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Original Article

Diagnostic role of urinary CA-2 in urinary stones and its prediction of complications

Yuanjing Leng[1], Haibin Zhou[1], Wenjuan Wang[2], Jiangling Fu[3]

Departments of [1]Urology, [2]Clinical Laboratory, Affiliated Hospital of Jiujiang College, Jiujiang 332000, Jiangxi, People's Republic of China; [3]Clinical Precision Medical Research Center, Affiliated Hospital of Jiujiang College, Jiujiang 332000, Jiangxi, People's Republic of China

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Abstract: Background: Carbonic anhydrase-2 (CA-2) is involved in the mineralization and calcification of organisms. Evidence suggests that CA-2 is associated with urolithiasis. However, the relationship between CA-2 and urinary stones remains unclear. The study aimed to assess the correlation of urinary CA-2 (uCA-2) level with the risk of urinary stones. Methods: A retrospective cohort study was conducted on patients with urinary stones and healthy subjects who presented to our hospital between March 2017 and November 2019 to determine the pretreatment uCA-2 level by enzyme linked immunosorbent assay (ELISA). Differences in uCA-2 levels between patients with urinary stones and healthy subjects were compared. Then, comparison between stone patients with complications and those without was carried out as well as correlation analysis to detect factors associated with biomarker expression. Results: Patients with urinary stones (n=118) were designated the urinary stones group and healthy subjects (n=42) were designated the healthy control group. The mean pretreatment uCA-2 level was significantly higher in cases than in controls (P=0.028). Furthermore, the uCA-2 level had a positive correlation with urinary stone-associated complications (R=0.379, P<0.001), especially pain (R=0.524, P<0.001) and hematuria (R=0.374, P<0.001). Receiver operating characteristic curve (ROC) analysis revealed that a uCA-2 level threshold of 10.94 ng/mL had 83.67% sensitivity and 68.12% specificity for predicting complications in patients with urinary stones. Conclusion: Excessive uCA-2 excretion is a major risk factor for urinary stones. Our findings suggest that uCA-2 may be used as a novel biomarker for the diagnosis of urinary stones and the prediction of its complications.

Keywords: Urinary stones, urine, carbonic anhydrase-2, enzyme linked immunosorbent assay, biomarker, analysis

Introduction

A number of possible metabolic [1-3], genetic [4-6], drug-induced [7, 8], and epidemiologic factors contributing to urolithiasis have been reported [9, 10], and understanding these factors may help determine the most appropriate therapy for each patient. However, there are no predictable biomarkers for the disease, and most patients are diagnosed only after symptoms have developed. Uroscopy is the most commonly used tool for diagnosing urinary calculi, which includes red blood cells, stone crystals, white blood cells, white blood cell esterase, and other findings [11]. However, in some cases, such as renal colic, the results of urine detection are relatively delayed and non-specific. Thus, it is still necessary for researchers to find non-invasive biomarkers that can be easily detected with relatively high accuracy. Although non-contrast computerized tomography (CT) is considered as the gold standard method for diagnosis of urolithiasis, it cannot provide sufficient information about nesting status because it does not use contrast agents. Therefore, it is necessary to combine some biomarkers with imaging techniques [12]. A few studies have identified an association between urinary calculi and carbonic anhydrase-2 (CA-2), suggesting that CA-2 is associated with urinary stones [5, 8].

CA-2, as one of the multiple forms of human α-CA, exists almost ubiquitously in the human body and has a relatively high expression level in the gastrointestinal tract, biliary tract, and kidneys [13]. The main function of CA-2 in the human body is to interconvert CO_2 and HCO_3^- to

