นิพนธ์ต้นฉบับ

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Intrarenal gene expression of monocyte chemoattractant protein-1 and interleukin-6 in nephrolithiasis

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Objectives : *It is known that crystal formation which takes place in supersaturated urine causes oxidative stress, tubular injury and inflammation in the kidney of nephrolithic rats. Up-regulations of monocyte chemoattractant protein-1 (MCP-1) and interleukin-6 (IL-6) by renal tubular cells challenged with lithogenic crystals have been also demonstrated. We aimed to investigate the intrarenal mRNA expressions of MCP-1 and IL-6 in patients with nephrolithiasis and evaluate whether their expressions associated with renal dysfunction and oxidative stress.*

Methods : *Twenty-nine patients with nephrolithiasis who underwent surgical removal of stone were recruited and collected stone-adjacent renal biopsy, blood and 24-hr urine specimens. Control renal tissues were taken from non-cancerous and cancerous portions of nephrectomy from patients with renal cancers (n=6). Control 24-hr urine samples were obtained from 30 healthy subjects. Corrected creatinine clearance (CCr), urinary N-acetyl-β-glucosaminidase (NAG) activity and 8-hydroxy-deoxyguanosine (8 -OHdG) were determined. The mRNA expressions of MCP-1 and IL-6 in renal tissue were measured by real time RT-PCR.*

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Results : *Nephrolithiasis patients excreted urinary NAG activity and 8-OHdG significantly greater than healthy controls. Intrarenal mRNA expressions of MCP-1 and IL-6 in stone-adjacent renal tissues were significantly lower than in cancerous renal tissues, but not statistically different to that in non-cancerous renal tissues. In stone-adjacent renal tissues, mRNA level of MCP-1 was significantly higher than that of IL-6; however, their expressions were significantly correlated to each other. Nephrolithiasis patients with compromised renal function (corrected CCr < 50 ml/min/1.73m^2) had significantly higher intrarenal levels of MCP-1 and IL-6 mRNA than those with preserved renal function. Likewise, intrarenal mRNA levels of MCP-1 and IL-6 in patients with high renal tubular damage (urinary NAG activity $\geq$ 5.32 U/g Cr) were significantly higher than those with low degree of renal tubular damage. No association between intrarenal mRNA expression and urinary 8-OHdG was observed.*

Conclusion : *Nephrolithiasis patients manifested a low-grade intrarenal inflammation. Increased intrarenal mRNA expression of MCP-1 and IL-6 was associated with enhanced glomerular impairment and renal tubular damage. This finding indicated that MCP-1 and IL-6, at least in part, contributed to the progression of nephrolithiasis.*

Keywords : *Nephrolithiasis, Inflammation, Kidney function, MCP-1, IL-6.*

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วัตถุประสงค์ : *เป็นที่ทราบดีแล้วว่าเมื่อเกิดผลึกในปัสสาวะที่มีความอิ่มตัวยวดยิ่งเป็นสาเหตุให้เกิดภาวะเครียดจากออกซิเดชั่น การบาดเจ็บที่เยื่อบุท่อไต และภาวะการอักเสบในหนูที่ทำให้เป็นนิ่ว รายงานการศึกษาในเซลล์เพาะเลี้ยงพบว่าเมื่อกระตุ้นเซลล์เยื่อบุท่อไตด้วยผลึกนิ่ว เซลล์จะสังเคราะห์โปรตีนเคโมแอดแทรคแตรนท์โปรตีน-1 (MCP-1) และอินเตอร์ลิวคิน-6 (IL-6) เพิ่มมากขึ้นด้วย การศึกษานี้ตรวจสอบการแสดงออกของ MCP-1 และ IL-6 mRNA ในเนื้อเยื่อไตของผู้ป่วยโรคนิ่วไต และประเมินว่ามีความสัมพันธ์กับความผิดปกติของการทำงานของไตและภาวะเครียดจากออกซิเดชั่นหรือไม่*

วิธีการ : *เก็บสารตัวอย่างเนื้อเยื่อไต เลือด และปัสสาวะ 24 ชั่วโมง จากกลุ่มตัวอย่างผู้ป่วยโรคนิ่วไตที่ได้รับการผ่าตัดเอานิ่วออก จำนวน 29 ราย เนื้อเยื่อไตควบคุมที่ใช้คือเนื้อเยื่อไตส่วนที่ไม่ใช่มะเร็งและเนื้อเยื่อไตส่วนที่เป็นมะเร็งที่ได้จากผู้ป่วยโรคมะเร็งไตที่ต้องตัดไตออก จำนวน 6 ราย สำหรับตัวอย่างปัสสาวะ 24 ชั่วโมงควบคุมเก็บจากกลุ่มผู้มีสุขภาพดี จำนวน 30 ราย ตรวจวัด corrected creatinine clearance (CCr), urinary N-acetyl-β-glucosaminidase (NAG) activity และ 8-hydroxy-deoxyguanosine (8-OHdG) สำหรับระดับ mRNA ของ MCP-1 และ IL-6 ในเนื้อเยื่อไตตรวจวัดโดยวิธี real time RT-PCR*

