J Huazhong Univ Sci Technol［Med Sci］
DOI 10.1007/s11596-011-0681-z 31(6):807-814,2011

Decreased Expression of Vitamin K Epoxide Reductase Complex Subunit 1 in Kidney of Patients with Calcium Oxalate Urolithiasis*

Bo HU (胡 波), Tao WANG (王 涛)#, Zhuo LIU (刘 卓), Xiaolin GUO (郭小林), Jun YANG (杨 俊), Jihong LIU (刘继红)#, Shaogang WANG (王少刚), Zhangqun YE (叶章群)
Department of Urology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

Summary: Urinary prothrombin fragment 1 (UPTF1) is a potent inhibitor of urinary stone formation. UPTF1 exerts such inhibitory effect by effective γ-carboxylation in which vitamin K epoxide reductase complex subunit 1 (VKORC1), the rate-limiting enzyme, is involved. This study examined the correlation between VKORC1 expression and calcium oxalate urolithiasis. The renal cortex samples were obtained from patients undergoing nephrectomy and then divided into 3 groups: urolithiasis group, control group A [hydronephrosis-without-stone (HWS) group], control group B (normal control group). The localization and expression of VKORC1 in renal tissues were determined by using immunohistochemistry, immunofluorescence microscopy, Western blotting and SYBR Green Ⅰ real-time reverse-transcription PCR. The rapid amplification of cDNA ends (RACE) were conducted to obtain the 3'- and 5'-untranslated region (UTR) of VKORC1. The results showed that VKORC1 was located in the cytoplasm of renal tubular epithelial cells. The expression of VKORC1 in the urolithiasis group was significantly lower than that in the other two control groups (P<0.05). Moreover, the 3'- and 5'-UTR sequence of the VKORC1 gene was successfully cloned. No insertion or deletion was found in the 3'- and 5'-UTR. However, a 171-bp new base sequence was discovered in the upstream of 5'-UTR end in the urolithiasis group. It was concluded that the decreased expression of VKORC1 may contribute to the development of calcium oxalate urolithiasis in the kidney.
Key words: calcium oxalate urolithiasis; vitamin K epoxide reductase complex subunit 1; vitamin K circle; urinary prothrombin fragment 1

Urolithiasis is a common disease with a lifetime prevalence of 10% in men and 5% in women[1]. About 80% of urinary stones are composed of calcium oxalate and variable amounts of calcium phosphate[2]. The physiochemical mechanisms of stone formation via precipitation, growth, aggregation, and concretion of various lithogenic salts in urine are still in dispute. Although numerous studies reported that some macromolecule proteins such as nephrocalcin, urinary osteopontin and urinary prothrombin fragment 1 (UPTF1) are closely related to the calcium oxalate crystallization[3–6], the mechanisms of calcium oxalate urolithiasis remain to be fully understood.

The predominant protein associated with calcium oxalate monohydrate (COM) is UPTF1. It is a potent inhibitor of calcium oxalate (CaOx) crystal growth and aggregation under inorganic conditions or in undiluted human urine *in vitro*, and is expressed in the most lithogenic regions of the nephron[7]. The inhibitory properties of UPTF1 are undoubtedly conferred by a domain located near its N-terminus, which contains 10 residues of γ-carboxyglutamic acid (Gla), through which UPTF1 can bind to the CaOx crystal surface[8, 9]. Gla is the only kind of amino acid that possesses such high affinity to calcium. And it is postulated that Gla plays an important role in calcium oxalate crystallization. A specific cytoplasmic posttranslational modification of glutamic acid to Gla is indispensable for the biological activities of UPTF1[10]. In these processes, vitamin K reduction is involved, and carbon dioxide and oxygen act as cofactors to Gla side chains, and vitamin K epoxide is generated as a by-product. Due to the limited availability of vitamin K in tissues *in vivo*, the vitamin K epoxide must be rapidly reduced again to vitamin K to sustain further γ-carboxylation reactions. Vitamin K epoxide reductase complex subunit 1 (VKORC1) is the only enzyme that has been shown to reduce the vitamin K epoxide and is the rate-limiting enzyme of vitamin K recycling[11]. It is also the rate-limiting enzyme for γ-carboxylation of the vitamin K dependent protein. Recently, the function of VKORC1 has become a subject of interest. In this study, the role of VKORC1 in calcium oxalate urolithiasis (COU) was examined to explore the possible mechanism of urolithiasis.

Bo HU, E-mail: hale1975@sohu.com
#Corresponding author, Tao WANG, E-mail: twang@tjh.tjmu.edu.cn; Jihong LIU, E-mail: jhliu@tjh.tjmu.edu.cn
*This project was supported by a grant from the National Natural Science Foundation of China (No. 30901482).

