Urine CA-2 as a biomarker for diagnosis urinary stone and prediction of its complications

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Abstract

PURPOSE

Carbonic anhydrase-2 (CA-2) plays a role in mineralization and calcification in organism. Strong evidence suggests that CA-2 is associated with urolithiasis. However, the relationship between CA-2 and urinary stone remains unclear. The study aimed to assess the association of urine CA-2 (uCA-2) level and the potential risk of urinary stone.

METHODS

From March 2017 to November 2019, a prospective cohort study was conducted on patients with urinary stones and healthy subjects to determine the pretreatment uCA-2 level detection by Enzyme linked immunosorbent assay (ELISA). The difference of uCA-2 level between patients with urinary stones and healthy subjects was compared. Then comparison between stone patients with complications and without complications was carried out as well as correlation analysis to detect factors associated with biomarker expression.

RESULTS

118 patients with urinary stones were into urinary stones group and 42 healthy subjects were into healthy control group. The mean pretreatment uCA-2 level was significantly higher in patients with urinary stones group than healthy controls group ($P=0.028$). Furthermore, The uCA-2 level was positive correlation with urinary stones complications ($R=0.379$, $P=0.000$), especially pain complications ($R=0.524$, $P=0.000$) and hematuria complications ($R=0.374$, $P=0.000$). Receiver operating characteristic curve (ROC) analysis that a uCA-2 level threshold of 10.94 ng/mL had 83.67% sensitivity and 68.12% specificity for predicting urinary stones complications.

CONCLUSIONS

Excessive uCA-2 excretion is a major risk factor for urinary stone. Our findings suggested that uCA-2 may be used as an unappreciated biomarker for the diagnosis urinary stone in patients and to predict its complications.

Introduction

A number of potential metabolic¹⁻³, genetic⁴⁻⁶, drug-induced^{7,8}, and epidemiological^{9,10} factors for urolithiasis have been reported. The knowledge of these factors contributes in determining the most appropriate therapy for each patient. Few studies have identified that urinary stone are related to carbonic anhydrase-2 (CA-2)^{5,8}, thereby suggesting that the CA-2 is associated with urinary stone.

CA-2, as one of the multiple forms of human α -CA, exists as almost ubiquitous in the human body and have a relatively high expression level in the gastrointestinal tract, biliary tract, and kidneys¹¹. The main

function of CA-2 in human is to interconvert CO_2 and HCO_3^- to maintain the acid/base balance in blood and other tissues and help transport CO_2 out of the tissues. Several studies have demonstrated that CA-2 and its isoenzymes are involved in the calcification in human tissues, including the blood vessel^{12,13} and the brain¹⁴, though their mineralization mechanisms have not been fully understood. Similarly, CA isoenzymes are also involved 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 stone is frequently associated with ectopic calcification, including vascular calcification and gallstone¹⁵⁻¹⁷. Generally, ectopic calcification follows the same pattern as physiological 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 biological systems. CA-2 and its isoenzymes also promote CaCO_3 formation, which in turn serves as the basis for $\text{Ca}_3(\text{PO}_4)_2$ deposition (hydroxyapatite).

Although some relevant evidence supported the association of CA-2 and calcification or mineralization, information on the role of CA-2 in the modification of the risk of urinary stone disease is scarce. In this study, we conducted a prospective clinical study to assess the association of the urine CA-2 (uCA-2) level and the potential risk of urinary stones.

Patients And Methods

A prospective study from March 2017 to November 2019 was conducted on patients with urinary stones were considered eligible to participate in the affiliated hospital of Jiujiang University. Patients diagnosed with urinary stones whose age is between 20 and 70 were considered eligible to participate in the affiliated hospital of Jiujiang University. All these objects correspond to the following conditions. (1) A non-contrast computerized tomography (CT) scan and color doppler ultrasound inspection proved an unilateral ureteral stone or kidney stone larger than 4 mm; (2) sCr was within normal limits and without disturbance of acid–base balance of water and electrolytes when diagnosed; (3) Patients understand the content of this study and voluntarily sign informed consent. The exclusion criteria were: (1) With double-J ureteral stent placement status; (2) With 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 status; (7) With immunodeficiency diseases; (8) With any malignant diseases; (9) with hypertension, diabetes, hypercholesterolemia, or any other chronic disease history. Description of calculi (including the location, maximum diameter, etc.) and hydronephrosis evaluated by noncontrast CT scan or ultrasound was recorded. Many healthy subjects, age and gender-matched, were selected during a routine physical examination during the same period, and 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 Jiujiang University Affiliated Hospital.