maintain the acid/base balance in blood and other tissues and to help transport CO_2 out of tissues. Several studies have demonstrated that CA-2 and its isoenzymes are involved in the calcification of human tissues, including blood vessels [14, 15] and the brain [16], though the mineralization mechanisms have not been fully elucidated. CA isoenzymes also participate in the calcification process of many biological systems, including bacteria-induced calcification, calcareous bone formation, and shell formation in shell-forming animals. Furthermore, the occurrence of renal stones is often associated with ectopic calcification, including vascular calcification and gallstones [17-19]. Generally, ectopic calcification follows the same pattern as physiologic bone mineralization, with an increase in carbonated hydroxyapatite on fibrous collagen. CA-2 and its isoenzymes catalyze the reversible hydration of CO_2 and participate in the calcification process in various biologic systems; they also promote $CaCO_3$ formation, which in turn serves as the basis for $Ca_3(PO_4)_2$ deposition (hydroxyapatite).

Although some relevant evidence supports the correlation of CA-2 with calcification or mineralization, information on the role of CA-2 in the modification of the risk of urinary stones is scarce. Accordingly, we conducted a retrospective clinical study to assess the association of the urine CA-2 (uCA-2) level with the risk of urinary stones.

Material and methods

Inclusion and exclusion criteria

A retrospective study was conducted on eligible patients (age range: 20-70) with urinary stones who presented between March 2017 and November 2019 to the Affiliated Hospital of Jiujiang University. All the included participants were in line with the following conditions: (1) A non-contrast CT scan and color doppler ultrasound inspection proved a unilateral ureteral stone or kidney stone larger than 4 mm; (2) Serum creatinine (sCr) was within normal limits, without disturbance of acid-base balance of water and electrolytes at diagnosis; (3) With complete clinical files. Exclusion criteria: those with (1) Double-J ureteral stent placement; (2) Chronic kidney disease; (3) Medical history of manual or instrumental urological intervention; (4) Urinary tract infection or bacteremia or sepsis; (5) Anatomical or pathological solitary renal; (6) Pregnancy; (7) Immunodeficiency diseases; (8) Malignant diseases; (9) Hypertension, diabetes, hypercholesterolemia, or any other chronic disease history. Description of calculi (including the location, maximum diameter) and hydronephrosis evaluated by noncontrast CT scan or ultrasound was recorded. In addition, age and gender-matched healthy subjects were selected during a routine physical examination during the same period; none of them had history of urinary stones or any known ectopic calcification. Detailed information of age, gender, height and weight was recorded. The study protocol was approved by the Ethics Committee of Affiliated Hospital of Jiujiang University.

Outcome measures

Before medical intervention, serum and midstream urine samples were collected from the study population and stored at 4°C during the entire sample collection period. Cell debris and particulate matter were obtained from serum samples by centrifugation at 3000 r/min and 4°C for 15 minutes and urine samples at 1500 r/min and 4°C for 10 minutes. The midstream urine was used to analyze uCA-2 and urine kidney injury molecule-1 (uKIM-1) levels using commercially available enzyme-linked immunosorbent assay (ELISA) kits following the manufacturer's protocol (Human CA-2 ELISA Kit (ab222881, Abcam, Cambridge, UK), Human KIM-1 ELISA Kit (ab235081, Abcam, Cambridge, UK)). Measurements of sCr, urea nitrogen (BUN), and Uric acid (UA) in plasma were performed at the department of laboratory medicine (Affiliated Hospital of Jiujiang College).

Statistical analysis

Continuous variables including age, body mass index (BMI), serum biochemistry, uCA-2 and uKIM-1 were expressed as the mean ± standard error of the mean. Enumerated data including gender were expressed as frequencies (%). Statistical analyses were performed using Graphpad Prism 7.00 (Graphpad Software Inc., La Jolla, CA, USA). The inter-group differences were tested using one-way ANOVA and the Student's t test was used to compare participants' age, BMI, sCr, sBUN and sUA; the Chi-square test was used to compare gender; the Mann-Whitney U test and Kruskal-Wallis H test