1 MATERIALS AND METHODS

1.1 Patients and Samples

Renal cortex specimens and clinical data were obtained from 155 patients who underwent nephrectomy for severe hydronephrosis (parenchymal thickness <1 cm on ultrasonography) with a non-functioning kidney or kidney cancer from November 2004 to April 2010 in Tongji Hospital, Wuhan, China. The patients were divided into three groups: urolithiasis group, control group A [hydronephrosis-without-stone (HWS) group] and control group B (normal control group). In urolithiasis group, patients with any systemic disorder, such as primary hyperparathyroidism, sarcoidosis, hyperthyroidism, glucocorticoid excess, renal tubular acidosis, hypercalciuria or hyperoxaluric states were excluded; by using infrared spectrophotometry after operation, the 65 patients were diagnosed as having COU (stones containing 70% calcium oxalate and more); of these 65 patients, there were 40 males and 25 females with their median age of 42 years (range 20–74 years). In control group A, 7 patients (3 males, 4 females) with congenital ureteropelvic junction obstruction (UPJO) and 16 patients (10 males, 6 females) with tuberculosis of kidney were enrolled; the median age of the patients was 45 years (range 29–54 years). In control group B, 67 samples of normal renal cortexes adjacent to the tumor were harvested; there were 37 males and 30 females with their median age of 55 years (range 21–77 years). Experimental protocols were complied with the guidelines of National Institutes of Health for human specimens and approved by the Ethics Committee of Tongji Hospital, China.

All samples were stored at –80°C until use. The renal cortex specimens were treated with RNAlater (Qiagen, Germany) according to the manufacturer's recommendations before being frozen for RNA extraction.

1.2 Immunohistochemistry and Immunofluorescence Microscopy

Specimens were fixed in 10% (v/v) aqueous formalin and embedded in paraffin. The embedded tissues were sectioned 4 mm in thickness and dried at 60°C for 1 h. The sections were deparaffinized, rehydrated, and microwaved for 5 min in 10 mmol/L citrate buffer (pH 6.0) twice. After cooling, the sections were washed in distilled H_2O for 20 min. Immunohistochemical analyses were performed by streptavidin-perosidase method with the primary antibody against the human VKORC1 (Santa Cruz Biotechnology, USA) used. In this method, a biotinylated secondary antibody is detected with horse radish peroxidase-conjugated streptavidin, and peroxidase activity with 3, 39-diamino-benzidine tetrahydrochloride. The tissues were counterstained with hematoxylin. Controls were performed in the same manner except that the primary antibody was replaced by PBS.

In immunofluorescence experiments, the sections were incubated with 3% H_2O_2 for minimizing endogenous peroxidase. In order to enhance antigen retrieval, the sections were bathed in citrate buffer solution (0.01 M, pH 6.0) and then heated in microwave oven for 10 min. After blocking with 5% normal goat serum, the sections were incubated overnight at 4°C with appropriate dilutions of the primary specific antibody, goat VKORC1 antibody (1:100, Santa Cruz Biotechnology Inc, USA). Sections were then washed with PBS 3 times. After reacted with fluorescein isothiocyanate (FITC) conjugated donkey anti-goat IgG (1:100, Santa Cruz Biotechnology Inc, USA) at 37°C for 40 min, the sections were washed with PBS 3 times and stained with DAPI (1:1000, Sigma-Aldrich Inc, USA). Finally, they were mounted in 50 g/L glycerin. As a negative control, PBS was used instead of primary antibody. Digital images were acquired using a confocal laser scanning microscope (Olympus FV500, Japan). The specimens were excited with a laser beam at a wavelength of 488 nm (FITC) and 360 nm (DAPI) respectively. Fluorescence micrographs were captured with the same exposure time using a Nikon camera and analyzed using image-pro plus 6.0 image processing software for the FITC fluorescent intensity.

1.3 Western Blotting

The tissue samples were lysed by protein extraction buffer, and the protein concentrations were measured using Bio-Rad protein assay reagent (Bio-Rad Laboratories, USA) following the manufacturer's instructions. Equal amounts of protein were subjected to SDS-PAGE. They were then transferred onto a polyvinylidene difluoride membrane (Amresco, USA) which was afterwards blocked with 5% (w/v) skim milk powder in TBS overnight at 4°C. The blocked membrane was incubated for 90 min with the primary goat monoclonal antibody against human VKORC1 (Santa Cruz Biotechnology, USA) at room temperature, then washed with TBS/T and incubated for 30 min with horseradish peroxidase conjugated mouse anti-goat IgG antibody (Santa Cruz Biotechnology, USA) and visualized by ECL (Sigma, USA). The expression of GAPDH was detected as internal control, and the results were analyzed by software.

1.4 RNA Extraction

The frozen tissues were weighed and ground to a fine powder using a cold pestle and mortar. Total RNAs were extracted using Tri-Reagent according to the manufacturer's instructions. Briefly, 1 mL of Tri-Reagent was added to 100 mg of the powdered tissues. The samples were thoroughly mixed by repeated pipetting at room temperature for about 10 min and then supplemented with 0.1 mL of 1-bromo-3-chloropropane per mL of Tri-Reagent, after which, they were shaken vigorously for 15 s and allowed to stand at room temperature for 20 min. This was followed by centrifugation at 12 000 g for 15 min at 4°C in a Beckman L8-70m Ultracentrifuge using SW-28 rotor, which separated the mixture into a lower red phase, an interphase and a colourless upper aqueous phase. RNA remaining exclusively in the aqueous phase, which usually comprised about 60% of the volume of Tri-Reagent, was used. The aqueous phase was transferred to a fresh tube and the RNA was precipitated by mixing it with isopropanol (0.5 mL of isopropanol per mL of Tri-Reagent). The samples were stored at room temperature for 30 min and centrifuged at 12 000 g for 10 min at 4°C. The supernatant was carefully removed and the RNA pellet was washed once with 75% ethanol (1 mL of 75% ethanol per mL of Tri-Reagent) by vortexing and subsequent centrifugation at 7500 g for 5 min at 4°C. The RNA pellet was air-dried for 5 min and solubilized in 40 μL of DEPC-treated

water. Its purity, concentration and integrity were determined by the spectrophotometric assay and agarose gel electrophoresis.