ผลการทดลอง : *กลุ่มผู้ป่วยโรคนิ่วขับออก urinary NAG activity และ 8-OHdG มากกว่ากลุ่มคนปกติอย่างมีนัยสำคัญ ระดับการแสดงออกของ MCP-1 และ IL-6 mRNA ในเนื้อเยื่อไตของผู้ป่วยโรคนิ่วไตต่ำกว่าเนื้อเยื่อไตส่วนที่เป็นมะเร็งอย่างมีนัยสำคัญ แต่ไม่พบความแตกต่างอย่างมีนัยสำคัญกับเนื้อเยื่อไตส่วนที่ไม่ใช่มะเร็ง ในเนื้อเยื่อไตของผู้ป่วยโรคนิ่วไตมีระดับการแสดงออกของ MCP-1 mRNA สูงกว่า IL-6 mRNA อย่างมีนัยสำคัญ อย่างไรก็ตามการแสดงออกของยีนทั้งสองมีความสัมพันธ์เชิงบวกต่อกัน กลุ่มผู้ป่วยโรคนิ่วไตที่มีประสิทธิภาพการทำงานของไตเสื่อมลง (corrected CCr < 50 ml/min/1.73m^2) มีระดับ MCP-1 และ IL-6 mRNA ในเนื้อเยื่อไตสูงกว่ากลุ่มผู้ป่วยโรคนิ่วไตที่มีประสิทธิภาพการทำงานของไตดีอย่างมีนัยสำคัญ เช่นเดียวกับกลุ่มผู้ป่วยโรคนิ่วไตที่มีภาวะการทำลายเซลล์บุท่อไตสูง (urinary NAG activity ≥ 5.32 U/g Cr) พบระดับ MCP-1 และ IL-6 mRNA ในเนื้อเยื่อไตสูงกว่ากลุ่มผู้ป่วยโรคนิ่วไตที่มีภาวะการทำลายเซลล์บุท่อไตต่ำอย่างมีนัยสำคัญ การศึกษานี้ไม่พบความสัมพันธ์ระหว่างระดับการแสดงออกของ MCP-1 และ IL-6 กับภาวะเครียดจากออกซิเดชั่นและการติดเชื้อในระบบทางเดินปัสสาวะ*

สรุปผล : *ผู้ป่วยโรคนิ่วไตมีระดับการอักเสบในไตในระดับต่ำ อย่างไรก็ตามการแสดงออกที่เพิ่มขึ้นของ MCP-1 และ IL-6 สัมพันธ์กับประสิทธิภาพการทำงานของไตที่ลดลงและภาวะการทำลายของเซลล์บุท่อไตที่สูงขึ้น ผลการศึกษานี้แสดงให้เห็นว่า MCP-1 และ IL-6 มีบทบาทเกี่ยวข้องกับการดำเนินของโรคนิ่วไต*

คำสำคัญ : *โรคนิ่วไต, การอักเสบ, ประสิทธิภาพการทำงานของไต, เคโมแอดแทรคแตรนท์โปรตีน-1, อินเตอร์ลิวคิน-6*

Kidney stone disease is one of the oldest ailments ever known in history of humankind. However, the mechanism(s) of stone formation in renal tissue is (are) still not fully understood. It is known that crystal formation, which takes place in chronically supersaturated urine, and crystal deposition in renal interstitium are prerequisite events in lithogenic process. Oxidative stress, renal tubular injury and inflammation are well recognized factors in the pathogenesis of kidney stone. [1-3]

The association between crystal deposition and inflammation has been intensively verified in experimental nephrolithiasis.[4-7] Crystals formed in tubular lumen, shift into renal interstitium and initiate inflammatory response by recruiting inflammatory leukocytes. Evidences showed that calcium oxalate crystals residing in the renal interstitium are mainly surrounded by monocytes, macrophages and multinucleated giant cells. [6-8] These cells encapsulate and dissolve the crystals. A study in ethylene glycol-induced nephrolithic rats demonstrated that free radical formation is increased in an early stage and persist in the kidney whilst the infiltration of leukocytes gradually increases in the late stage of stone formation. [9, 10] It is known that the leukocytes infiltration causes by chemotactic factors such as monocyte chemoattractant proteins-1 (MCP-1).

A line of evidences from cell culture model confirmed that MCP-1 is up-regulated in oxalate/ crystals-challenged renal tubular cell lines and its expression attenuates by the intervention of antioxidants. [11-15] Crystal-induced up-regulation of MCP-1 and osteopontin (OPN) was observed in renal fibroblast cells. [16] The authors propose that exposure of high oxalate ions and calcium oxalate crystals to renal tubular epithelial cells or fibroblasts stimulate the production of MCP-1 and OPN to attract inflammatory cells into renal intersitium leading to interstitial inflammation.

MCP-1 or CCL2, a member of CC chemokine subfamily, is a small protein that functions through its receptor, CCR2. A hallmark feature of chemokines is chemotaxis property. MCP-1 is a powerful and specific chemotactic factor for the recruitment of monocytes, macrophages as well as lymphocytes. [17] An over-expression of MCP-1 was found in various renal diseases, and its up-regulation is associated with the extent of mononuclear cell infiltration. [18, 19] Thus, MCP-1 is suggested to be responsible for leukocytes influx which leads to tubulointerstitial inflammation and renal damage.

Interleukin-6 (IL-6), originally identified as a T-cell-derived B-cell differentiation factor, is a pleiotropic cytokine having important roles in the regulation of immune response, inflammation and hematopoiesis. [20] It is suggested to play an important role in development and progression of renal cell carcinoma.[21] Increased intrarenal expression of IL-6 has been demonstrated in patients with IgA nephropathy, which is correlated with the disease severity. [22, 23] IL-6 is also up-regulated in oxalate-treated renal epithelial cells. [24] Additionally, patients with urolithiasis had an elevated urinary IL-6. [25] The increased expression and secretion of IL-6 has been proposed to play an important role in the progression of hyperoxaluric urolithiasis.

