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Original Article

Clinical value of TRPV5 combined with OPN in the diagnosis of urinary calculi

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Abstract: In order to provide a reliable theoretical basis for the diagnosis and prevention of urinary calculi, this study investigated the clinical significance of the expression of transient receptor potential cation channels, vanilloid subfamily member 5 (TRPV5) and osteopontin (OPN) in calcium-contained renal calculi. Renal cortex specimens were collected from 44 patients with calcium-contained renal calculi and 40 normal subjects without calculi who were admitted to our hospital from February 2018 to December 2019. The morphological differences as well as the differences in the mRNA and protein levels of TRPV5 and OPN were compared between the two groups by immunohistochemistry, Western blot and fluorescent quantitative polymerase chain reaction. Data were analyzed by ROC curve to facilitate the diagnosis of urinary calculi. We found that the mRNA and protein levels of TRPV5 in renal tissue in the observation group were significantly lower than the control group ($P < 0.05$), whereas the mRNA and protein levels of OPN were significantly higher in the observation group ($P < 0.05$). The area under the curve (AUC) for the expression levels of TRPV5 and OPN proteins in renal tissue in the diagnosis of urinary calculi were 0.816 and 0.748, respectively. The optimal cutoff values of TRPV5 and OPN by the maximum Jordan index were 0.45 (sensitivity 88.20%, specificity 80.40%) and 0.90 (sensitivity 79.20%, specificity 81.10%), respectively. The AUC area of the combined diagnosis was 0.893, which was superior to individual indexes. These data suggested that TRPV5 combined with OPN has high sensitivity and specificity in the diagnosis of urinary calculi, and has a high clinical value.

Keywords: TRPV5, OPN, urinary calculi, diagnosis

Introduction

Urinary calculi are solid particles in the urinary system, with a global incidence of about 5%-15%. About 1.2%-6% of Chinese people are plagued by calculi [1], and its incidence is rising in recent years. Urinary calculi are the main cause of urinary tract obstruction and infection [2, 3]. At present, the clinical treatment of calculi includes extracorporeal lithotripsy, open surgical lithotripsy and nephrolithotripsy, ureteroscopy, etc. combined with ultrasound, laser, barometric ballistic and other techniques. However, a high rate of postoperative recurrence is still found clinically regardless of the procedure [4, 5]. Therefore, intensive study and clarification of the pathogenesis of urinary calculi are of great significance for the early diagnosis and prevention of urinary calculi.

Transient receptor potential cation channels, vanilloid subfamily member 5 (TRPV5), a cell membrane protein of urinary calcium reabsorption, functions in the regulation of urinary calcium levels and maintenance of calcium balance in the body. It has been found that decreased activity or down-regulated expression of TRPV5 can cause increased urinary calcium levels, and play a crucial role in the occurrence and progression of calcium-contained renal calculi [6]. Osteopontin (OPN) is a member of the secreted phosphorylated multifunctional glycoprotein family, which is widely present in a variety of human tissues. Studies have confirmed that OPN combined with calcium ions has an inhibitory effect on the formation of urinary calculi, and the involvement of OPN in cell migration has the capability of inhibiting the formation of calcium oxalate crystals [7]. The latest findings showed that the expression of OPN was significantly down-regulated in the renal tissue of rats with calculi, and it was considered that OPN has certain diagnostic value for calculi [8]. To date, there are no relevant reports of

Table 1. Comparison of general data between the two groups

Group	n	Gender		Age (year)
		Male	Female	
Control group	40	25	15	44.20±10.02
Observation group	44	29	15	46.05±11.10
t/χ^2		0.106		0.799
P		0.745		0.427

TRPV5 and OPN in human urinary calculi in China.

In this study, renal cortex specimens were collected from 44 patients with calcium-contained renal calculi and 40 healthy people without calculi who were admitted to our hospital from February 2018 to December 2019. In order to provide a reliable theoretical basis for the diagnosis and prevention of urinary calculi, we investigated the clinical significance of the expression of transient receptor potential cation channels, vanilloid subfamily member 5 (TRPV5) and osteopontin (OPN) in calcium-contained renal calculi. This study provided a reliable theoretical basis for the diagnosis and prevention of urinary calculi.