Total RNA was extracted from frozen renal cortex samples of COU patients (n= 65), HWS patients (n=23) and renal tumor patients (n=67) by using TRI-reagent (Invitrogen, USA) and using a DNA-free kit (Takara, Japan) to remove contaminated DNA.

1.5 SYBR Green Ⅰ Real-time PCR

The PCR of VKORC1 was performed by using the SYBR Premix EX Taq kit (Takara, Japan) with appropriate primers. The total RNA was then reverse-transcribed to cDNA in 20 μL at 37°C for 15 min and at 85°C for 5 s by using PrimeScript RT reagent kit (Takara, Japan). cDNA equivalent to 100 ng total RNA was used to perform real-time PCR. Every reaction was performed in triplicate using 2 μL cDNA, 10 μL SYBR Premix EX Taq mixture, 0.8 μL primers in a final volume of 20 μL. Samples were amplified using the following profile: an incubation at 95°C for 30 s to pre-denature the DNA, 40 cycles of denaturation at 95°C for 5 s followed by annealing and extension at 55°C for 20 s. Relative gene expression was calculated by using the comparative CT method.

To correct the differences in RNA quality and quantity, Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene and a no template sample as a negative control. GAPDH levels were used to normalize target gene mRNA values in respective cDNA samples. The relative quantitation of VKORC1 gene was defined by the $2^{-\Delta\Delta Ct}$ method as described previously[12]. This method was based on the assumption that the amplification efficiencies of the target (VKORC1) and the reference (GAPDH) genes were approximately equal. To verify this condition, we examined ΔCt ($Ct_{VKORC1}-Ct_{GAPDH}$) according to template dilution. A cDNA (sample No.1) preparation was diluted over a 1000-fold range. For each dilution sample, amplifications were performed using primers and SYBR Green Ⅰ for VKORC1 and GAPDH. Therefore, a polt of the log cDNA dilution versus ΔC_T was made. The log cDNA copy number of a target gene that was automatically normalized to the 6-carboxy-X-rhodamine (ROX) internal passive reference (logΔRn) was plotted as a function of the cycle number. The cycle number at which the signal crossed the midlinear portion of the logΔRn cycle function was defined as the cycle threshold (Ct). Because the input cDNA copy number and Ct are inversely related, a sample that contains more copies of the template will have a data line that crosses the Ct at an earlier cycle compared with one containing fewer copies of the template. By using the No.1 sample as the calibrator, the data of the other groups are presented as the fold change in gene expression normalized to an endogenous reference gene and relative to the No.1 sample. Relative quantification of the mRNA expression levels of the target gene was $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct=(Ct_{VKORC1}-Ct_{GAPDH})_{others}-(Ct_{VKORC1}-Ct_{GAPDH})_{calibrator}$.

1.6 3'-Rapid Amplification of cDNA Ends (RACE) of VKORC1

cDNA fragment was synthesized by reversely transcribing 1 μg total RNA with 3'-Full Race Core Set Ver. 2.0 (TaKaRa, Japan). 3'-RACE primer 3P1 (5'-GGCGGAACCTGGAGATAATG-3') and nested primer 3P2 (5'-ATTGTTAGGTTGCCTGCGGACA-3') were designed and synthesized according to the known sequences of human VKORC1 gene in the GenBank. Primary 3'-RACE PCR was performed with 3P1 and 3' RACE outer primer (5'-TACCGTCGTTCCACTAGTGATTT-3') in a total volume of 50 μL containing cDNA fragment, and was pre-denatured at 94°C for 3 min, followed by 30 cycles of amplification (30 s denaturation at 94°C, 30 s annealing at 55°C, and 2 min extension at 72°C). The product of the primary PCR was used as a template for nested PCR performed with primer 3P2 and 3'-RACE inner primer (5'-CGCGGATCCTCCACTAGTGATTTCACTATAGG-3'). The product was purified and sequenced.

1.7 5'-RACE of VKORC1

After dephosphorylation, "removal of cap reaction" and a link with 5'-RACE adaptor, cDNA fragment was synthesized by reversely transcribing 1 μg total RNA with 5'-Full Race kit (TaKaRa, Japan). 5'-RACE primer 5P1 (5'-AGTGTGTAGAAGATGCAACCGA-3') and nested primer 5P2 (5'-TGGATTGATTGAGGATGCTGTC-3') were designed and synthesized according to the known sequences of human VKORC1 gene in the GenBank. Primary 5'-RACE PCR was performed with 5P1 and 5'-RACE outer primer (5'-CATGGCTACATGCTGACAGCCTA-3') in a total volume of 50 μL containing cDNA fragment, and was pre-denatured at 94 °C for 3 min, followed by 30 cycles of amplification (30 s denaturation at 94°C, 30 s annealing at 55°C, and 2 min extension at 72°C). The product of the primary PCR was used as a template for nested PCR performed with primer 5P2 and 5'-RACE inner primer (5'-CGCGGATCCACAGCCTACTGATGATCAGTCGATG-3'). The product was purified and sequenced.

