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ORIGINAL PAPER

Urinary chemokines/cytokines are elevated in patients with urolithiasis

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Abstract The prevalence of urolithiasis in the general population has been increasing recently. The inflammatory responses may play an important role in the development of urolithiasis. We aimed to investigate whether the urine inflammatory cytokine and chemokine profiles from patients with urolithiasis can be used as prognostic markers for urolithiasis. Multiplex immunoassays were used to simultaneously detect five inflammatory cytokines and five inflammatory chemokines in urine collected from 29 patients and 38 sex and age-matched healthy volunteers. After adjusting for urinary creatinine, urinary levels of interleukin-8 (IL-8), regulated on activation, normal T cell expressed and secreted, monocyte chemoattractant protein-1, interferon-gamma (IFN-γ)-inducible 10-kDa protein, monokine induced by IFN-γ and IL-6 were significantly increased in patients compared with healthy controls. However, concentrations of urinary IL-1β, IL-10, IL-12, and tumor necrosis factor-alpha were not significantly different between those of patients and healthy controls. Using receiver operating characteristics curve analysis, we found that the adjusted IL-8 level of 6.2 pg/mg creatinine can reach a sensitivity of 90% and specificity of 68% to detect urolithiasis. Our data showed that urinary stones are associated with a cascade of inflammatory responses, including chemokine secretion, and urinary IL-8 levels. In addition, the elevation of urinary IL-8 could be a useful biomarker in healthy screening and clinical follow-up of urolithiasis.

Keywords Urolithiasis · Chemokines · Cytokines · IL-8

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Introduction

Urolithiasis, a condition involving the development of stones in the kidney, bladder, and/or urinary tract, is a common disease with a prevalence of 5–10% worldwide. It is a rapidly increasing universal problem with an important influence on the health care system. Urolithiasis is a multifactorial disease, and its underlying etiology is not yet well understood. The risk factors for

developing urinary stones include genetics, age, sex, geography, seasonal factors, diet and occupations [1]. There is no predictive biomarker for the disease and most patients are diagnosed after the development of symptoms. A reliable biomarker for urolithiasis could lead to earlier diagnosis, treatment, and better monitoring of the disease course.

Urine is normally supersaturated with oxalate ions and calcium. Under suitable conditions, it will lead to the formation of calcium oxalate crystals [2]. These crystals can be retained in the kidneys via binding to the tubular cells [3], and then aggregate to form large ones. Thus, crystal growth, aggregation and retention are all important aspects of the development of urolithiasis. During certain hyperoxaluric conditions, retained crystals move into the interstitium to induce non-infectious inflammation [4]. Several studies have shown that renal stones could stimulate renal cells to secrete inflammatory mediators in vitro, such as and monocyte chemoattractant protein-1 (MCP-1 also known as CCL2) [5], tumor necrosis factor-alpha (TNF-α) [6] and interleukin-6 (IL-6) [7]. Thus, the inflammatory responses of renal tissue play an important role in the disease process of urolithiasis [8]. However, very few studies have examined in detail the urinary inflammatory cytokines and chemokines in the patients with urolithiasis.

During inflammation, cytokines and chemokines play an important role in linking innate and adaptive immunity. Tissue macrophages or dendritic cells stimulated by toll-like receptor (TLR) can secrete various chemokines, such as IL-8 (also known as CXCL8), regulated on activation, normal T cell expressed and secreted (RANTES, also known as CCL5), macrophage inflammatory protein-1alpha (MIP-1α also known as CCL3), and MIP-1β (also known as CCL4) [9]. IL-8/CXCL8 is the key chemoattractant for neutrophils, while RANTES/CCL5, MIP-1α/CCL3, and MIP-1β/CCL4 are chemotactic factors for immature dendritic and natural killer (NK) cells. NK cells are an important source of interferon-gamma (IFN-γ), which induces the production of monokine induced by IFN-γ (Mig also known as CXCL9) and IFN-γ inducible 10-kDa protein (IP-10 also known as CXCL10). In turn, Mig/CXCL9 and IP-10/CXCL10 attract activated T cells to inflamed sites [10]. Furthermore, macrophages and endothelial cells can produce MCP-1/CCL2 to attract additional macrophages [11, 12], while immature dendritic cells stimulated by pathogens can produce IL-12, TNF-α, and IL-10 [13]. These secreted inflammatory mediators were examined in detail in the context of urolithiasis in the present study. We compared the concentrations of five inflammatory cytokines and five inflammatory chemokines in the urine between the patients with urolithiasis and healthy controls.

