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Inflammatory and fibrotic proteins proteomically identified as key protein constituents in urine and stone matrix of patients with kidney calculi

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ABSTRACT

To uncover whether urinary proteins are incorporated into stones, the proteomic profiles of kidney stones and urine collected from the same patients have to be explored. We employed 1D-PAGE and nanoHPLC-ESI-MS/MS to analyze the proteomes of kidney stone matrix ($n = 16$), nephrolithiatic urine ($n = 14$) and healthy urine ($n = 3$). We identified 62, 66 and 22 proteins in stone matrix, nephrolithiatic urine and healthy urine, respectively. Inflammation- and fibrosis-associated proteins were frequently detected in the stone matrix and nephrolithiatic urine. Eighteen proteins were exclusively found in the stone matrix and nephrolithiatic urine, considered as candidate biomarkers for kidney stone formation. S100A8 and fibronectin, representatives of inflammation and fibrosis, respectively, were up-regulated in nephrolithiasis renal tissues. S100A8 was strongly expressed in infiltrated leukocytes. Fibronectin was over-expressed in renal tubular cells. S100A8 and fibronectin were immunologically confirmed to exist in nephrolithiatic urine and stone matrix, but in healthy urine they were undetectable. Conclusion, both kidney stones and urine obtained from the same patients greatly contained inflammatory and fibrotic proteins. S100A8 and fibronectin were up-regulated in stone-baring kidneys and nephrolithiatic urine. Therefore, inflammation and fibrosis are suggested to be involved in the formation of kidney calculi.

1. Introduction

Boyce and colleagues firstly demonstrated an altered electrophoretic pattern of urinary proteins (particularly α-globulin fraction) from patients with urinary calculi, and a 3–13 times higher total excretion of urinary proteins in patients than in healthy controls [1]. Later, they reported that cellular elements were abundantly found in decalcified calculi, and this organic matrix comprised approximately 2.5% (w/w) of the dried-weight stone [2]. It is currently accepted as a rule that without organic matrix the stone is hardly formed. Also, it is well recognized that cellular biomolecules released into urine, particularly proteins, play a significant role in lithogenesis.

Proteomic technology has been employed to identify protein constituents in urine and stone matrix of patients with urolithiasis. Cadieux et al. used surface-enhanced laser desorption/ionization-time-of-flight mass spectrometry (SELDI-TOF MS) for urinary protein profiling (midstream urine) and demonstrated that the ratio of p67 (albumin) to unidentified p24 proteins in urolithiasis patients ($n = 25$) was higher than in non-stone forming controls ($n = 25$) [3]. Chutipongtanate et al. discovered a new stone urinary stone inhibitor, trefoil factor 1, using anion exchange chromatography coupled with matrix-assisted laser desorption/ionization (MALDI)-TOF MS and electrospray ionization-quadrupole-time-of-flight (ESI-QTOF) MS [4]. Wai-Hoe et al. identified differential urinary proteins (midstream urine) from 50 healthy, 30 nephrolithiasis and 35 recurrent nephrolithiasis subjects using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and liquid chromatography (LC)-MS/MS [5], and showed that albumin and immunoglobulins were the most abundant proteins in the patients' urine. The first quantitative proteomic comparison by Wright et al. [6] between urine from urolithiasis patients ($n = 57$) and non-stone forming patients with unrelated benign urological conditions ($n = 57$) showed that proteins involved in carbohydrate metabolism were uniquely found in the stone forming group. They also demonstrated that the level of urinary ceruloplasmin was significantly higher in the stone group than in the non-stone forming group, and that ceruloplasmin acted as a promoter of calcium oxalate (CaOx) crystallization [6].

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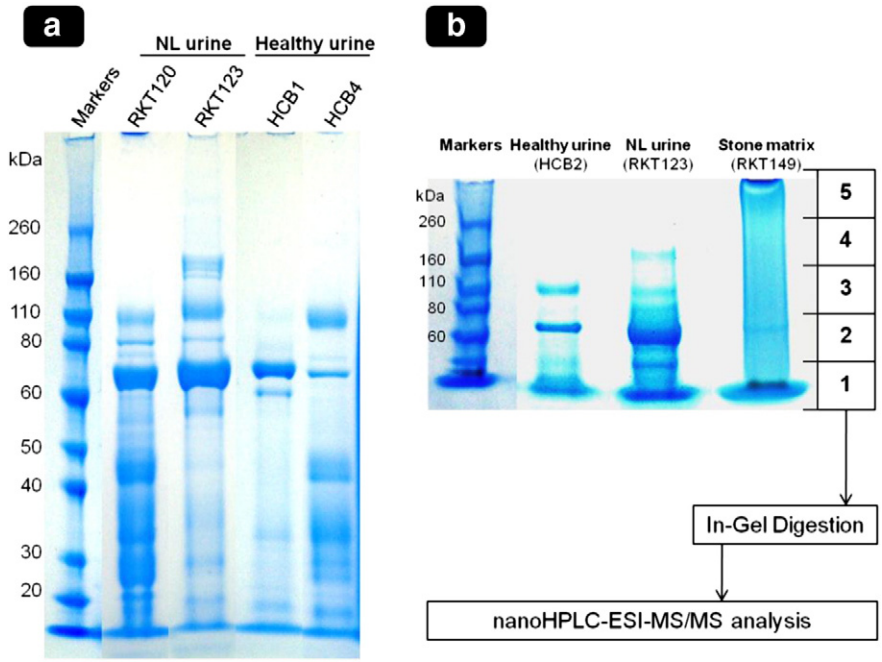