Comparative and bioinformatic analyses of obtained VKORC1 gene nucleotide sequence were carried out online at http://www.ncbi.nlm.nih.gov. The nucleotide sequence was analyzed by using the Sequencher software, and sequence comparison was conducted through database searches using the BLAST program (http://www.ncbi.nlm.nih.gov).

1.8 Statistical Analysis

SPSS17.0 was used for statistical analysis, and ANOVA was performed to compare the mean values. A probability value of 95% (P<0.05) was used to determine significance.

2 RESULTS

2.1 Expression of VKORC1 Protein

Immunohistochemistry revealed that granular cytoplasmic staining of various intensities was confined mainly to the tubular epithelial cells (fig. 1) both in the urolithiasis and control groups. The green immunofluorescence of VKORC1 was seen in the cytoplasm of renal tubular epithelial cells not only in the urolithiasic group but also in the control groups (fig. 2). Both in the immunohistochemistry and immunofluorescence, no positive staining was observed in the samples in which the first antibody was omitted to serve as a negative control.

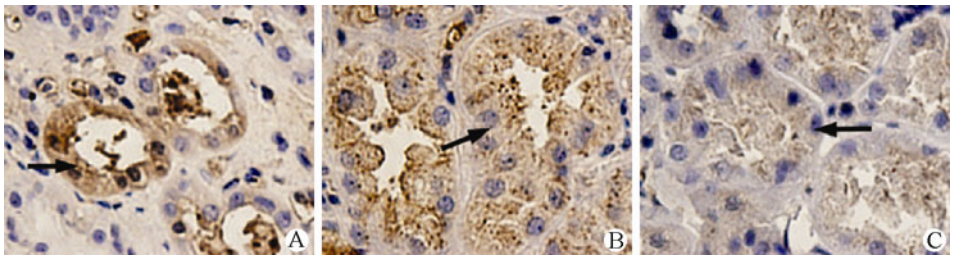

Fig.1 Immunostaining of VKORC1 in human kidney tissues (SP, ×400)
A: Control group A; B: Control group B; C: Urolithiasis group. The arrows indicate the tubular epithelial cells.

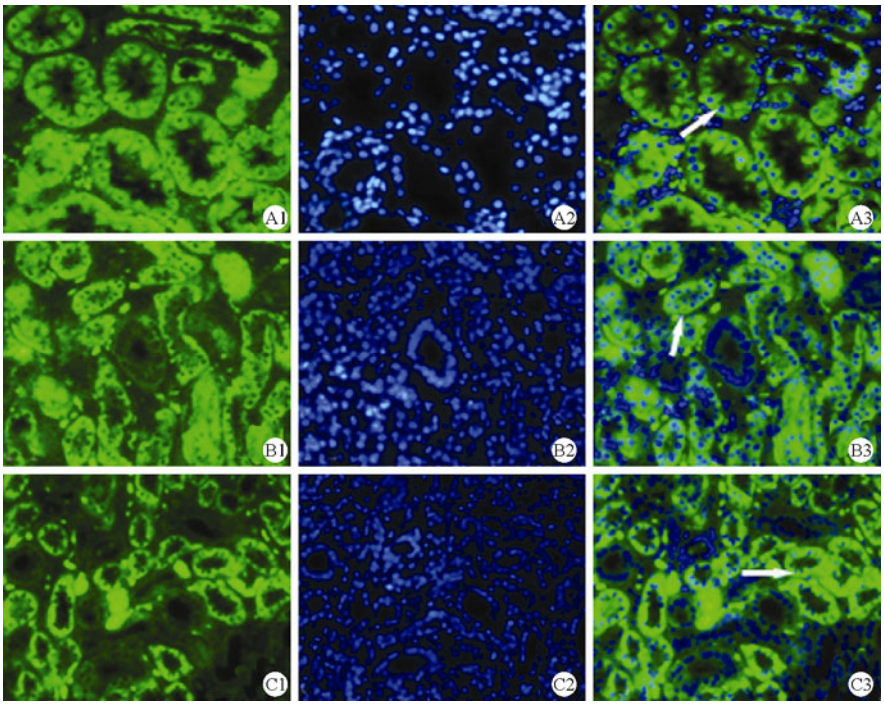

Fig. 2 Localization of VKORC1 in the renal section by immunolabeling (×200)
VKORC1 expression was visualized by labeling with fluorescein isothiocyanate (FITC) conjugated donkey anti-goat IgG (A1, B1, C1, *green*); cell nuclei were labeled using the DAPI labeling (A2, B2, C2, *blue*). The overlay of both images is shown in fig. 2A3, B3, C3. Arrows indicate the renal tubular epithelial cells.

