urinary trefoil factor, and nephrocalcin [27]. However, a crucial limitation of proteomics is that it cannot identify without subject selection, whether a observed protein is the "cause/promoter" or the "effect/result", especially in studies of humans that collect samples from patients with urolithiasis who have already formed urinary stones and scars. The present study, which recruited the children of patients with urolithiasis and healthy volunteers who were supposed to have no stone, could provide more information regarding possible stone promoting proteins.

Although our preliminary report showed that Tamm–Horsfall protein, albumin, and light chain immunoglobulins were increased in the children of patients with urolithiasis, we gained only a limited insight because they are common proteins in urine. Our findings are consistent with those of Merchant et al., who reported that Tamm–Horsfall protein, albumin, α-globulin, and γ-globulin are the abundant proteins in urine, and the major protein components of the stone matrix [28]. Tamm–Horsfall protein is the most abundant glycoprotein in urine, it is synthesized and secreted by distal tubular epithelial cells, and usually acts as a stone inhibitor. Patients with recurrent urinary stones are usually deficient in Tamm–Horsfall protein. Tamm–Horsfall protein might promote stone formation if its structure lacks anionic sialic acid subunits [29, 30]. Sialic acid appears to be important to modulate urinary protein function in lithogenesis, because PF1 protein also needs sialylation to function normally [31]. Abnormal Tamm–Horsfall protein structure predisposes calcium oxalate stone formation [32, 33]. Further analysis of Tamm–Horsfall structure in Thai patients with urolithiasis is essential to verify its function in these patients.

By contrast, albumin and light chain immunoglobulins are rich in blood plasma and can be filtered and reabsorbed in nephrons. We propose that the elevation of these proteins in patients with urolithiasis urine reflects the impairment of PTEC function. Further investigation of urinary proteomics and PTEC function in the children of patients with urolithiasis to determine inherited stone-promoting factors and to develop new stone prophylaxis

protocols for first-degree relatives of patients with urolithiasis is warranted from the results of the present study.

Conclusion

First-degree relatives of patients with urolithiasis had significantly elevated levels of urinary protein and albumin excretion compared with their normal counterparts. Tamm–Horsfall protein, albumin, κ and λ-2 light chain immunoglobulin were identified. These proteins might be associated with stone pathogenesis or indicated the proximal tubular cell dysfunction corresponding to the urolithiasis.

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Conflict of interest statement

The authors have no conflicts of interest to declare.

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