Fig. 1. 1D-SDS-PAGE (4–12%) of urinary and stone matrix proteins. a, Long run electrophoresis (200 V, 45 min) to compare overall distribution of proteins from neprolithiasis (NL) and healthy urine. b, For proteomic analysis, electrophoresis was done at 200 V for 10 min. Each lane, the electrophoresed gel was equally cut into five pieces as indicated. In-gel digest was performed to generate tryptic peptides. The peptides were further subjected to nanoHPLC-ESI-MS/MS for mass spectrum analysis.

Proteomics of kidney stones has been extensively investigated. Two-dimensional gel-based analysis by Kaneko et al. revealed 3 significant proteins, osteopontin, prothrombin and protein z in a CaOx renal stone sample [7,8]. Their subsequent study in CaOx stones ($n = 2$) found 5 additional proteins, including uromodulin, albumin, defensin, lysozyme and calgranulin A [9]. Based on SDS-PAGE, reverse phase (RP)-HPLC and MALDI-TOF-MS, Mushtaq et al. identified myeloperoxidase, α-defensin and calgranulin in CaOx kidney stones ($n = 40$) [10]. Later, Chen et al. analyzed 3 bands (27, 18 and 14 kDa) of proteins extracted from CaOx stones ($n = 10$) using nanoscale capillary LC-MS/MS, and identified several proteins involved in the inflammatory process [11]. Canales et al. employed RP-HPLC and MS/MS to show that several proteins involved in cell injury and inflammation were identified in pure CaOx monohydrate (COM) stones ($n = 7$) [12]. Merchant et al. identified 158 proteins in 5 calcium kidney stones, and osteopontin, myeloperoxidase, lactotransferrin, nucleolin and HSP90 were immunologically validated [13]. Priyadarshini et al. reported a novel stone inhibitory protein, phosphate cytidylyltransferase 1, in CaOx kidney stones [14]. Canales et al. showed that protein profiles of CaOx and of calcium phosphate (CaP) stones were rather similar, as inflammatory proteins were common abundant components [15]. Recently, Jou et al. profiled proteins in pure uric acid stones ($n = 5$) using RP-nanoHPLC and MS/MS and found inflammatory proteins as primary constituents [16]. Taken together, both urinary and stone matrix proteomes suggest that the inflammatory reaction is involved in the development of kidney stones. However, no study investigates the proteomic profiles of urine and stones obtained from the same patients. Furthermore, no validation of the proteomic findings is carried out in the stone-bearing renal tissues.

In this study, we investigated for the first time proteomes of kidney stone and urine samples obtained from the same patients. The proteomic profiles of the three stone types (CaOx, uric acid and struvite) were compared. In addition, expression of the selected proteomically-abundant proteins in renal biopsy and urine samples was immunologically validated.

2. Materials and methods

2.1. Patients and specimens

Sixteen patients with kidney calculi were admitted to Khon Kaen Hospital, Khon Kaen province, Thailand, and three healthy individuals were recruited for the study. All patients had positive plain abdominal film and/or intravenous urogram (IVU) for calculi in the kidney. Patients with anomalous kidney and other urinary tract diseases i.e. horseshoe kidney, polycystic kidney, congenital vesicoureteral reflux, neurogenic bladder and any malignancies were excluded. Healthy condition was confirmed by direct interview and/or previous medical evaluation reports. Written-informed consent was obtained from all participants before specimen collection, and the research protocol was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University.

Midstream spot morning urine samples were collected between 8 am and 11 am. In patients, urine was collected on the day before surgical removal of stones. Renal function was estimated by plasma creatinine (Cr) and Cr clearance (CCr). The corrected CCr adjusted for a body surface area of 1.73 m^2 was calculated [17]. Stone specimens were collected during surgery. Stone samples were washed, dried, grounded into powder and kept at −80 °C. The stone composition was analyzed by Fourier transform-infrared spectroscopy.

Renal biopsy specimens from the patients were collected (wedge-resection at surgery) by urologists in accordance with standard procedures. The biopsy was taken only for research purpose. All biopsied tissues were taken from stone-bearing kidneys, and both renal cortex and medulla near the stones were collected. Control renal sections were obtained from non-pathological regions of kidneys removed because of tumors. The biopsied tissue was immersed in 10% formalin buffer. Tissue processing was carried out according to routine histological protocols. Serial paraffin-embedded sections were cut at 4 μm for histological analysis.