Western blotting showed that the relative value of VKORC1 expression was 0.327±0.055 in the urolithiasis group, significantly lower than that in the other 2 control groups (control group A: 0.913±0.090, control group B: 0.983±0.117, $P<0.05$) (fig. 3). No significant difference in VKORC1 expression was found between the control groups ($P>0.05$).

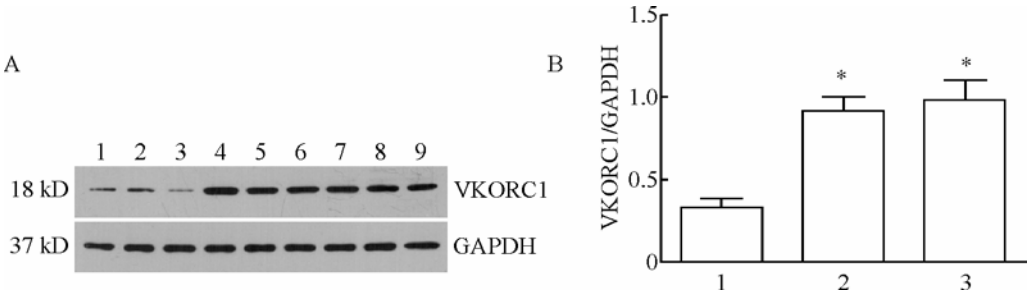

Fig. 3 Western blot analysis (A) of VKORC1 in COU patients and controls, and the quantification (B)
Lanes 1–3: Urolithiasis group; Lanes 4–6: Control group A; Lanes 7–9: Control group B. 1: Urolithiasis group; 2: Control group A; 3: Control group B; $^{*}P<0.05$ *vs.* urolithiasis group

2.2 Expression of VKORC1 mRNA

The average amount of total RNA isolated from155 samples of kidney tissues was 80 μg/100 mg of tissue. The ratio of A_{260} to A_{280} for all samples ranged from 1.68 to 1.95, confirming that the purity of each sample was satisfactory. Further, the ratios of the intensities of their 28S and 18S ribosomal RNA bands were greater than 2, demonstrating that no significant degradation of RNA

samples occurred during isolation.

As shown in fig. 4A, the slope of the line was –0.0045. Because the absolute value of the slope was close to zero, the efficiencies of the target and reference gene transcription were similar, and the ΔΔCt method could be used to analyze the data. According to Ct values in the urolithiasis group, control group A and control group B, the ΔCts were obtained as 3.444±1.271, 0.732±0.387 and 0.835±0.443, respectively. VKORC1 mRNA expression was significantly lower in urolithiasis patients than that in the control group A and B by using the $2^{-\Delta\Delta Ct}$ methods (fold: 6.063±5.275 , 26.324±10.632 and 27.167±14.248, $P<0.05$; fig. 4B). Moreover, the expression in the control group A was similar to that in the control group B and there was no significant difference ($P>0.05$).

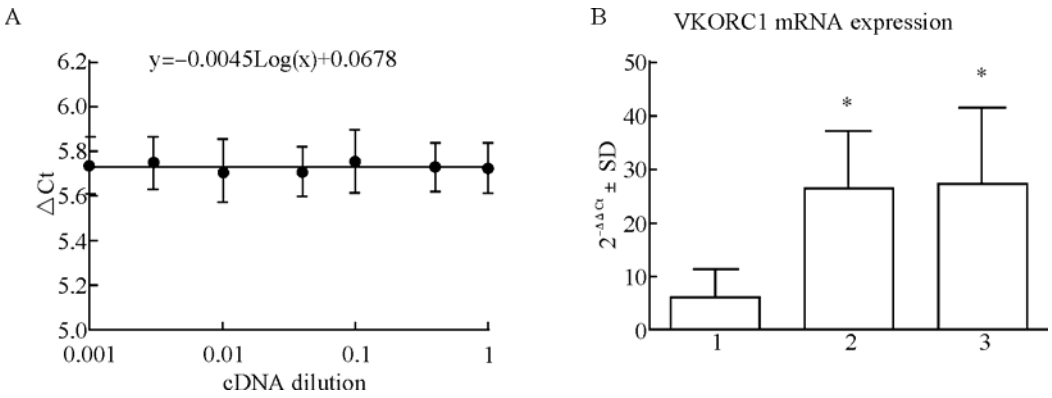

Fig. 4 Quantitative analysis of VKORC1 mRNA expression by real-time RT-PCR

A: The mean ΔCt (Ct_{VKORC1}– Ct_{GAPDH}) values were plotted against the relative concentration of cDNA. The slope of the fitted line was then determined (n=3). The amplification efficiencies of the target (VKORC1) and the reference (GAPDH) genes were approximately equal (the absolute value of the slope was close to zero). B: VKORC1 mRNA expression in the different groups. 1: Urolithiasis group; 2: Control group A; 3: Control group B; $^{*}P<0.05$ *vs.* urolithiasis group