Materials and methods

Study subjects

We conducted a case control study between 2005 and 2006 in Kaohsiung Medical University Hospital. Patients who were diagnosed as having upper urinary tract calcium urolithiasis during this period were enrolled in this study. Briefly, calcium urolithiasis was diagnosed by ultrasonography or radiography. No case was found by X-ray to have radiolucent stones or by clinical evaluation to have cystine or uric acid stones. If stone specimens were removed by surgery or obtained after medical treatment or shock-wave lithotripsy, composition of the stones was confirmed by infrared spectroscopy (Spectrum RX I Fourier Transform-Infrared System, Perkin-Elmer, USA) [14].

Normal controls were randomly selected from subjects receiving general health examinations at the same hospital during the same period. The controls had no past history of urinary stone disease and no clinical findings of stones, confirmed by plain abdominal X-ray and abdominal ultrasound. Both cases and controls were excluded if they had a history of chronic urinary tract infection, renal failure, chronic diarrhea, gout, renal tubular acidosis, autoimmune diseases, primary and secondary hyperparathyroidism, cancer or took medications which would influence inflammatory process, such as corticosteroid. All study subjects were living in southern Taiwan. The study protocol was approved by the Institutional Review Board of Kaohsiung Medical University Hospital. Each subject provided signed informed written consent.

Urine collection and biomarker determination

After overnight fasting, all subjects provided a one-spot urine sample from midstream of the first void for urinary biomarker determination. Urine samples were kept at 4°C and centrifuged at 1,000*g* for 10 min. To exclude subjects with subclinical infectious urinary inflammation, subjects whose urinary C-reactive protein (CRP) levels >0.1 mg/l were excluded from this study. The urine with neutrophils and macrophages examined by microscopy was also excluded.

The supernatant was then divided into aliquots and stored at −70°C. We used multiplex immunoassay to detect inflammatory cytokines and chemokines. Inflammatory cytokine and chemokine concentrations were measured by Cytometric Bead Array (BD Biosciences, San Diego, CA, USA), which contains microparticles that are dyed to different fluorescence intensities of approximately 650 nm. Each particle (capture bead) is coated with monoclonal antibodies against one of cytokines (IL-1β, IL-6, IL-10, TNF-α, or IL-12p70) or chemokines

(IL-8/CXCL8, RANTES/CCL5, Mig/CXCL9, MCP-1/CCL2 or IP-10/CXCL10) selected for this study. The cytokines, after being bound to their respective beads, are directly detected using a mixture of corresponding antibodies conjugated with phycoerythrin (PE), which emits at 585 nm. The mixture of capture beads was incubated with standards (recombinant cytokines) or test samples, followed by PE-conjugated detection antibodies to form sandwich complexes, according to the manufacture's instructions. The samples were then run on a FACSarray flow cytometer and analyzed using the Becton-Dickinson Cytometric Bead Array software. The median fluorescence intensity in 585 nm is proportional to the concentration of the recombinant cytokine. Cytokine and chemokine concentrations of samples were determined from the standard curves. The minimum thresholds for detection of each protein in this assay are as follows: IL-1β, 3.6 pg/ml; IL-6, 2.5 pg/ml; IL-10, 3.3 pg/ml; TNF-α, 3.7 pg/ml; IL-12p70, 2 pg/ml; IL-8/CXCL8, 0.2 pg/ml; RANTES/CCL5, 1 pg/ml; Mig/CXCL9, 2.5 pg/ml; MCP-1/CCL2, 3 pg/ml; and IP-10/CXCL10, 3 pg/ml.

Statistics

To reduce dilution effects, we first adjusted the concentrations of urine cytokines/chemokines for urine creatinine levels in each subject, which then were expressed as the mean ± SD (pg/mg creatinine). We checked the distribution of each biomarker in patients and controls separately. Age and gender effects were also tested on these biomarkers. We calculated the area under the receiver operating characteristics (ROC) curve for each biomarker, and used it as an index to determine which biomarker had a greater diagnostic power to distinguish patients from controls. We also dichotomized biomarker levels to evaluate the differences of their levels between patients and controls by the chi-square test. The cutoff value to dichotomize each cytokine and chemokine was the mean + 2 SD calculated from controls. Significance was defined as a two-side P value <0.05. Statistical comparisons of data among groups of controls and patients were performed with the non-parametric Mann–Whitney U test.