Table 1. Participants and event characteristics

Variable	Urinary stones group (n=118)	Healthy control group (n=42)	*P* value
Ages (years)	42.07±8.23	41.52±8.70	0.718
Gender [male n (%)]	79 (66.95)	26 (61.90)	0.574
BMI (Kg/m^2)	23.18±1.95	22.51±2.26	0.068
sCr (μmol/L)	67.06±9.29	64.58±9.20	0.139
sBUN (mg/dL)	13.15±3.07	13.68±3.36	0.350
sUA (μmol/L)	237.23±57.87	239.35±77.65	0.853
uCA-2 (ng/mL)	18.29±22.78	8.49±3.86	0.028#
uKIM-1 (ng/mL)	1.36±0.79	1.24±0.82	0.457

Note: BMI, Body Mass Index; sCr, serum Creatinine; sBUN, serum Urea Nitrogen; sUA, serum Uric Acid; uCA-2, urine Carbonic Anhydrase-2; uKIM-1, urine Kidney Injury Molecule-1. #P<0.05 by Mann-Whitney U test.

Table 2. Participants and event characteristics in patients with or without complications

Variable	With complication group (n=69)	Without complication group (n=49)	*P* value
Age (years)	41.87±8.14	41.97±8.10	0.958
Gender [male n (%)]	48 (69.6)	31 (63.3)	0.473
BMI (Kg/m^2)	22.84±1.87	22.98±2.03	0.700
sCr (μmol/L)	66.96±9.15	67.07±8.94	0.948
sBUN (mg/dL)	13.32±2.85	13.61±3.12	0.602
sUA (μmol/L)	238.55±60.34	239.22±69.58	0.956
uCA-2 (ng/mL)	20.18±7.36	18.03±8.78	0.030#
uKIM-1 (ng/mL)	1.29±0.87	1.25±0.95	0.813

Note: BMI, Body Mass Index; sCr, serum Creatinine; sBUN, serum Urea Nitrogen; sUA, serum Uric Acid; uCA-2, urine Carbonic Anhydrase-2; uKIM-1, urine Kidney Injury Molecule-1. #P<0.05 by Mann-Whitney U test.

were used to compare uCA-2 and uKIM-1 levels. Correlations of uCA-2 and uKIM-1 with urinary stone-associated complications were studied by Spearman correlations or partial correlations analysis. Receiver operating curve (ROC) analysis was carried out to evaluate the ability of uCA-2 and uKIM-1 to predict complications in patients with urinary stones. The area under the curve (AUC) was derived from receiver operating curve (ROC) analysis. Significance was set at P<0.05.

Results

Demographic and clinical characteristics of participants

Among the 160 participants included in the present study, there were 103 (64.37%) males and 57 (35.63%) females. Of them, 118 patients with urinary stones were classified into the urinary stone group and 42 healthy subjects were classified into the healthy control group. In terms of detailed calculi location of the 118 patients, 29 (24.58%) were ureteral stones and 89 (75.42%) were kidney stones; the average stone size in the urinary stones group was 10.86±4.24 mm (5-32 mm). The mean age of all participants was (41.93± 8.33) years old (range: 20-68). The demographic and clinical characteristics of all participants are reported in **Table 1**. There were no significant differences between cases and controls in age, gender, BMI, sCr, sBUN, or sUA (all P>0.05).

Participants and event characteristics in patient with or without complications

Table 2 shows the nonparametric univariate analysis of the characteristics between the two groups. The two groups did not differ significantly in pretreatment uKim-1 level.

Moreover, the mean pretreatment uCA-2 level was significantly higher in the urinary stones group than in the healthy control group (**Figure 1**). In terms of complications in the urinary stones group, 40.67% of patients developed hematuria, 24.57% experienced pain, and 35.59% had hydronephrosis. Almost 41.52% of patients had no stone complications. In the comparison of participants and event characteristics in patients with or without complications, uCA-2 levels were found to be significantly different between patients with and without complications.

uCA-2 and uKIM levels in urinary stone patients with complications

Urinary stone-related complications were associated with a high uCA-2 excretion, whereas no association was observed with uKim-1 excretion (**Figure 2**). The uCA-2 level was significantly higher in patients with hematuria compared to those without hematuria in the urinary stone