2.3 3'-RACE and 5'-RACE of VKORC1

The 3'- and 5'-UTR sequence of the obtained VKORC1 gene in the urolithiasis and control groups was successfully cloned. After comparing and analyzing, it was found that the obtained VKORC1 gene nucleotide sequences in the control groups were accordant with the known sequence of VKORC1 (GenBank accession No: NM_024006.4). In the urolithiasis group, 3'-RACE and 5'-RACE generated a 299-bp fragment and a 422-bp fragment respectively (fig. 5). By alignment and assembling of these sequences, the full-length cDNA sequence of the obtained VKORC1 was deduced and confirmed by sequencing. The full-length cDNA of VKORC1 was 1174 bp, and it contained a 492-bp open reading frame (ORF, +398–+889), with a 3'-UTR of a 279-bp down-stream of the stop codon and a 5'-UTR of a 397-bp up-stream of the start codon. The VKORC1 nucleotide sequence and the deduced amino acid sequence were compared with known sequence of VKORC1 (GenBank accession no: NM_024006.4) through database searches using the BLAST program (http: //www.ncbi.nlm.nih.gov). There was no insertion or deletion in the 3'- and 5'-UTR, but a 171-bp new base sequence was identified in the upstream of 5'-UTR end (fig. 6).

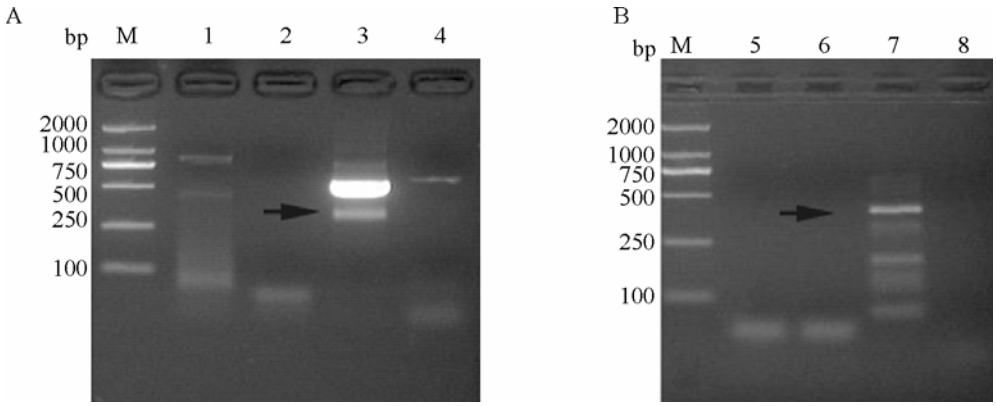

Fig. 5 The result of rapid amplification of cDNA end-polymerase chain reaction (RACE-PCR)

The products of 3'-RACE (A) and 5'-RACE (B) represented the results of 3'- RACE-PCR and 5'-RACE-PCR of VKORC1. 3'-RACE and 5'-RACE generated a 299-bp fragment and a 422-bp fragment respectively (as indicated by arrows). Lane M: DNA Marker; Lane 1: 1st RT-PCR product of 3'-RACE; Lane 2: M-MLV(–) control; Lane 3: 2nd RT-PCR product of 3'-RACE; Lane 4: M-MLV(–) control; Lane 5: 1st RT-PCR product of 5'-RACE; Lane 6: M-MLV(–) control; Lane 7: 2nd RT-PCR product of 5'-RACE; Lane 8: M-MLV(–) control