Materials and methods

General data

A total of 44 patients with calcium-contained renal calculi who were admitted to our hospital from February 2018 to December 2019 were selected as the observation group. Urinalysis results (**Table 1**) showed that the urinary calcium level was > 300 mg/24 h. Postoperative analysis results of stone composition showed calcium-contained calculi. A small amount of renal cortex specimen was collected during surgery. Forty patients with normal urinary calcium levels who underwent related surgery for other diseases at the same time were selected as the control group, and a small amount of renal cortex specimens were also collected during surgery. All specimens were sampled, placed in EP tubes and stored at -80°C until use. In the observation group, there were 24 male patients and 20 female patients with an average age of 42.34±10.87 and an average disease course of 1.66±1.25 years. In the control group, there were 19 male patients and 21 female patients with an average age of 42.45±11.02 and an average disease course of 1.57±1.26 years. No statistical significance was found in terms of gender, age, disease course and other general information when comparing the two groups (P > 0.05). All patients gave informed consent to participate in this study.

Main materials

In the current study, the main materials are shown in **Table 2**, and all other analytical purified reagents were purchased domestically.

Preparation of tissue section

Specimen tissue sections: 2.5 mm × 2.5 mm renal tissue was cut and placed in 4% paraformaldehyde solution for 24 h; they were soaked in 50%, 70%, 80%, and 95% ethanol solutions for 12 h, in pure alcohol for 2 h, and then put in xylene plus ethanol for 0.5 h; renal tissue specimens were transferred to xylene plus paraffin (65°C, 0.5 h) followed by level I, II, and III paraffin for penetration, with the duration of 60 min at each level; later, these tissues were embedded and then cut into slices (4-5 μm).

Immunohistochemistry

Specimen tissues were soaked in xylene solution for 10 min followed by anhydrous ethanol, 95% and 75% ethanol solutions for 5 min, respectively; sections were soaked in 0.01 mol/L citric acid at 100°C for 60 s, heated in a microwave oven for about 20 min, and rinsed with phosphate buffer solution (PBS) three times for 15 min in total; primary antibodies against TRPV5 and OPN were incubated with tissue sections overnight at 4°C, and rinsed with PBS five times for 15 min in total; secondary antibody was incubated with tissue at 37°C for 60 min, rinsed with PBS, developed with diaminobenzidine (DAB) for 180 s at room temperature, protected from the light, stained with hematoxylin for 5 min, and then rapidly placed in hydrochloric acid alcohol solution to soak for 0.5 h; they were rinsed with tap water, soaked in 80%, 90%, anhydrous ethanol for 10 min, permeabilized with xylene for 5 min, mounted with neutral resin, and observed and photographed under an optical microscope.

Real-time polymerase chain reaction

Thirty mg of each specimen was weighed and quickly ground by adding a small amount of liquid nitrogen, and total RNA was extracted after

Table 2. Main materials used in the study and their respective manufacturers

Material	Manufacturer
TRPV5 antibody	Cell Signaling Technology
OPN antibody	Cell Signaling Technology
β-actin antibody	Abcam
BCA protein quantitation kit	CoWin Biosciences
Goat anti-rabbit secondary antibody	CoWin Biosciences
Goat anti-mouse secondary antibody	CoWin Biosciences
DAB kit	Aithen Biology
Trizol kit for the extraction of RNA	Beijing Tianmo Sci&Tech Development Co., Ltd.
Reverse transcription kit	Takara
Real-time PCR kit	Takara
TRPV5 primer	Sangon Biotech
OPN primer	Sangon Biotech