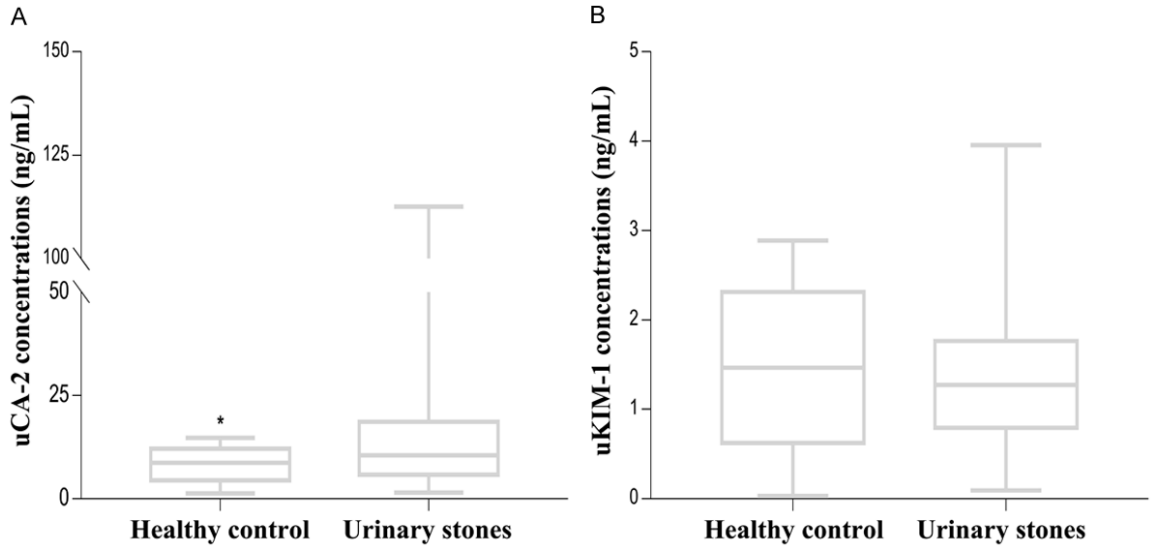

Figure 1. uCA-2 and uKIM levels in patients with urinary stones and healthy subjects by ELISA. *P<0.05 as evaluated using Mann-Whitney U test. uCA-2, urine carbonic anhydrase-2; uKIM-1, urine kidney injury molecule-1.

group and healthy subjects [mean (30.04±31.19) ng/mL vs. (10.24±7.45 or 8.49±3.86 ng/mL), respectively, each P<0.001]. As for pain, the uCA-2 level was also significantly higher in patients suffering from pain than those without pain in urinary stones group and in healthy controls [mean (37.84±32.47) ng/mL vs. (11.92±13.69 or 8.49±3.86 ng/mL), respectively, each P<0.001]. Interestingly, patients with pain had ureteral stones (76.67%) with a smaller calculi size (8.5±2.87 mm). As for hydronephrosis, the uCA-2 level was also significantly higher in patients with hydronephrosis versus those without hydronephrosis in the urinary stone group and healthy controls [mean (22.54±23.21) ng/mL vs. (15.95±22.35 or 8.49±3.86 ng/mL), respectively, each P< 0.001]. A non-significantly higher uKIM-1 level excretion was found in patients with hematuria, pain and hydronephrosis compared to the urinary stone patients without complications as well as controls.

The uCA-2 level had a positive correlation with urinary stone-related complications (R=0.379, P<0.001), especially pain (R=0.524, P<0.001) and hematuria (R=0.374, P<0.001). Spearman correlations or partial correlation analysis showed that there was no statistical correlation between uKim-1 level and urinary stone-associated complications.