Results

The clinical characteristics of patients with urolithiasis are shown in Table 1. Representative data of chemokine levels using cytometric bead arrays are shown in Fig. 1. One outlier was found in the control group who had an IL-8/CXCL8 level >5 group-specific SD, therefore his data on IL-8/CXCL8 (adjusted value of 275.46 pg/mg creatinine) was not analyzed. The mean levels of IL-12p70, IL-10 and TNF-α were

Table 1 Clinical information for control subjects and urolithiasis patients included in this study

	Normal control	Urolithiasis
Case no.	38	29
Age[a] (range) (year)	48 ± 16 (18–74)	54 ± 15 (26–80)
Sex (female/male)	11/27	8/21
Single/multiple stones	NA	17/12
Unilateral/bilateral	NA	21/8
Stone in kidney (%)	NA	35
Stone in ureter (%)	NA	83
Recurrent (%)	NA	28

NA Not applicable

[a] Mean ± standard deviation

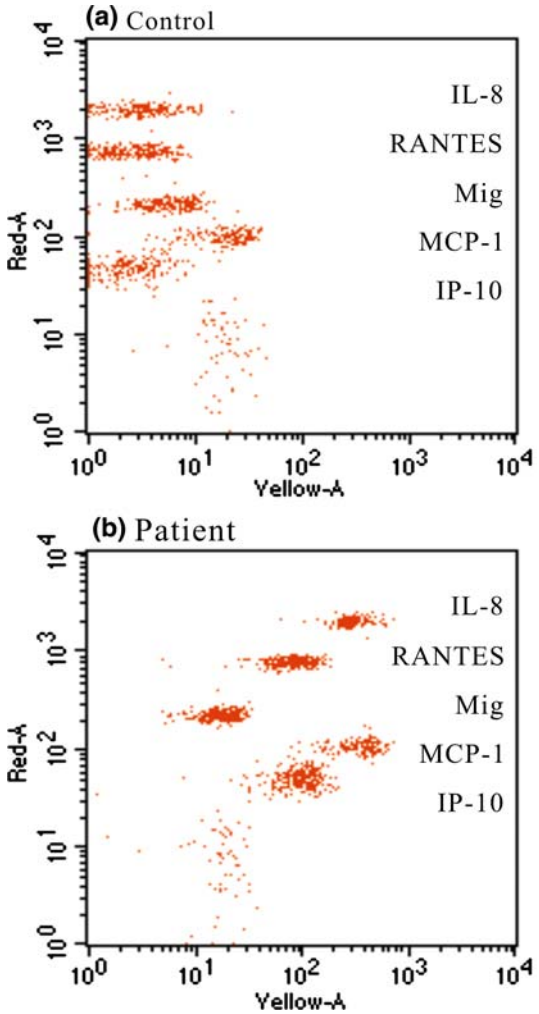

Fig. 1 Representative data for cytometric bead array chemokine from controls (**a**) and patients with urolithiasis (**b**). *Dot* plots yellow-A (585 nm) versus red-A (650 nm) representing different chemokines are indicated in the figure by five discrete microparticle dye intensities along the *y*-axis. The *x*-axis shows the values for sample fluorescence intensities, which reflects the concentrations of the various biomarkers

below the detectable values in both patients and controls (Table 2). IL-6 levels were below the detectable threshold in controls. The range of the creatinine-adjusted value of each biomarker is shown in Table 2. The ROC area was significant for the creatinine-adjusted levels of IL-8/CXCL8 (area = 0.84, $P < 0.001$), Mig/CXCL9 (area = 0.76, $P < 0.001$), and RANTE (area = 0.66, $P = 0.024$). Based on the ROC curve, the cutoff point for the adjusted IL-8/CXCL8 level was 6.2 pg/mg creatinine. According to this cutoff point of IL-8/CXCL8, the diagnostic sensitivity and specificity can reach 90 and 68%, respectively.

The other approach was to dichotomize every biomarker using an arbitrary cutoff point of mean + 2 SD. Using the chi-square test, IL-8/CXCL8, RANTES/CCL5, MCP-1/CCL2, IP-10/CXCL10 and IL-6 were significantly elevated in patients compared to controls (Fig. 2). Among these biomarkers, 41% of patients had at least three biomarkers elevated simultaneously, and 92% of those also had IL-8/CXCL8 level increased significantly. On the other hand, among those patients with elevated IL-8/CXCL8 levels ($n = 16$, 55%), 69% of patients had at least three of those biomarkers elevated. However, only 28% of patients have normal biomarker levels (Fig. 2 and data not shown). Thus, the subgroup of patients with elevated levels of those biomarkers might not be random.