Although renal inflammation has been proposed to mediate the development of nephro-lithiasis, this hypothesis has not been corroborated in the stone-containing renal tissues of the patients. In

the present study, we investigated the intrarenal mRNA expressions of MCP-1 and IL-6 in patients with kidney calculi and evaluated whether their transcript expressions are associated with renal tubular damage and glomerular dysfunction.

Subjects and Methods

Subjects

Thirty healthy subjects and 29 nephrolithiasis patients were recruited for the study. The healthy group consisted of 11 males (37 %) and 19 females (63 %). They had normal physical examination and urinalysis. Nephrolithiasis group consisted of 9 males (31 %) and 20 females (69 %). All nephrolithiasis patients admitted at Khon- kaen Hospital, Khon Kaen Province, and King Chulalongkorn Memorial Hospital, Bangkok and had positive KUB x-ray for opaque calculi in the kidney. Written informed consents were obtained from all participants prior to specimen collection and the research protocol was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University.

Renal biopsy and 24-hour urine specimens

Renal biopsy samples were obtained from nephrolithiasis patients who underwent stone removal by open surgery. Control renal tissues were taken from non-cancerous and cancerous portions of nephrectomy specimens from patients with localized renal cancers. Renal tissues were immediately submerged in RNA stabilization solution (RNA*later*®, Ambion, USA) after taking from the patients' kidney, placed at 4°C overnight and stored at -80°C until the RNA extraction were performed.

24-hr urine specimens were collected from healthy and nephrolithiasis subjects using thymol as preservative and the urine volume was measured. In nephrolithiasis patients, the urine specimens were collected pre-operatively. The urine samples were kept at -20°C until testing. Since 24-hr creatinine level varies among different laboratories, the normal value can range from 0.5 to 2.0 g/day. In this study, any specimens with urinary creatinine < 0.5 g/day were regarded as inadequate collection and excluded.

Assessments of kidney dysfunction, renal tubular injury and oxidative stress

The glomerular function was estimated by creatinine clearance (CCr). The corrected CCr was calculated and adjusted for body surface area (BSA) of 1.73 m^2. The BSA was computed according to the formula:

$$\text{Body surface area} = \frac{[\text{body weight (kg)} \times 4] + 7}{90 + \text{body weight (kg)}}$$

Urinary β-*N*-acetylglucosaminidase (NAG) activity, an indicator of renal tubular injury was measured by spectrophotometric method. Urinary 8-OHdG as a biomarker of oxidative stress was determined by competitive enzyme-linked immunosorbent assay (ELISA) (New 8-OHdG Check ELISA kit, JaICA, Japan). ELISA procedure was performed according to the manufacturer's instructions.

Urinalysis was performed and the patients with nephrolithiasis were subdivided into two groups, i.e., those with pyuria and those with negative result. Pyuria was defined as urinary white blood cells > 5 cells/HP, or urine test strip positive for leukocytes and nitrite, or both. Based upon these criteria, nephrolithiasis patients with positive pyuria were accounted for 62 % (18/29) whereas the negative ones were 38 % (11/29).

RNA isolation and two-steps real-time reverse transcription- polymerase chain reaction (real time RT-PCR)

Total RNA isolation from renal tissues was carried out using SV Total RNA Isolation System (Promega, WI, USA). The RNA concentration was determined by spectrophotometer (Smart Spec 3000, Biorad, USA) and all samples were diluted to 50 ng/ μl RNA.

The ImProm-I ™ Reverse Transcription System (Promega, WI, USA) was used for the reverse transcription step. The total RNA of 100 ng (2 μl) mixed with 0.5 μg Random Primers (1 μl) was heated at 70°C for 15 min then chilled on ice for 5 min. The resultant was added with 17 μl of reverse transcription mix (final conc.: 1x reaction buffer, 3 mM $MgCl_2$, 0.5 mM dNTP mix, 20 U ribonuclease inhibitor, 1 μl ImProm-II ™ Reverse trascriptase). The cDNA synthesis reaction was performed at the following condition: 25°C for 5 min, 42°C for 1 hr and 70°C for 15 min.

In real-time PCR step, 2 μl of cDNA template, 10 ml of 2x QuantiTect SYBR Green PCR Master Mix (provided final conc. of 2.5 mM $MgCl_2$) (Qiagen, Germany), 1 μl of specific primers (final conc. of 0.5 μM) (Table 1) and 7 μl of nuclease-free water were mixed and placed into a capillary tube (Roch Applied Science, Germany). The real-time PCR reaction was performed in a LightCycler machine (Roche Diagnostics). PCR amplicon was checked by melting curve analysis and verified by 2 % agarose gel electrophoresis.

The relative quantification of gene expression was determined by the $2^{-\Delta\Delta Ct}$ method. Crossing point (Cp), a representative of threshold cycle (Ct), of each sample was computed by LightCycler Software 4.05. The ΔCp was calculated from Cp of target minus Cp of reference and the $\Delta\Delta$Cp was calculated by the subtraction of ΔCp of calibrator from ΔCp of sample. The reference gene used in this study was β - 2 microglobulin (B2M) and the calibrator was one of non-cancerous renal tissues. The $2^{-\Delta\Delta Cp}$ value of each sample indicated a relatively normalized ratio of gene expression.

Statistical analysis

Descriptive statistics were used to summarize the characteristics of the subjects. Data were presented as mean ± standard deviation (SD) for variables with normal distribution and as median (interquartile range; IQR) for variables with skewed distribution. Two independent groups were compared

Table 1. Specific primers used for real-time PCR.