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

Continuous variables including age, body mass index index (BMI), serum biochemistry, uCA-2 and uKIM-1 were expressed as the mean±standard error of the mean. Enumeration data including gender was expressed as frequency (%). Statistical analysis was performed using Graphpad Prism 7.00 (Graphpad Software Inc., La Jolla, CA, USA). The intergroup differences were tested using One-way ANOVA and Student's *t* test was used → compare participants age, BMI, sCr, sBUN and sUA; Chi-square test was used to compare gender; Mann-Whitney U test and Kruskal-Wallis H test were used to compare uCA-2 and uKIM-1. Associations between uCA-2 and uKIM-1 and urinary stones complications were studied by Spearman correlations or partial correlations analysis. Receiver operating curve (ROC) analysis was used to evaluate the ability of uCA-2 and uKIM-1 to predict urinary stones complications. Area under the curve (AUC) was derived from receiver operating curve (ROC) analysis. Significance was considered at P<0.05.

Results

Among the 160 participants in the present study, including 103 (64.37%) were males and 57 (35.63%) females. 118 patients with urinary stones were classified into urinary stones group and 42 healthy subjects were classified into healthy control group. All the 118 urinary stones patients, In terms of detailed calculi location, 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 (5-32mm). Total mean age of all participants was 41.93±8.33 years (20-68 years), the demographic and clinical characteristics of patients and healthy subjects are reported in **Table 1**. There were no significant differences between patients and healthy controls in age, gender, BMI, sCr, sBUN, and sUA.

Table 1 also shows nonparametric univariate analysis of the relationship of pretreatment uCA-2 and uKim-1 between the two groups. Participants of urinary stones group and healthy control group did not significantly differ in pretreatment uKim-1 level. Moreover, the mean pretreatment uCA-2 level was significantly higher in patients with urinary stones group than healthy controls group (**Fig. 1**).

The prevalence of stones complications of urinary stones group had 40.67% hematuria, 24.57% pain and 35.59% hydronephrosis. Almost 41.52% patients were detected absent stone complications. Stones complications was associated with a high uCA-2 level excretion, whereas no association was observed with uKim-1 level excretion (**Fig. 2**). As for hematuria complications the uCA-2 level was significantly

higher than without hematuria in urinary stones 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 $P=0.000$]. As for pain complications the uCA-2 level was also significantly higher than without pain in urinary stones group and healthy subjects group [mean 37.84 ± 32.47 ng/mL vs (11.92 ± 13.69 or 8.49 ± 3.86 ng/mL), respectively, each $P=0.000$]. To be interesting, patients with pain complications were ureteral stone (76.67%) and had smaller calculi size (8.5 ± 2.87 mm). As for hydronephrosis complications the uCA-2 level was also significantly higher than without hydronephrosis in urinary stones group and healthy subjects group [mean 22.54 ± 23.21 ng/mL vs (15.95 ± 22.35 vs 8.49 ± 3.86 ng/mL), respectively, each $P=0.000$]. The non-significantly higher uKIM-1 level excretion was found in hematuria, pain and hydronephrosis complications among the other two groups.

The uCA-2 level was positive correlation with urinary stones complications ($R=0.379$, $P=0.000$), especially pain complications ($R=0.524$, $P=0.000$) and hematuria complications ($R=0.374$, $P=0.000$). Spearman correlations or partial correlation analysis showed that there was no statistical correlation between uKIM-1 level and urinary stones complications.

The predictive value of uCA-2 and uKIM-1 levels to predict urinary stones complications was analyzed using a ROC curve (**Fig. 3**). ROC analysis of uCA-2 yielded an area under the curve (AUC) of 0.786 for total complications, 0.720 for hematuria, 0.852 for pain and 0.683 for hydronephrosis (respectively, each $P=0.000$); There were no significant correlation in ROC analysis of uKIM-1 yielded for any complications. These values between uCA-2 and uKIM-1 predicted urinary stones complications were significantly different. Table 2 shows the sensitivity and specificity of uCA-2 level to predict urinary stones complications. The optimal cut-off value of the uCA-2 level was chosen based on the maximum value of the Jordan index, and the indicated that uCA-2 level was a reliable predictor. Furthermore, we determined that a uCA-2 level threshold of 10.94 ng/mL had 83.67% sensitivity and 68.12% specificity for predicting urinary stones complications.

Discussion

Our prospective study included 118 patients with urinary stone and 42 healthy individuals. Substantial evidence indicated that patients with urinary stone exhibited increased uCA-2 level was found in urine by ELISA. The key finding in this study is that an significant difference in uCA-2 excretion by ELISA analysis could discriminate patients with urinary stones from healthy controls. If our results are supported by many prospective validation studies, such a test might be clinically beneficial in the surveillance of high-risk patients, such as patients with metabolic syndrome¹⁻⁴, inflammatory bowel disease¹⁸ and accepted bariatric surgery¹⁹ et al. High risk of stone recurrence patients may also reap the benefits of our results.