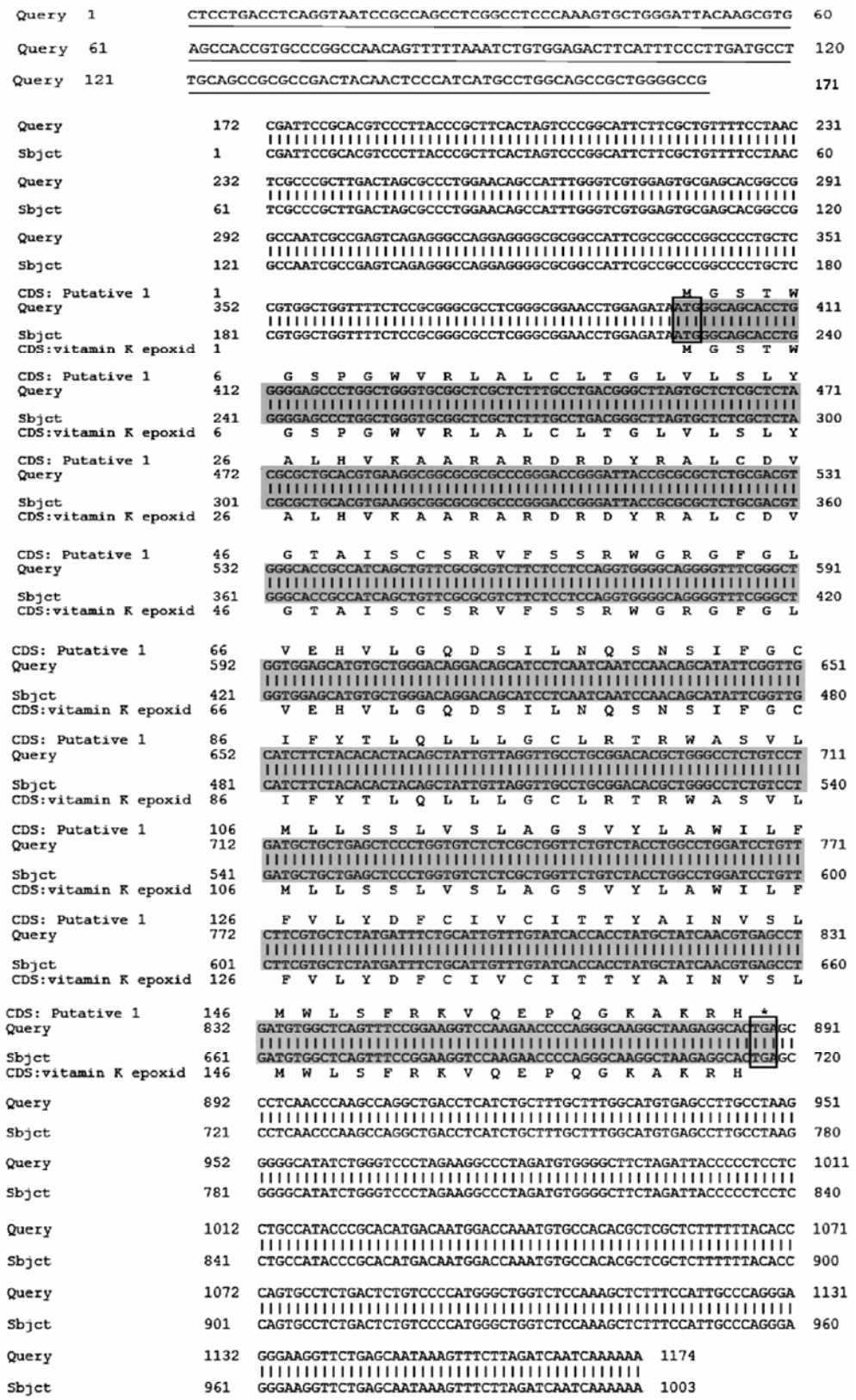

Fig. 6 Alignment of nucleotide sequence and deduced amino acid sequence

The obtained VKORC1 nucleotide sequence and deduced amino acid sequence were compared with the known sequence of VKORC1 using the BLAST program. The ORF was indicated by a gray background. The start and stop codon were boxed. Part of the newly acquired 5'-UTR nucleotide sequence was underlined. The full-length cDNA of the obtained VKORC1 was 1174 bp, and it contained a 492-bp ORF (+398 to +889), with a 3'-UTR of a 279-bp downstream of the stop codon and a 5'-UTR of a 397-bp upstream of the start codon. Though there were no insertion or deletion in the 3'- and 5'-UTR, 171 bp new bases were extended in the upstream of 5'-UTR end. Query: Obtained VKORC1; Sbjct: Known VKORC1 (GenBank accession No: NM_024006.4)

3 DISCUSSION

Our study demonstrated that VKORC1 expression was significantly down-regulated in kidneys of urolithiasis patients, suggesting that VKORC1 may play a role in UPTF1-mediated urolithiasis inhibition. On the basis of the results of Western blotting and real time PCR, we speculated that the down-regulation of VKORC1 expression in the urolithiasis patients might result in the hypo-activity of the reductase, which leads to a decrease in reduced vitamin K, and a decreased rate of carboxylation by the γ-glutamyl carboxylase and the production of under-carboxylated UPTF1.

The VKORC1 activity was first reported in 1974 and the gene was cloned in 2004[13]. Our study showed that the VKORC1 protein was highly expressed in the cytoplasm of renal tubular epithelial cells both in kidneys of urolithiasis patients and control subjects. To exclude the possibility that the change in the expression of VKORC1 in COU patients is caused by renal cortical atrophy (cortical thickness <1 cm), we also investigated the VKORC1 expression in the HWS patients in this study. Two control groups were set up in terms of the different thickness of the renal cortex: HWS group (cortical thickness <1 cm) and normal control group (cortical thickness ≥1 cm). Although various pathological factors, such as other obstructive factors (UPJO or renal tuberculosis), might lead to the decreased expression of VKORC1 theoretically, our study found that there was no significant difference in the VKORC1 expression between the normal control and HWS groups, and the expression of VKORC1 protein was decreased significantly in the urolithiasis group as compared with the two control groups (P<0.05). Meanwhile, the real-time PCR also showed the expression of VKORC1 mRNA in the renal cortex in the urolithiasis group was much lower than that in the control groups.

Moreover, to understand the regulatory mechanism of VKORC1 protein expression, the naturally occurring VKORC1 gene variants (i.e., variants encompassing mutations in nontranslated regions of the gene) were detected in our study for these variants have important influences on the downstream function of vitamin K dependent proteins, including UPTF1, which has been suggested to play a role in the pathogenesis of urolithiasis[14]. RACE is a procedure for amplification of nucleic acid sequences from a messenger RNA template between a defined internal site and an unknown sequence at either the 3'- or the 5'-end of the mRNA. The products generated by the 3' and 5' RACE procedures may be connected to generate full-length cDNAs.