Target mRNA	Primer sequences	Annealing Temp. (°C)	Size (bp)	Accession No.
MCP-1	F: 5'-tcagccagatgcaatcaatg-3'	55	120	S71513
	R: 5'-gcttctttgggacacttgct-3'			
IL-6	F: 5'-ttctccacaagcgccttc-3'	55	69	NM_000600
	R: 5'-gaaggcagcaggcaacac-3'			
B2M	F: 5'-aggctatccagcgtactcca-3'	55	112	NM_004048
	R: 5'-tcaatgtcggatggatgaaa-3'			

by two-sample *t*-test or Mann-Whitney test where appropriate. Difference between three independent groups was assessed by Kruskal-Wallis test followed by Sidak multiple-comparison test. Spearman's rank correlation test was performed to determine the association between MCP-1 and IL-6 expressions. Statistical analyses were performed using STATA version 8.0 (StataCorp., College Station, TX). A two-sided P < 0.05 was considered significant.

Results

Three groups of subjects were recruited, healthy controls or group 1 (n=30), nephrolithiasis patients or group 2 (n=29) and renal cancer patients or group 3 (n=6). The demographic data of these three groups are shown in Table 2. Group 1 consisted of 11 males (37 %) while group 2 and group 3 consisted of 9 (45 %) and 2 (33 %) males, respectively. The mean ages of the three groups were 41.43 ± 10.34, 50.56 ± 11.20 and 64.50 ± 18.26 years, respectively. Since renal biopsy taken from healthy subjects is not ethically feasible, renal tissue samples both from cancerous and non-cancerous regions obtained from the renal cancer group were employed to compare the mRNA expression with the renal stone group.

Urine volume and creatinine excretion were not significantly different between nephrolithiasis and healthy groups (Table 3). Nephrolithiasis patients excreted 8-OHdG significantly greater than healthy subjects (median (IQR): 23.53 (24.51) vs. 4.32 (4.93) μg/g Cr, P < 0.001). Likewise, urinary NAG activity in nephrolithiasis patients was significantly higher than in healthy controls (median (IQR): 5.32 (8.35) *vs.* 3.02 (2.28), P < 0.001).

Table 2. Basic characteristics of subjects.

Characteristics	Subject groups		
	Healthy	**Nephrolithiasis**	**Renal cancers**
n	30	29	6
Gender (M:F)	11 : 19	9 : 20	2 : 4
Age (mean ± SD)	41.43 ± 10.34	50.86 ± 11.20	64.50 ± 18.26
BMI (mean ± SD)	22.56 ± 3.16	23.08 ± 2.54	21.28 ± 4.92

BMI: Body mass index

Table 3. Renal tubular damage and oxidative stress compared between nephrolithiasis patients and healthy controls.

Urinary variables	Disease status		P value
	Healthy	**Nephrolithiasis**	
24-hr urine volume (ml)	1780 (1000)	1900 (1250)	0.500
Creatinine (g/day)	0.99 (0.52)	0.93 (0.40)	0.404
NAG activity (U/g Cr)	3.02 (2.28)	5.32 (8.35)	<0.001*
8-OHdG (μg/g Cr)	4.32 (4.93)	23.53 (24.51)	<0.001*

Data are presented as median (interquartile range). P values were obtained from Mann-Whitney test;

* indicates statistical significance.

The relative mRNA expression of MCP-1 and IL-6 compared among three sources of renal tissues, stone-adjacent renal tissues, non-cancerous renal tissues and cancerous renal tissues, is shown in Fig. 1. Intrarenal mRNA level of MCP-1 in cancerous renal tissues were significantly higher than that in stone-adjacent renal tissues (P = 0.037) but not in non-cancerous renal tissues (P = 0.877). Expressions of MCP-1 mRNA in stone-adjacent and non-cancerous renal tissues were not statistically different (P = 0.242). IL-6 mRNA level in cancerous renal tissues was significantly greater than in both stone-adjacent (P = 0.006) and non-cancerous renal tissues (P = 0.041). The mRNA expression of IL-6 compared between stone-adjacent and non-cancerous renal tissues were not significantly different (P = 1.000).