We next examined whether the levels of those inflammatory mediators are associated with the clinical features of urolithiasis. As shown in Fig. 3a, we found recurrent cases had higher levels of IL-8/CXCL8 (mean ± SD, 129.9 ± 95.6 pg/mg creatinine), but not other mediators, than first attack patients (50.7 ± 80.4 pg/mg creatinine). Although there was no significance between these two groups ($P = 0.059$), we found around 75% of recurrent patients have high IL-8/CXCL8 levels above the arbitrary cutoff point compared to 33% of first attack patients. On the other hand, all of the biomarker levels were not related to single or multiple stones (Fig. 3b, only IL-8/CXCL8 level shown). In addition, IL-8/CXCL8 level (Fig. 3c) and other biomarker levels in patients with ureteral stones were not significantly different from those with renal stones.

Discussion

Our study investigated several inflammatory biomarkers and found that the urinary concentrations of cytokines and chemokines in the patients with urolithiasis are significantly higher than those in normal subjects. The significant biomarkers include IL-6, IL-8/CXCL8, RANTES/CCL5, MCP-1/CCL2, Mig/CXCL9 and IP-10/CXCL10. Among them, IL-8/CXCL8 is the most sensitive marker to distinct patients from controls. The data suggest a link between urinary stones and inflammation in the urinary tract. Although it is unclear whether inflammation contributes to urinary stone formation or vice versa, our findings may still provide a tool to detect high risk individuals, especially for the recurrent cases.

In our study, IL-8/CXCL8 is found to be the most sensitive marker to distinct patients from controls. If we use the adjusted IL-8 level of 6.2 pg/mg creatinine as the cutoff point to detect urolithiasis, the diagnostic sensitivity and specificity can reach 90 and 68%, respectively. In addition, the elevation of urinary IL-8 level is noted in patients regardless of stone numbers or locations (Fig. 3b, c). Therefore, it might be applied to healthy screening in order to detect subjects with high risk of having urolithiasis. Patients with urolithiasis tend to have high recurrence rate and need to receive regular follow-up. The recurrence rate has been reported approximately 10% at 1 year, 33% at 5 years, and 50% at 10 years [15]. We also find that

Table 2 The mean, standard deviation and range of each biomarker

	Case Mean[b] ± SD (range)	Control Mean[b] ± SD (range)
IL-8/CXCL8[a]	104.66 ± 159.70 (0–729.75)	7.76 ± 8.90 (0–33.82)
RANTES/CCL5	18.56 ± 31.57 (0–144.78)	4.69 ± 3.06 (0–14.04)
Mig/CXCL9	222.91 ± 175.01 (0–738.37)	177.57 ± 149.03 (0–665.20)
MCP-1/CCL2	707.00 ± 985.27 (0–3504.28)	146.73 ± 95.66 (30.35–500.83)
IP-10/CXCL10	687.71 ± 774.24 (0–3236.31)	257.09 ± 244.09 (0–904.06)
IL-1β	8.91 ± 15.69 (0–60.41)	4.59 ± 10.43 (0–44.15)
IL-6	11.42 ± 17.84 (0–65.66)	1.61 ± 2.25 (0–7.22)
IL-10	Below the detectable level	Below the detectable level
TNF-α	Below the detectable level	Below the detectable level
IL-12p70	Below the detectable level	Below the detectable level