homogenization to determine OD values using a spectrophotometer. The cDNA was synthesized according to standard procedures at 42°C for 60 min, 70°C for 10 min, 4°C for 10 min, and stored at -80°C. Two ng of cDNA was used for real-time fluorescent quantitative PCR reaction at 94°C for 5 min, 94°C for 30 s, 58°C for 40 s, and 72°C for 20 s for 35 cycles; and 72°C for 5 min. Primer pairs and product length for each gene are as following: GAPDH forward CCATGGGGAAGGTGAAGGTC, GAPDH reverse AGTGATGGCATGGACTGTGG, 548 bp; TRPV5 forward GATAAACGAGAGGCTCGCCA, TRPV5 reverse AGGCACCAACCCTGAAGATG, 340 bp; OPN forward AGCAGAATCTCCTAGCCCCA, OPN reverse ACGGCTGTCCCAATCAGAAG, 509 bp. Data analysis results were corrected for the glyceraldehyde-phosphate dehydrogenase (GAPDH), the relative expression levels of TRPV5 and OPN mRNA were calculated using the 2-ΔΔCt method, and independent experiments were performed in triplicates.

Western blot

Fifty mg of specimen was used, with removed fat and connective tissue and was dissected into fragments on ice; later, 1 ml of lysis buffer was added for resuspension, subjected to ultrasonication, and centrifuged at 4°C (12,000 r/min, 10 min) to determine the total concentration of protein. Thirty μg of each sample was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Later, the resulting protein was transferred to a PVDF membrane by the semi-dry method, and blocked with 5% non-fat milk for 60 min at room temperature. Primary antibodies against TRPV5 and OPN were incubated with tissue at 4°C overnight, with GAPDH as an internal reference. Membranes were rinsed with tris-buffered saline tween-20 (TBST), and secondary antibodies were incubated for 60 min at room temperature. The expression level of protein was analyzed using Quanity-One software.

Statistical analysis

SPSS 21.0 statistical software was used to process the study data. The quantitative data were expressed as $\bar{x} \pm sd$, and analyzed by *t*-test; the qualitative data were expressed as the frequency or percentage, and analyzed by chi-square test; the clinical value of TRPV5 combined with OPN detection in the diagnosis of urinary calculi was analyzed by the ROC curve. $P < 0.05$ suggested a statistically significance.

Results

Immunohistochemical staining

OPN was expressed in the renal proximal tubule, distal convoluted tubule, Henle's loop, and collecting tubule in the two groups. In the control group, the amount of OPN was low, which showed weak expression on the surface of epithelial cells in the medullary descending branch and distal convoluted tubule. In the observation group, OPN showed strong expression in renal tissue, and significant expression in the medullary descending branch, proximal convoluted tubule, distal convoluted tubule and collecting tubule (**Figure 1**). In contrast, the

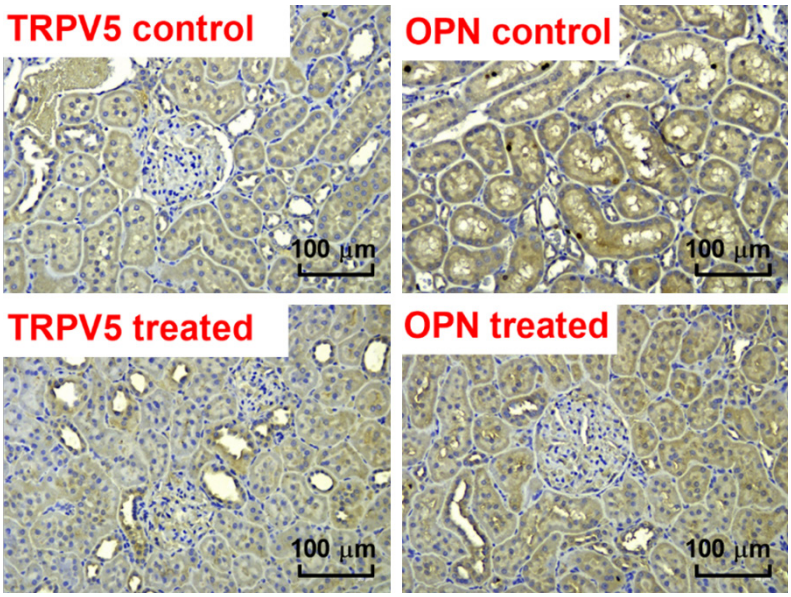

Figure 1. The expression of TRPV5 and OPN in renal tissues of the control and treated groups (DAB, × 40).