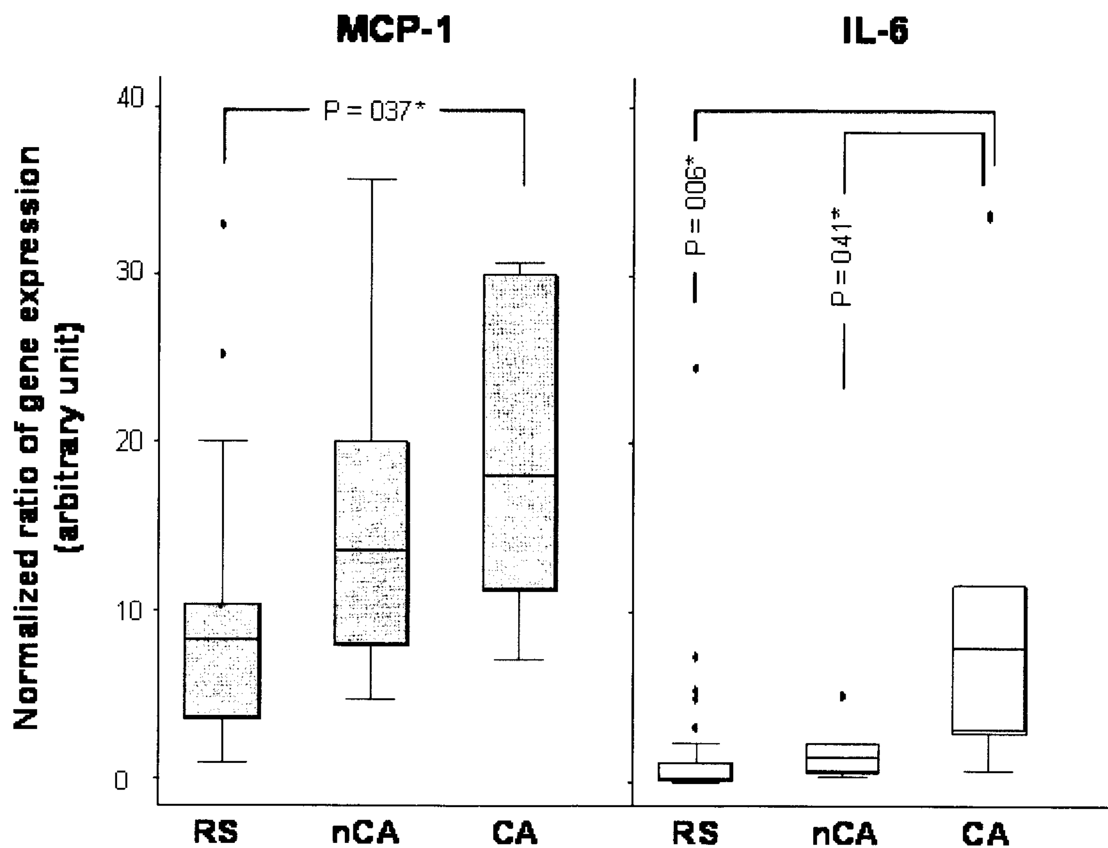

Figure 1. Box-Whisker plot of normalized ratios of MCP-1 and IL-6 mRNA expressions compared between the three sources of renal tissues, stone-adjacent renal tissues (RS), non-cancerous renal tissues (nCA) and cancerous renal tissues (CA). Boxes depict 10th, 25th, 50th, 75th and 90th percentiles. Intrarenal mRNA expression of MCP-1 in cancerous renal tissues was significantly higher than only in stone-adjacent renal tissues whereas intrarenal mRNA expression of IL-6 in cancerous renal tissues was significantly higher than both in stone-adjacent and non-cancerous renal tissues.

In nephrolithiasis group, ΔCp value of IL-6 was significantly higher than that of MCP-1 ($P < 0.001$) (Fig. 2). Since a lower ΔCp value signifies a higher mRNA level, our data indicated that mRNA level of MCP-1 in stone-adjacent renal tissues was greater than that of IL-6. To examine the relationship between MCP-1 and IL-6 expressions in nephrolithiasis patients, scatter plot and Spearman's rank correlation test were carried out. A strong positive correlation between MCP-1 and IL-6 mRNA levels with Spearman's rho of 0.76 ($P < 0.001$) was revealed (Fig. 3).

In an attempt to find the association between MCP-1 and IL-6 expression and renal dysfunction in nephrolithiasis patients, we subdivided the patients regarding to the corrected CCr into two groups: compromised and preserved kidney function groups. Patients with corrected CCr < 50 ml/min/1.73m^2 were defined as compromised kidney function whereas those with corrected CCr $\geq$ 50 ml/min/1.73m^2 were tagged as preserved kidney function. The intrarenal mRNA levels of MCP-1($P = 0.023$) and IL-6 ($P = 0.041$) in patients with compromised kidney function were significantly higher than in those with preserved kidney function (Fig. 4).

Whether the expression of MCP-1 and IL-6 was associated with degree of renal tubular damage in nephrolithiasis patients was also evaluated. The patients were re-grouped according to the level of urinary NAG activity into two groups: patients with high and low renal tubular damage. Since the reference

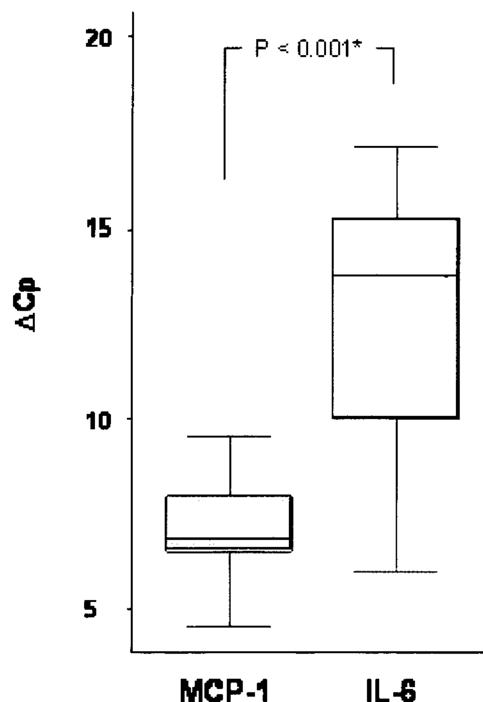

Figure 2. Box-Whisker plot compared ΔCp values between MCP-1 and IL-6 in nephrolithiasis group. Boxes depict 10th, 25th, 50th, 75th and 90th percentiles. The data indicated that intrarenal mRNA expression of MCP-1 in nephrolithiasis patients was significantly higher than that of IL-6. The ΔCp values were obtained from Cp of target genes minus Cp of B2M. A lower ΔCp value indicates a higher mRNA expression.