[a] The outlier was removed

[b] pg/mg creatinine

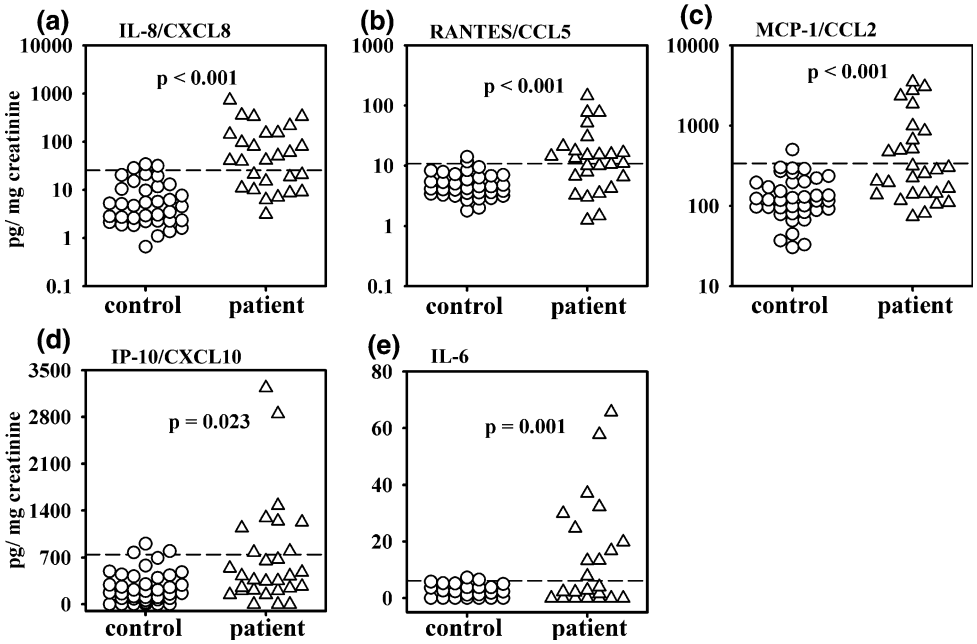

Fig. 2 Comparison of urinary chemokine and cytokine concentrations in patients with urolithiasis and normal subjects. *Spots* represent the values of creatinine-adjusted chemokine/cytokine concentrations. The *horizontal dashed lines* indicate the normal range cutoff of each biomarker, expressed as the mean + 2 SD of the control group (cutoff values: 25.56 pg/mg creatinine for IL-8/CXCL8, 10.81 pg/mg creatinine for RANTES/CCL5, 338.05 pg/mg creatinine for MCP-1/CCL2, 745.27 pg/mg creatinine for IP-10/CXCL10, 6.11 pg/mg creatinine for IL-6). The difference between patients and controls was assessed by the chi-square test

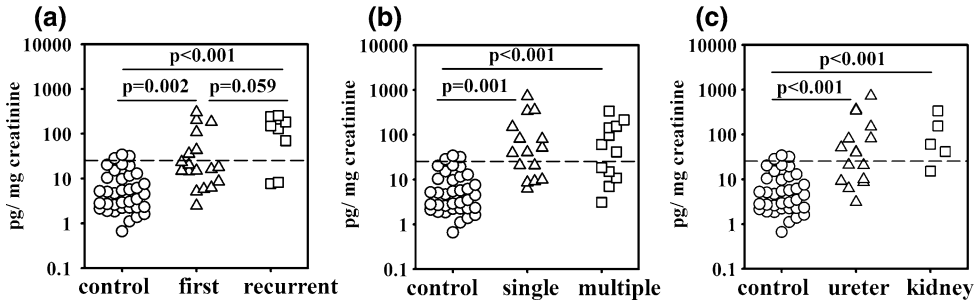

Fig. 3 Comparisons of IL-8/CXCL8 level in different groups of patients with urolithiasis and controls. Results are shown as the values of creatinine-adjusted IL-8/CXCL8 concentrations in controls ($n = 37$) and first attack ($n = 21$) or recurrent patients ($n = 8$) (**a**) and patients with single ($n = 17$) or multiple stones ($n = 12$) (**b**) or patients with ureteral or renal stones (**c**).The *horizontal dashed lines* indicate the normal range cutoff of IL-8/CXCL8, expressed as the mean + 2 SD of the control group (cutoff values: 25.56 pg/mg creatinine). $P < 0.05$ was considered as significant by the Mann–Whitney U test

recurrent stone cases have higher urinary IL-8 levels than normal controls ($P < 0.001$) and first attacked patients ($P = 0.059$) (Fig. 3a). Those findings might imply that evaluation of urinary IL-8 level could be useful in clinical follow-up to detect recurrence, and have potential to identify patients with high risk to be recurrence. However, a long-term follow-up study is still needed to confirm our results.