Predictive value of uCA-2 and uKIM-1 levels for urinary stone-associated complications

The predictive value of uCA-2 and uKIM-1 levels to predict urinary stone-associated complications was analyzed using a ROC curve (**Figure 3**). ROC analysis of uCA-2 yielded an AUC of 0.786 for total complications, 0.720 for hematuria, 0.852 for pain, and 0.683 for hydronephrosis (each P<0.001). The ROC analysis revealed no significant correlation between uKIM-1 and any complications. The values of uCA-2 and uKIM-1 in predicting urinary stone-associated complications differed significantly. **Table 3** shows the sensitivity and specificity of uCA-2 level to predict urinary stone-related complications. The optimal cut-off value of the uCA-2 level was chosen based on the maximum value of the Youden index, and the results indicated that uCA-2 level was a reliable predictor. Furthermore, we determined that a uCA-2 level threshold of 10.94 ng/mL had a sensitivity of 83.67% and a specificity of 68.12% for predicting urinary stone-related complications.

Discussion

This retrospective study included 118 patients with urinary stone and 42 healthy individuals. Substantial evidence has indicated that patients with urinary stone exhibited increased uCA-2 levels as detected by ELISA. The key find-

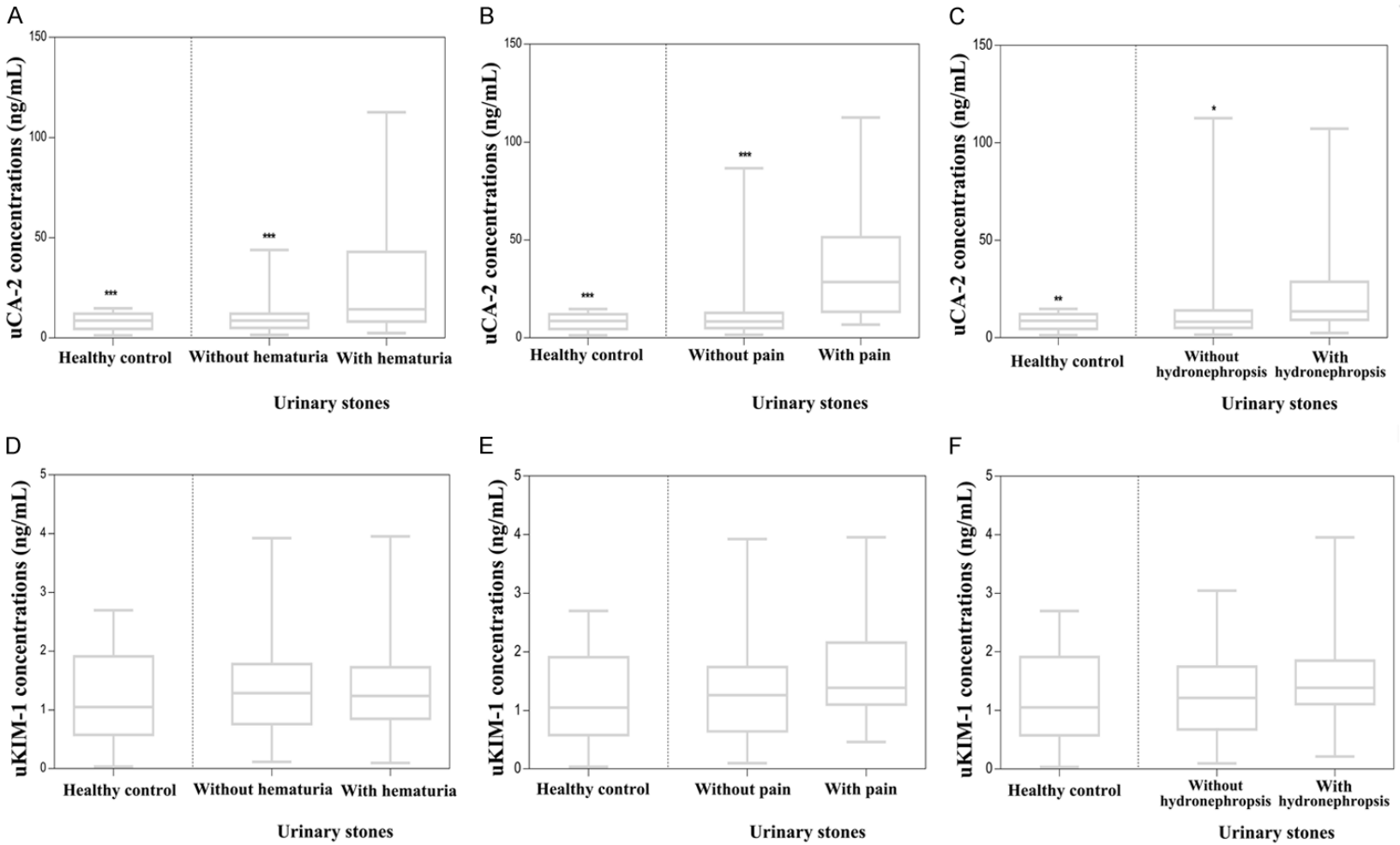

Figure 2. uCA-2 and uKIM levels in patients with stone complications and healthy subjects by ELISA. *P<0.05, **P<0.01, ***P<0.001 as evaluated using a Kruskal-Wallis H test. uCA-2, urine Carbonic Anhydrase-2; uKIM-1, urine Kidney Injury Molecule-1.