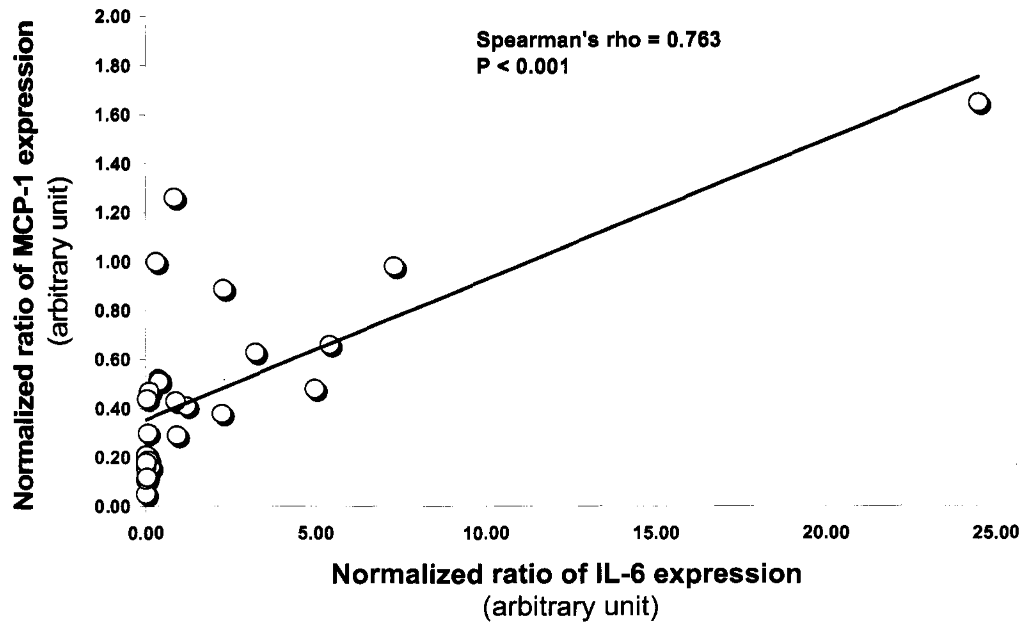

Figure 3. Relationship between intrarenal mRNA expressions of MCP-1 and IL-6 in nephrolithiasis patients. The intrarenal mRNA level of MCP-1 was significantly correlated to that of IL-6 with a Spearman's rho of 0.763 (n = 29, $P < 0.001$).

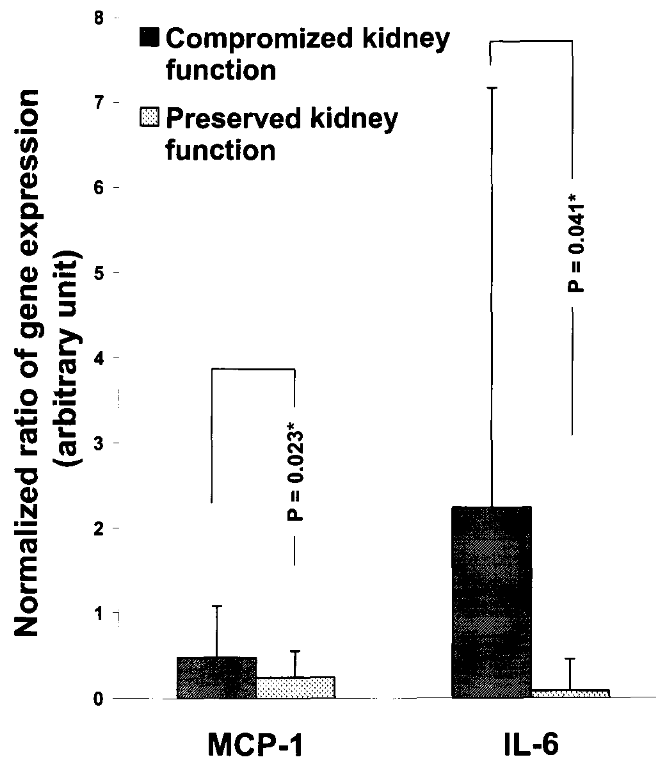

Figure 4. Comparison of intrarenal mRNA expression of MCP-1 and IL-6 between nephrolithiasis patients with compromised and preserved kidney function. Intrarenal mRNA levels of MCP-1 and IL-6 in patients with compromised kidney function (corrected CCr < 50 ml/min/1.73m^2) were significantly greater than in those with preserved kidney function. Bars and error bars denote median values and IQR, respectively.

value of urinary NAG activity has not been clinically documented, to minimize the bias we used 50th percentile or median as a cutoff. Patients with urinary NAG activity ≥ 5.32 U/g Cr were defined as high renal tubular damage whereas those with urinary NAG activity < 5.32 U/g Cr were considered as low degree of renal tubular damage. The intrarenal transcript levels of MCP-1 (P = 0.005) and IL-6 (P = 0.012) in patients with high renal tubular damage was significantly higher than in those with low renal tubular damage (Fig. 5).

To see the correlation between MCP-1 and IL-6 expressions and oxidative stress, the nephrolithiasis patients were sub-grouped into patients with high oxidative stress (urinary 8-OHdG ≥ 23.53 μg/g Cr) and those with low oxidative stress (urinary 8-OHdG < 23.53 μg/g Cr) (Table 4). The mRNA expressions of MCP-1 and IL-6 showed no statistical difference between the two groups. When the patients were reclassified, regarding their pyuria status, mRNA expressions of MCP-1 and IL-6, they showed no significant difference between those with positive pyuria and those with negative result (Table 4).