The small, hard mineral deposits that characterize urinary stone can cause severe pain, hematuria, obstruction or (and) hydronephrosis. The diagnosis of urinary stone depends on imaging examination and lacks effective biomarker, which means it is difficult to predict the risk recurrence of urinary stone. The AUA guideline has proposed that millions of patients newly diagnosed with urinary stone could be

advised to a screening evaluation consisting to reduce the risk of stone recurrence²⁰. To achieve this goal, more advanced diagnostic approaches have to be developed and applied to earlier detection of urinary stone. The available clinical data today support the conclusion that leads to a decreased risk of stones recurrence of asymptomatic patients, because of an early dietary or drug intervention^{21,22}.

To improve understanding on the significance of the excessive production of uCA-2 in patients with urinary stone, we evaluated data from our prospective database on patients with urinary stone and assigned uCA-2 level to patients with or without stone-related complications according to appropriate symptoms and signs. Our data showed that uCA-2 were significantly increased excretion in patients with urinary stone develop one or more complications. All 3 complications showed a high level of concordance and pain complication demonstrated superiority over the others with regard to predicting uCA-2 excretion.

To trace the phenomenon of higher uCA-2 level in patients with urinary stone develop one or more complications, our goal in this study was to describe the unclear effect of stone formation. The results of uCA-2 excretion and stone complications interactions had emphasized potential factors to pathophysiological mechanism, such as crystal-membrane interaction, microbial-stone interaction, or other possible interactions. The data show that the non-significantly higher uKIM-1 level excretion was found in hydronephrosis complications among the other two groups. Our results are seemingly at variance with a previous study evaluated that uKIM-1 as a biomarkers of acute renal injury was significantly higher in stone patients with hydronephrosis compared to without hydronephrosis²³. The difference results may be explained by the variation of participants inclusion criteria.

The excretion of uCA-2 by the bacteria in patients with urinary stone is also a potentially important factor. Twenty bacterial genera have significant differences in relative abundance between urinary stone patients and healthy subjects. These findings may provide new and non-invasive potential biomarkers for the diagnosis of kidney stone²⁴. *Escherichia coli* is one of most commonly detected pathogenic microorganisms attached to renal stone²⁵, which also actively secrete PH-dependent CA-2²⁶. Both CA and urease in bacteria play a synergic role in promoting CaCO_3 precipitation²⁷. Microbial urease is a Ni-containing enzyme found in various microorganisms that hydrolyzes urea to NH_4^+ and CO_2 , whereas carbonic anhydrase transforms CO_2 into HCO_3^- . In *Bacillus megaterium*, calcite precipitation is driven by the coupled activity of both urease and carbonic anhydrase enzymes. The precipitation of CaCO_3 , which predominantly consists of calcite crystals, by microbial carbonic anhydrase has been documented²⁸. CA-2 may decrease the stone inhibitory protein through its potential effect on the g-glutamyl carboxylase reaction by providing CO_2 . CA-2 may also supply CO_2 to g-glutamyl carboxylase that catalyzes glutamate residue carboxylation in some proteins, thereby enhancing their biological activity. 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 osteocalcin²⁹. The relationship between CA-2 and other stone-promoting or -inhibiting factor will be the focus of our study in the future.

Although we have a larger number of samples, the overall sample size is still small. The study did not follow up healthy participants with a 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 is difficult to evaluate in urine because the 24 h urine biochemical analysis was not measured.

Conclusion

Excessive CA-2 excretion in urine is a major risk factor for urinary stone. Our findings suggested that urinary CA-2 may be used as an unappreciated biomarker for the diagnosis renal stone in patients and to predict its complications.

Declarations

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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

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Tables

Table 1. Participants and event characteristics

Variables	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 NO. (%)]	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.			
# by Mann-Whitney U test			

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

Variables	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%

Figures

Figure 1

uCA-2 and uKIM level detected in patients with urinary stones and healthy subjects by ELISA. * $P \leq 0.05$ as evaluated using Mann-Whitney U test.

Figure 2

uCA-2 and uKIM level detected in subset of patients with stones complications and healthy subjects by ELISA. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ as evaluated using Kruskal-Wallis H test.

Figure 3

Area under the curve values for receiver operator characteristic (ROC) curves for uCA-2 and uKIM level predict stone complications. a Summary stone complications of ROC curve. b ROC curve for uCA-2 and uKIM in hematuria. c ROC curve for uCA-2 and uKIM in pain. d ROC curve for uCA-2 and uKIM in hydronephrosis.