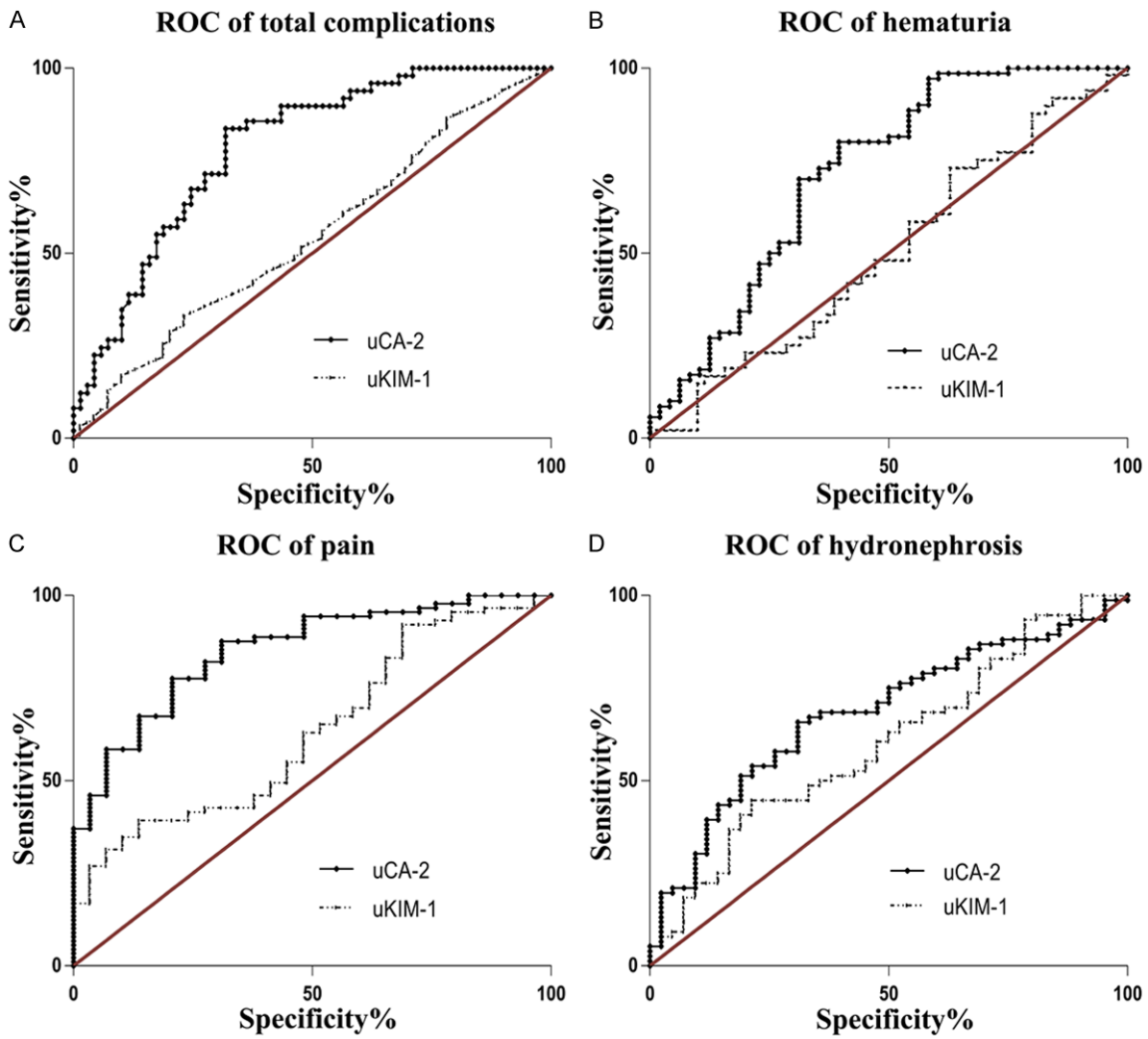

Figure 3. Area under the receiver operator characteristic (ROC) curve value for uCA-2 and uKIM for predicting stone complications. A. ROC curve analysis of total stone complications. B. ROC curve analysis of uCA-2 and uKIM in hematuria. C. ROC curve analysis of uCA-2 and uKIM for pain. D. ROC curve analysis of uCA-2 and uKIM in hydronephrosis. uCA-2, urine Carbonic Anhydrase-2; uKIM-1, urine Kidney Injury Molecule-1.

Table 3. Cut-off values, positive predictive values (PPV), sensitivity and specificity of uCA-2 to predict urinary stone-related complications

Variable	Cut-off value (ng/ml)	PPV	% Sensitivity	% Specificity
Total complications	<10.94	81.03%	83.67%	68.12%
Hematuria	<9.029	51.47%	52.86%	72.92%
Pain	<13.01	53.49%	77.53%	79.31%
Hydronephrosis	<10.94	53.70%	65.79%	69.05%

Note: uCA-2, urine Carbonic Anhydrase-2; PPV, Positive Predictive Values.

ing of this study was that a significant difference in uCA-2 excretion can discriminate patients with urinary stones from healthy controls. If our results are supported by many prospective validation studies, a uCA-2 excretion test might be clinically beneficial for the surveillance of high-risk patients, such as patients with metabolic syndrome [1-4], inflammatory bowel disease [20] and accepted bariatric surgery [21]. Patients at high risk of stone recurrence may also benefit from our results.

Small, hard mineral deposits that characterize urinary stones can cause severe pain, hematuria, obstruction or (and) hydronephrosis. The

diagnosis of urinary stones depends on imaging examination and lacks effective biomarker, which makes it difficult to predict the recurrence of urinary stones. The American Urological Association (AUA) guidelines recommend that millions of patients newly diagnosed with urinary stone be screened for evaluation to reduce the risk of stone recurrence [22]. To achieve this goal, more advanced diagnostic approaches have to be developed and applied to earlier detection of urinary stones. Available clinical data support the conclusion that asymptomatic patients have a reduced risk of stone recurrence due to early dietary or drug interventions [23, 24].

To better understand the significance of excessive uCA-2 production in patients with urinary stones, we evaluated data from our database on patients with urinary stones and assigned uCA-2 levels to patients with or without stone-related complications based on appropriate symptoms and signs. Our data showed that uCA-2 excretion was significantly increased in urinary stone patients with one or more complications. All the three complications showed a high level of consistency, and pain demonstrated superiority over the other two with regard to predicting uCA-2 excretion.