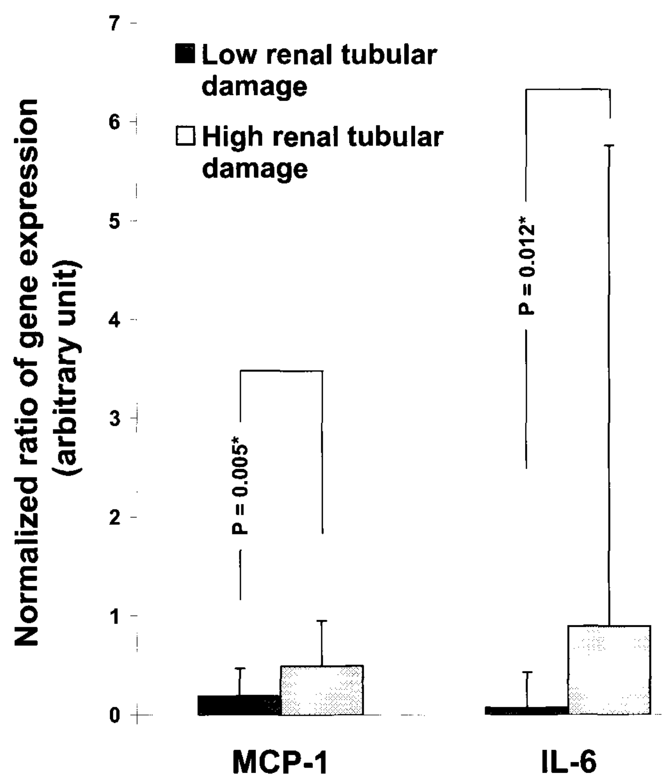

Figure 5. Comparison of intrarenal mRNA expression of MCP-1 and IL-6 between nephrolithiasis patients with low and high renal tubular injury. Intrarenal mRNA levels of MCP-1 and IL-6 in patients with high renal tubular injury (urinary NAG activity ≥ 5.32 U/g Cr) were significantly higher than in those with low renal tubular injury. Bars and error bars denote median values and IQR, respectively.

Table 4. Intrarenal mRNA levels of MCP-1 and IL-6 by oxidative stress severity and pyuria status.

Neprolithiasis classification	n	Normalized ratio of gene expression	
		MCP-1	IL-6
Oxidative stress status			
- Low	15	0.44 (0.36)	0.10 (0.82)
- High	14	0.36 (0.44)	0.64 (2.21)
P value		0.983	0.190
Pyuria			
- Negative	11	0.38 (0.36)	0.10 (2.22)
- Positive	18	0.42 (0.44)	0.34 (1.16)
P value		0.380	0.574

Data are presented as median (interquartile range). P values were obtained from Mann-Whitney test.

Discussion

The present study investigated the mRNA expression of MCP-1 and IL-6 in the kidney of patients with nephrolithiasis, which employed renal tissues from non-cancerous and cancerous portions of nephrectomy as controls. The mRNA levels of MCP-1 and IL-6 in stone-containing kidney tissues were significantly lower than in cancerous renal tissues but not in non-cancerous renal tissues. Urinary profile showed that nephrolithiasis patients had high oxidative stress and increased renal tubular damage. An increased renal tubular damage as well as impaired glomerular function was associated with increased mRNA expression of MCP-1 and IL-6.

It is well recognized that oxidative stress mediates the kidney stone pathogenesis. Elevated urinary level of oxidatively modified substances has been documented in nephrolithiasis patients and the oxidative stress severity was correlated with renal tubular damage. [3, 26, 27] The urinary NAG activity has been widely used as a sensitive marker of renal tubular injury in kidney diseases including nehrolithiasis. [3, 26-28] Consistently, our present data show that urinary levels of 8-OHdG and NAG activity in nephrolithiasis patients were significantly higher than that in healthy controls. This emphasizes that kidney stone patients have high oxidative stress and increased damage of renal proximal tubules.

The mechanism of human stone formation is mostly deduced from experimental nephrolithiasis studies. Intrarenal oxidative damage and inflammation are common pathological pathways detected in the nephrolithic rats. Lithogenic crystals, formed in urine under persistently supersaturated condition, are internalized by renal tubular cells to be dissolved in lysosomes, and the remaining crystals are exocytosed into renal interstitium where crystals deposit, which is called Randall's plaque. This plaque moves toward renal papilla and is considered to serve as stone nidus. [29] Huang et al. demonstrated in nephrolithic rats that generation of free radicals occurred in an early stage and persevered while the infiltration of leukocytes (CD45 positive cells) progressively increased in the late stage. [9, 10] In addition, de Water et al. showed

that interstitial crystals were mostly surrounded by ED1 positive cells (specific for monocytes and macrophages) and multinucleated giant cells. [6, 7] These cells function to remove the crystals via a sterile inflammatory process by which crystals were phagocytosed, slowly dissolved within the cells then released into the extracellular environment. [8, 30] The histological changes included glomerular damage, tubular dilation and necrosis and enlargement of the interstitium were obviously seen in rats with prolong crystal-induced condition.[5] The attraction of inflammatory cells is believed to be responsible by chemotectic proteins and the over-production of MCP-1 chemokine and IL-6 cytokine by crystals-exposed renal tubular cells has been demonstrated.[11-15, 24] We hypothesize that MCP-1 and IL-6 are up-regulated in the kidney of nephrolithiais patients and their rise is contributed to renal dysfunction.