Table 3. Comparison of relative expression of TRPV5 and OPN mRNA in renal tissue between the two groups ($\bar{x} \pm s$)

Group	n	TRPV5	OPN
Control group	40	0.90±0.25	1.70±0.24
Observation group	44	0.46±0.05	3.25±0.60
t		11.430	15.260
P		< 0.001	< 0.001

Table 4. Comparison of the protein levels of TRPV5 and OPN in renal tissue between the two groups ($\bar{x} \pm s$)

Group	n	TRPV5	OPN
Control group	40	0.90±0.26	0.72±0.11
Observation group	44	0.32±0.05	1.10±0.32
t		14.510	7.134
P		< 0.001	< 0.001

expression level of TRPV5 protein in renal tissue of patients in the observation group was lower than the control group.

Comparison of the relative expression of TRPV5 and OPN mRNA in renal tissue between the two groups

The relative level of TRPV5 mRNA in renal tissue in the observation group was significantly lower than that in the control group ($P < 0.05$), whereas the level of OPN mRNA was significantly higher than that in the control group ($P < 0.05$) (**Table 3**).

Comparison of the expression levels of TR-PV5 and OPN protein in renal tissue between the two groups

The expression level of TRPV5 protein in renal tissue in the observation group was significantly lower than that in the control group ($P < 0.05$), whereas the expression level of OPN protein was significantly higher than that in the control group ($P < 0.05$) (**Table 4** and **Figure 2**).

Analysis of the expression levels of TRPV5 and OPN proteins in renal tissue for the diagnosis of urinary calculi by the ROC curve

The area under the curve (AUC) for the expression levels of TRPV5 and OPN proteins in renal tissue in the diagnosis of urinary calculi were 0.816 and 0.748, respectively. The optimal cutoff values of TRPV5 and OPN by the maximum Jordan index were 0.45 (sensitivity 88.20%, specificity 80.40%) and 0.90 (sensitivity 79.20%, specificity 81.10%), respectively. The AUC area of the combined diagnosis was 0.893, which was superior to individual indexes (**Figure 3** and **Table 5**).

Discussion

The main components of urinary calculi include struvite, calcium oxalate, calcium phosphate oxalate mixture and cystine calculi, most of which are calcium oxalate calculi, including calcium phosphate oxalate mixture [9, 10]. “Three highs and one low” (hypercalciuria, hypercalcemia, hyperoxaluria, and hypocitraturia) is primarily associated with urinary calcium-contained calculi, and abnormal calcium metabolism in the body, and is an important factor in the formation of calcium-contained calculi in the urinary tract [11]. Additionally, hypercalciuria is one of the important risk factors for the formation of calcium oxalate and calcium phos-