To trace the phenomenon of higher uCA-2 levels in urinary stone patients developing one or more complications, this study tried to describe the unclear effect of stone formation. The results of uCA-2 excretion interacting with stone-related complications highlighted factors that may influence pathophysiology, such as crystal-membrane interactions, microbial-stone interactions, or other possible interactions. CA-2 regulates acid-base homeostasis. Recent evidence has shown that there is a correlation between cell control of acid-base status and the innate defense in the kidney [25]. However, our data showed non-significantly higher uKIM-1 excretion in patients with hydronephrosis compared to the other two groups. Our results appear to be at odds with a previous study that evaluated uKIM-1 as a biomarker of acute renal injury and was significantly higher in stone patients with hydronephrosis compared to those without hydronephrosis [26]. The differingresults may be explained by the variation in participants' inclusion criteria.

The excretion of uCA-2 by the bacteria in patients with urinary stones is also a potentially important factor. The relative abundances of 20 bacterial genera differed significantly between urinary stone patients and healthy subjects. These findings may provide new and non-invasive potential biomarkers for the diagnosis of kidney stones [27]. *Escherichia coli*, one of most commonly detected pathogenic microorganisms attached to renal stones [28], can also actively secrete PH-dependent CA-2 [29]. CA and urease in bacteria play a synergic role in promoting $CaCO_3$ precipitation [30]. Microbial urease is a Ni-containing enzyme found in various microorganisms that hydrolyzes urea to $NH4^+$ and CO_2, whereas carbonic anhydrase transforms CO_2 into HCO_3^-. In *Bacillus megaterium*, calcite precipitation is driven by the coupled activity of urease and carbonic anhydrase enzymes. The precipitation of $CaCO_3$, which predominantly consists of calcite crystals, by microbial carbonic anhydrase has been documented [31]. CA-2 may decrease stone inhibitory proteins through its potential effect on the g-glutamyl carboxylase reaction by providing CO_2. CA-2 can also supply CO_2 to G-glutamyl carboxylase, which catalyzes glutamate residue carboxylation in some proteins, thereby enhancing their biological activity [32, 33]. Several proteins that require this post-translational carboxylation to become biologically active may be involved in the calcification processes in human tissues, including matrix Gla protein, growth arrest-specific protein 6, Gla-rich protein, and osteocalcin [34]. The relationship between CA-2 and other stone-promoting or -inhibiting factors will be the focus of our studies in the future.

This research still shows room for improvement. Although the number of samples is large, the overall sample size remains small. Besides, the study did not follow up healthy controls with relatively increased uCA-2 excretion. Stone formation is a dynamic process that cannot easily be captured by a midstream urine collection. Finally, the relationship among uCA-2, Ca, oxalic acid, and P excretion was difficult to evaluate in urine because the 24 h urine biochemical analysis was not performed.

Conclusion

Excessive CA-2 excretion in urine is a major risk factor for urinary stones. Our findings suggested that uCA-2 may be used as a novel biomarker for the diagnosis of renal stones and the prediction of complications in patients.

Acknowledgements

A preprint has previously been published [35] as "Urine CA-2 as a biomarker for diagnosis urinary stone and prediction of its complications" on Research Square (https://www.researchsquare.com/article/rs-59394/v1). This work was supported by Science and Technology Support Project of Jiangxi Health and Family Planning Commission (No. 20204287).

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

Disclosure of conflict of interest

None.

Address correspondence to: Jiangling Fu, Clinical Precision Medical Research Center, Affiliated Hospital of Jiujiang College, Jiujiang 332000, Jiangxi, People's Republic of China. Tel: +86-0792-2173516; E-mail: Fu_jiangling@126.com; Yuanjing Leng, Departments of Urology, Affiliated Hospital of Jiujiang College, Jiangxi, Jiujiang 332000, Jiangxi, People's Republic of China. Tel: +86-0792-2173516; E-mail: herolat_leng@126.com

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