Our results show that mRNA levels of MCP-1 and IL-6 in stone-adjacent renal tissues are significantly lower than in cancerous renal tissues. This indicates that nephrolithiasis patients have a lower extent of intrarenal inflammation than renal cancer patients. However, the mRNA expressions of MCP-1 and IL-6 between stone-adjacent and non-cancerous renal tissues are not significantly differerent. This is not a totally unexpected result. It is possibly due to the use of inappropriate normal control of the renal tissue. The control non-cancerous renal tissue used in this study was taken from a vicinity area of tumor mass and the histological staining to verify normal situation was not applied. As shown in Fig. 1, the mRNA expression of MCP-1 between cancerous and non-cancerous renal tissues was not significantly different indicating that non-cancerous renal tissue has some degree of pathological condition. Other studies in various renal diseases showed no mRNA and protein expressions of MCP-1 in their evaluated normal renal tissues. [31-33] These suggest that our non-cancerous renal tissue control is not a truly non-pathological renal tissue. Based upon our present findings, we conclude that the kidney of nephrolithiasis patients encompasses an active low-grade inflammation as equivalent as an adjacent area of the malignant kidney. The low-grade inflammatory response in nephrolithiasis patients may be caused by persistent oxidative stress and mechanical irritation of stones and it may relevant to the progression of disease; however these speculations need further investigation.

In nephrolithiasis renal tissues, our data demonstrate that intrarenal mRNA expression of MCP-1 is significantly greater than that of IL-6 (Fig. 2). This may implied that MCP-1 has an active role in the renal inflammation more than IL-6. We believe that these two inflammatory mediators partly contribute in the pathogenesis and progression of kidney stone. Combined with other studies in the literature, expression of MCP-1 and IL-6 by renal tubular cells may be a cellular defense in response to oxalate/crystal exposure as well as to mechanical irritation by stones. Exposed/irritated renal tubular cells generate reactive oxygen species (ROS), which modulate an array of gene expression including MCP-1 and IL-6. MCP-1 is likely to exert chemotactic property to attract inflammatory cells, mainly monocytes and macrophages, into the renal interstitium or where a formed stone causes inflammatory activation. These infiltrated cells, in turn, enhance the production of chemokines, cytokines as

well as extracellular matrix proteins leading to inflammatory amplification, consequently, causing interstitial damage and loss of renal function. [24]

The precise biological role of IL-6 in nephrolithiasis is not known. IL-6 is a multifunctional cytokine involving epithelial cell growth and differentiation, inflammation, immune response, hematopoiesis and the acute-phase response. It was suggested to be produced by mesagial cells and inflamed renal cells rather than infiltrated leukocytes. [24] Perhaps, IL-6 plays a role in the healing process to stimulate the proliferation of renal tubular cells in order to replace the damaged renal cells. Additionally, L-6 is known as a stimulating factor for bone resorption. It enhances hypercalcemia via increasing a pool of osteoclast precursor, which is mediated by parathyroid hormone-related protein. [34] To see a clue of its bone resorptive role in our renal stone patients, the association between intrarenal IL-6 mRNA and circulating as well as urinary calcium should be further explored.

We examined whether an increased expression of inflammatory mediators in nephrolithiasis patients associated with renal dysfunction. The data demonstrate that patients with lower corrected CCr and those with higher urinary NAG activity had significantly higher intrarenal transcripts of MCP-1 and IL-6. This indicates that increased intrarenal expression of MCP-1 and IL-6 is associated with enhanced glomerular dysfunction and renal tubular damage. An over-expression of intrarenal MCP-1 was reported in various renal diseases such as idiopathic membranous nephropathy [31], reflux nephropathy [33], lupus nephritis [19], congenital obstructive nephropathy [32] and IgA nephropathy [18] moreover its up-regulation is associated with the extent of leukocyte infiltration. Thereby, MCP-1 is suggested to plays an important role in leukocytes influx and consequently tubulointerstitial inflammatory damage. Increased intrarenal expression of IL-6, mainly in glomerular and vascular endothelial locations, has been demonstrated in patients with IgA nephropathy, which is correlated with the disease severity. [22, 23] Furthermore, urinary IL-6 levels could be used as a predictor of worse renal outcome in patients with IgA nephropathy. [35] An elevation of urinary IL-6 in patients with urolithiasis has already been reported. [25] We conclude that increased intrarenal inflammation, indicated by high MCP-1 and IL-6 mRNA levels, in renal stone patients contribute to the glomerular impairment and renal tubular damage.

In this study we did not find a significant relationship between urinary 8-OHdG excretion and intrarenal gene expression. ROS-induced gene expression is well recognized and the expression of MCP-1 and IL-6 in renal tubular cells is suggested to mediate via ROS. [11, 12, 15, 24] Since ROS can attack all kinds of cellular biomolecules, urinary 8-OHdG represents only ROS that assails on DNA, may not represent the entire amount of ROS generated in the renal tissues. This might be the explanation for this insignificant association; this, however, may be attributed by the small sample size in the study. No association between the level of MCP-1 and IL-6 expressions and pyuria found in this study might indicate that the expression of these genes is irrespective to infection, a sterile inflammation. The extent of their transcript expression may be primarily caused by lithogenic crystals and stones.

In conclusion, nephrolithiasis patients manifested a low-grade intrarenal inflammation. Increased intrarenal mRNA expression of MCP-1 and IL-6 was associated with enhanced glomerular dysfunction and renal tubular damage. The determination of MCP-1 and IL-6 in urine should be further investigated if it is clinically useful as a non-invasive mean for monitoring the progression of kidney stone disease.

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