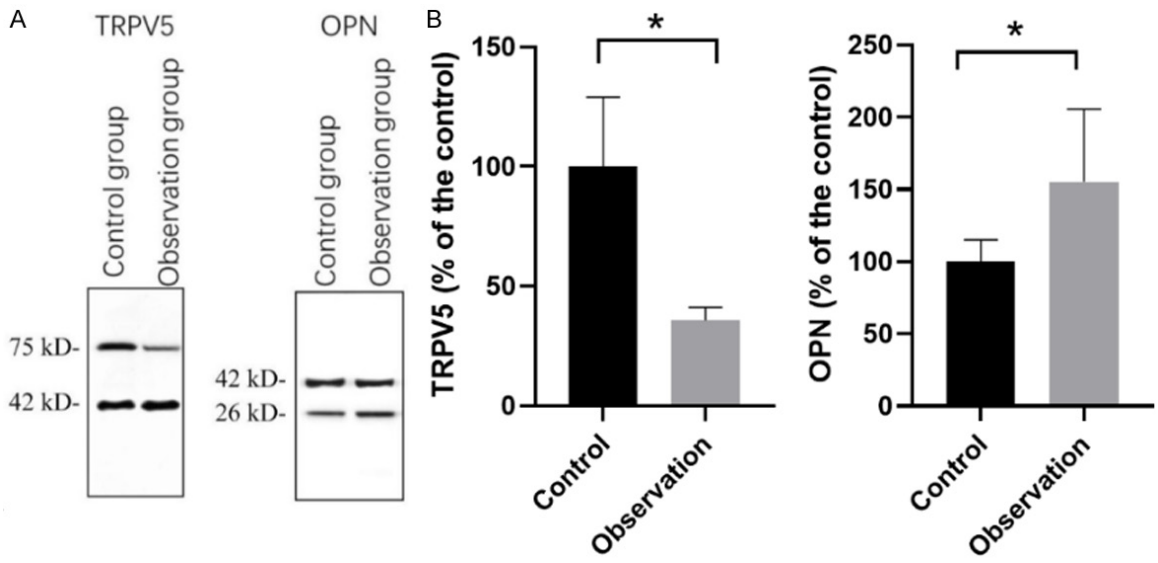

Figure 2. The relative protein levels of TRPV5 and OPN in the control and observation group. A. Representative western blot images of TRPV5 (75 kDa) and OPN (26 kDa). β-actin (42 kDa) was used as internal control. B. The relative protein levels of TRPV5 and OPN compared with the control group (n≥40). * indicates $P < 0.001$.

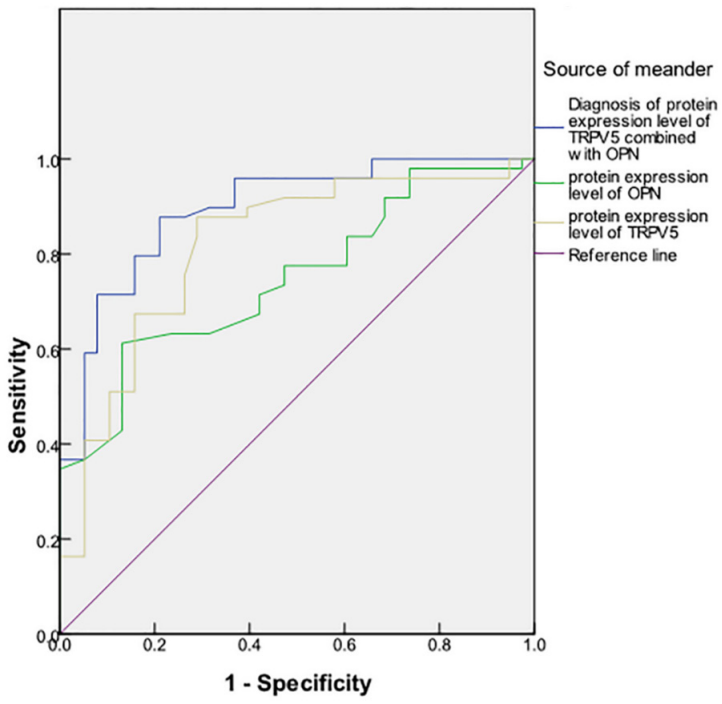

Figure 3. ROC curve analysis of the protein levels of TRPV5 and OPN in the renal tissues for the diagnosis of urinary calculi.

phate stones. The specific molecular mechanism of urinary calculi remains unclear. Therefore, the search for relevant diagnostic markers is an important method to effectively prevent the occurrence of calculi except for good dietary habits and regular physical examination.