The overall translation rate is also affected by the characteristics of the 5'-UTR, such as the 5'-UTR length and the start-site consensus sequences, the presence of secondary structure, the upstream AUGs, the upstream open reading frames (uORFs) and the internal ribosome entry sites (IRES). In addition, 5'-UTR contains sequences that function as binding sites for regulatory proteins. Similarly, 3'-UTR also contains a multitude of translational regulatory elements[15]. The impairment of any of these features in the mRNAs can alter translational regulation, leading to various diseases or increasing disease susceptibility. In this study, the obtained nucleotide sequence of VKORC1 in the urolithiasis patients was compared with the known sequence of VKORC1 through database search using the BLAST program (http://www.ncbi.nlm.nih.gov). The result showed that there was no insertion or deletion in the 3'- and 5'-UTR, and a 171-bp new base sequence was identified in the upstream of the 5'-UTR end. The length of the 5'-UTR can influence the translation efficiency because a navigating ribosome needs to reach the AUG through a highly structured 5'-UTR. On the basis of the RACE result, we concluded that that the down-regulated expression of VKORC1 in the urolithiasis patients wasn't significantly associated with the 3'-UTR. Whether the newly discovered base sequence of the 5'-UTR affects the translation of mRNA and expression of VKORC1, or the activity of the initiation region needs to be further studied.

In this study, the rigorous criteria were established to exclude the patients with calcium oxalate urolithiasis who suffered any other systemic disorders concomitantly. However, urolithiasis has a multifactorial origin, and dietary, geographic and ethnic factors may affect the lithogenesis[16]. An interesting question was raised from the findings of the present study: does the low expression of VKORC1 occur in COU patients from different geographic regions with different habitual diet patterns? Because the majority of the patients enrolled in this study came from a relatively limited geographical area with roughly the same diet structure, we can't answer this question according to the results obtained in our study. A large-scale study will be warranted in the future.

In conclusion, our study showed that the expression of VKORC1 was decreased in renal tissues of patients with calcium oxalate urolithiasis, indicating that VKORC1 may play an important role in the formation of calcium oxalate stones. Further studies are required to confirm the impacts of the 171-bp new base sequence in the 5'-UTR on the activity of VKORC1.

REFERENCES

1 Hall PM. Nephrolithiasis: treatment, causes, and prevention. Cleve Clin J Med, 2009,76(10):583-591

2 Worcester EM, Coe FL. Clinical practice: calcium kidney stones. N Engl J Med, 2010,363(10):954-963

3 Kumar V, Peña de la Vega L, Farell G, *et al.* Urinary macromolecular inhibition of crystal adhesion to renal epithelial cells is impaired in male stone formers. Kidney Int, 2005,68(4):1784-1792

4 Walton RC, Kavanagh JP, Heywood BR, *et al*. The association of different urinary proteins with calcium oxalate hydromorphs. Evidence for non-specific interactions. Biochimica et Biophysica Acta (BBA), 2005,1723 (1):175-183

5 Basavaraj DR, Biyani CS, Browning AJ, *et al*. The role of urinary kidney stone inhibitors and promoters in the pathogenesis of calcium containing renal stones. EAU-EBU Update Series, 2007,5(3):126-136

6 Kumar V, Farell G, Lieske JC. Whole urinary proteins coat calcium oxalate monohydrate crystals to greatly decrease their adhesion to renal cells. J Urol, 2003,170(1):221-225

7 Grover PK, Miyazawa K, Coleman M, *et al*. Renal prothrombin mRNA is significantly decreased in a hyper-oxaluric rat model of nephrolithiasis. J Pathol, 2006, 210(3):273-281

8 Grover PK, Ryall RL. Inhibition of calcium oxalate crystal growth and aggregation by prothrombin and its fragments *in vitro*: relationship between protein structure and inhibitory activity. Eur J Biochem, 1999,263(1):50-56
9 Grover PK, Ryall RL. Effect of prothrombin and its activation fragments on calcium oxalate crystal growth and aggregation in undiluted human urine *in vitro*: relationship between protein structure and inhibitory activity. Clin Sci (Lond), 2002,102(4):425-434
10 Liu J, Chen J, Wang T, *et al*. Effects of urinary prothrombin fragment 1 in the formation of calcium oxalate calculus. J Urol, 2005,173(1):113-116
11 Gong X, Gutala R, Jaiswal AK. Quinone oxidoreductases and vitamin K metabolism. Vitam Horm, 2008,78 (1):85-101
12 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using realtime quantitative PCR and the 2 (−Delta Delta C (T)) method. Methods, 2001,25 (4):402-408
13 Stafford DW. The vitamin K cycle. Thromb Haemost, 2005,3(8):1873-1878
14 Berkner KL, Runge KW. The physiology of vitamin nutriture and vitamin K-dependent protein function in aerosclerosis. Thromb Haemost, 2004,2(12):2118- 2132
15 Gray NK, Wickens M. Control of translation initiation in animals. Annu Rev Cell Dev Biol, 1998,14(1):399-458
16 Sayer JA. The genetics of nephrolithiasis. Nephron Exp Nephrol, 2008,110(2):37-43

(Received Mar. 25, 2011)