Signs of inflammation have been associated with the presence of stone in the urinary tract. Evidence indicates that inflammation due to urolithiasis is different from inflammation caused by infectious agents. Studies by Rhee and colleagues [7] demonstrated that urinary levels of inflammatory cytokines such as IL-1α, IL-1β and IL-6 were markedly elevated in bacterial infection; however, only the IL-6 level was increased in urolithiasis. We had a similar finding in the present study. In our study, we excluded the patients with infection based on clinical evidence and urinary levels of CRP >0.1 mg/l. Our results showed that several inflammatory chemokines and IL-6 in the urine of the patients with urolithiasis were significantly higher compared with the control subjects, but levels of IL-1β were not different between the cases and controls. This finding implies that urolithiasis may be associated with chronic subclinical non-infectious inflammation.

Our data showed that the levels of TNF-α, IL-10 and IL-12p70 were undetectable in both patients and controls. This finding could be due to no increased production of these biomarkers in urolithiasis. However, it is also possible that these cytokines act mainly as local inflammatory mediators, and are too diluted to be detected in the urine. As for chemokines monitored in this study, they belong to

the subset of inducible chemokines, which are up-regulated during inflammatory responses [16]. The major function of inducible chemokines is to selectively control the chemotaxis of leukocytes to the inflamed tissues. Thus, the urinary levels of most inducible chemokines are higher than those of inflammatory cytokines even in normal individuals, as shown in Table 2. Accordingly, although our study did not demonstrate increased levels of TNF-α, IL-10 and IL-12p70, they may be still involved in the non-infectious inflammation of urolithiasis.

Due to the complexity and diversity of cells within the kidney, it is difficult to examine which cells play a major role in the recruitment of immune cells to the hyperoxaluric kidney in vivo. On the other hand, in vitro cell culture studies have provided important information. Previous studies have shown that tubular epithelial cells are a rich source of inducible chemokines including IL-8/CXCL8, RANTES/CCL5 and MCP-1/CCL2 [5, 17]. Renal stones may contain appreciable amounts of endotoxin, and can stimulate epithelial cells to secrete chemokines via TLR4 [18]. In fact, renal tubular cells can express several types of TLRs, including TLR1, 2, 3, 4 and 6, but not TLR5 and 9 [19]. Induction of RANTES/CCL5 and MCP-1/CCL2 by renal tubular cells has been demonstrated after stimulation by TLR4 and TLR2 [19]. However, the in vivo role of tubular epithelial cells and renal tubular cells in chemokine production should be further examined.

Data from our study demonstrate a significant increase of IL-8/CXCL8, RANTES/CCL5, Mig/CXCL9, MCP-1/CCL2, IP-10/CXCL10 and IL-6 urinary levels in patient compared with control subjects. These chemokines/cytokines can be secreted by tubular epithelial cells and renal tubular cells, as well as particular immune cell subsets. We speculate that dendritic cells, NK cells, monocytes, T cells and neutrophils may be involved in the non-infectious inflammation of urolithiasis. For example, RANTES/CCL5 is a chemoattractant for immature dendritic cells and NK cells. Immature dendritic cells can also produce RANTES/CCL5, IP-10/CXCL10, IL-8/CXCL8, MIP-1α/CCL3 and MIP-1β/CCL4 in response to agonists of TLR2 and TLR4 [9]. In addition, IFN-γ secreted by NK cells can also induce the production of IP-10/CXCL10 and Mig/CXCL9 from resident tissue cells. IP-10/CXCL10 and Mig/CXCL9 will then guide activated T cells back into the inflamed tissues [10]. The early production of these chemokines from tubular epithelial cells and dendritic cells is essential in shaping the immune response in kidney. Early expression of IP-10/CXCL10 induced by endogenous danger signals, such as trauma or hypoxia, was found to be important for the influx of T and NK cells [20]. IL-8/CXCL8 is known for neutrophil recruitment to an inflammatory site. MCP-1/CCL2 not only recruits monocytes but memory T cells and NK cells to mediate proinflammatory effects [21]. MCP-1/CCL2 also can stimulate tubular epithelial cells to secrete IL-6 and express intercellular adhesion molecule-1 [22]. Thus, renal damage induced by stones can initiate a 'chemokine to cytokine to chemokine' cascade, which may play a significant role in the disease process of urolithiasis.

In conclusion, the present study showed that urinary IL-8/CXCL8 is a potential biomarker for the detection of urolithiasis, especially in healthy screening and clinical follow-up. Although our study cannot tell whether the elevated level of IL-8/CXCL8 is the cause or the result of urolithiasis, it still has the potential to become a prognostic biomarker. A follow-up study on high risk individuals may provide a better inside to the role of IL-8/CXCL8 in the development of urolithiasis.

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