OPN, procalcitonin, and Tamm-Horsfall protein have an inhibitory effect on stones and are present in large amounts in the urine. If these stone inhibitors are in abnormal amounts or in unbalanced proportions, it can lead to the formation of renal calculi [12]. OPN is a multifunctional phosphorylated glycoprotein that is widely expressed in the human and secreted by the epithelium on the surface of Henry's loop, proximal convoluted tubule and renal papillae. It has a high affinity for calcium oxalate crystals and serves as an inhibitor in the formation and aggregation of calcium oxalate crystals in urine [7, 13]. Studies have shown that OPN does not normally bind to αvβ3 integrin on the basement membrane surface of renal tubular epithelial cells [14], whereas in pathological conditions such as epithelial cell injury, it can disturb cell polarity and drive αvβ3 integrin to adhere to the inner surface of the renal tubular lumen along with the OPN binding site on the surface of cal-

Table 5. Analysis of the expression levels of TRPV5 and OPN proteins in renal tissue for the diagnosis of urinary calculi by the ROC curve

Parameters	AUC area	Cutoff value	Sensitivity (%)	Specificity (%)	*P* value	95% *CI*	
						Lower limit	Upper limit
Expression level of TRPV5 protein in renal tissue	0.816	0.45	88.20	80.40	< 0.001	0.723	0.908
Expression level of OPN protein in renal tissue	0.748	0.90	79.20	81.10	< 0.001	0.647	0.849
TRPV5+OPN detection	0.893	-	92.60	83.80	< 0.001	0.826	0.960

cium oxalate crystals, thereby prompting the formation and accumulation of calcium oxalate crystals to cause stone [15]. Meanwhile, the results of this study showed that the relative expression of OPN mRNA and protein in renal tissues of stone-forming patients were significantly higher than that of non-stone forming patients ($P < 0.05$).

The study indicated that, mediated by the TRPV5, calcium ions can enter the cytoplasm from the apical membrane of the epithelial cells of the distal tubules and collecting tubules of the kidney, diffuse to the extracellular basement membrane in association with relevant carrier proteins, and enter the blood circulation from the basement membrane via NCX1 and PMCA1b transport [16, 17]. The active calcium reabsorption in the distal tubules and collecting tubules of the kidneys of TRPV5-deficient rats was found to be significantly reduced, while the urinary calcium excretion was six times higher than that of the control group [18, 19]. There was another study indicating that the expression level of TRPV5 protein in renal tissue of stone-forming rats was significantly lower than that of non-stone-forming rats [20]. In the present study, it was found by immunohistochemistry, western blot and qPCR that the expression level of TRPV5 protein in renal tissue in the observation group was lower than that in the control group ($P < 0.05$), which suggested that the abnormal expression of TRPV5 was closely related to the formation of stones, and TRPV5 has the potential to serve as a marker for the early diagnosis of stones. Furthermore, our study demonstrated by the ROC curve and Jordan index that TRPV5 combined with OPN detection has high sensitivity and specificity in the diagnosis of urinary calculi, which is of great clinical significance.

Our study still had certain limitations: (1) the sample size was small, and the expression of TRPV5 and OPN varied among populations, so the results may have a certain bias; (2) the study was a single center, and the precision of the quantitative index in this study was lacking, which may result in false positives or false negatives. Therefore, it is still necessary to expand the sample size and combine with DNA sequencing to accurately analyze the correlation between the expression of TRPV5 and OPN and stones, so as to provide a scientific basis for the clinical diagnosis of stones by the expression levels of TRPV5 and OPN. Moreover, the sampling method used in this study uses invasive procedures causing increased patient discomfort, increased wound care, and longer healing period, which may limit the clinical application of this study.

In conclusion, the relative expression of TRPV5 in renal tissue of patients with urinary calculi is significantly down-regulated, whereas the relative expression of OPN is up-regulated. TRPV5 combined with OPN has high sensitivity and specificity in the diagnosis of urinary calculi, and as such has a high clinical value.

Ethic statement

This study was approved by the Medical Ethical Committee of Hengshui People's Hospital and it is in line with the Declaration of Helsinki.

Disclosure of conflict of interest

None.

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