Association of Vitamin D Receptor Gene Polymorphisms with Calcium Oxalate Calculus Disease*

WANG Shaogang (王少刚), LIU Jihong (刘继红), HU Shaoqun (胡少群), YE Zhangqun (叶章群)

Department of Urology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030

Summary: To study the relationship between polymorphism of vitamin D receptor (VDR) allele with formation of calcium oxalate calculus and find the predisposing genes of calcium oxalate calculus, we screened out 150 patients who suffered from calcium oxalate calculus. 36 of them had idiopathic hypercalciuria according to analysis of calculus component and assay of urine calcium. The polymorphisms of VDR gene TaqI, ApaI and FokI were detected using PCR-RFLP technique and the correlation were analyzed between the polymorphism and urinary calculus or between the polymorphism and hypercalciuria. The difference in each genotypic frequency of the allele of promoter FokI between calculus group and healthy group or between idiopathic hypercalciuria calculus group and health group was significant. The content of 24-h urine calcium of those who had genotype ff was obviously higher than that of those who have other genotypes in the same group. There was no significant difference in the polymorphism of gene ApaI and TaqI between each two groups. It is concluded that hypercalciuria and calcium oxalate calculus were related to the polymorphism of VDR gene's promoter FokI allele, but it had nothing to do with the polymorphism of gene ApaI and TaqI. The genotype ff was a candidate heredity marker of calcium calculus disease.

Key words: gene polymorphism; vitamin D receptor; calcium oxalate calculus; hypercalciuria

Urinary calculus is a common disease that is closely related to the disorder of calcium metabolism and has the tendency of polygenic inheritance. But up to now its predisposing genes have yet to be identified. Vitamin D receptor (VDR) can regulate the metabolism of calcium and phosphorus in the body. Its allele polymorphisms are widely used to detect genetic characteristics of some diseases such as osteoporosis, rickets and secondary hyperparathyroidism[1-6]. In recent years, some researchers believed that there might be some relationship between VDR allele polymorphisms and urinary calculus or hypercalciuria. Therefore, we examined several familiar polymorphism sites of VDR gene with calcium oxalate calculus and hypercalciuria sufferers and analyzed the relation between each allele polymorphism and calcium oxalate calculus formation.

1 MATERIALS AND METHODS

1.1 Subjects

The subjects were divided into 3 groups. The patients in the first group had calcium oxalate calculus; the second had stones with hypercalciuria and the third group were healthy controls. 150 patients were selected who suffered from calcium oxalate calculus with normal urine calcium and were treated in our department from Nov. 2000 to Nov. 2001. There were 89 men and 61 women, with age ranging from 18—73 years, mean 43.6±16.4. All patients were eliminated from hypercalcinemia, hyperuricemia, hyperlithuria and hyperparathyroidism by using blood and urine biochemical exam plus blood parathyrin exam. The possible hypercalciuria was excluded by 24-h urine calcium content (<0.1 mmol/kg). The calculus specimen was of calcium oxalate calculus as revealed by chemical analysis (The reagent kits were provided by the Department of Urology, Beijing University). The patients had no chronic urinary tract infection and renal function insufficiency. The second group had calcium oxalate calculus with 24-h urine calcium>0.1 mmol/kg including 22 men and 14 women. Ages was from 22—54 years, with a an average of 36.0±11.7. This group had no abnormality in blood and urine except hypercalciuria. The control group included 80 healthy volunteers (58 men and 22 women). The age ranged from 20—79 years, with the average being 49±19.6. Subjects in this group had no family history of calculus and renal calcification. The ultrasonic examination and urinalysis revealed no abnormalities in urinary tract.

1.2 Methods

1.2.1 Reagents Peripheral blood DNA separation kit, TaqDNA polymerase and buffer, 4d-NTPs, TaqI restriction enzyme, ApaI restriction enzyme, FokI restriction enzyme, DNA retrieving

WANG Shaogang, male, born in 1968, M.D., Ph.D.

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glass pearls kit and 100 bp's product laddermarker were all products of MBI Fermentas company (Canada). The primer sequence was synthesized by the Shanghai Bioengineering Company on the basis of published VDR gene sequence by GeneBank, by using DNAman program.

1.2.2 Methods DNA was extracted from peripheral blood by using DNA separation kit. A PCR system of gene polymorphisms of the promoter Fok1 contained DNA template, primers, 1 × Taq polymerase buffer (1.5 mmol/L $MgCl_2$) and 0.5 μl Taq polymerase (final volume: 50 μl). The forward primers were 5′-ACTGACTCTGGCTCTGAC-3′ and the reverse primers were 5′-CACCTTGCTTCT TCTCCC-3′. The PCR amplification cycling parameters included an initial denaturation at 94 ℃ for 5 min, then denaturation at 94 ℃ for 30 s, annealing at 57.5 ℃ for 45 s and extension at 72 ℃ for 45 s. The cycle was repeated 35 times, followed by a extension step at 72 ℃ for 7 min.

Detection of ApaI and TaqI polymorphisms used both the forward primers 5′-CAGAGCATG-GACAGGGAC-3′ and the reverse primer 5′-AACAGCAACTCCTCAC-3′. The PCR system was the same as that for FokI. The cycling included an initial denaturation at 94 ℃ for 7 min, then denaturation at 94 ℃ for 30 s, annealing at 54 ℃ for 45 s, and extension at 72 ℃ for 45 s. The cycle was performed 40 times with a final extension at 72 ℃ for 10 min. To increase purity, we cut gel after electrophoresis and retrieved DNA fragment by using DNA retrieving glass pearls. The specific restriction enzyme (5 μl) and its buffer was added into the products of PCR, which was stored overnight at 37 ℃. Then DNA fragments were separated by gel electrophoresis.

2 RESULTS

2.1 Genotype of VDR

After gel electrophoresis, there was only one band (with a length of 745 bp) if there was no site for ApaI enzyme on allele of homozygote, and the genotype of the homozygote was designated as "AA". There would be 3 bands (The length of them was 745 bp, 533 bp and 220 bp) if one site was present on allele of heterozygote, which was designated as "Aa". Two bands would appear if two sites were on allele of homozygote (The length of them was 533 bp, 220 bp), which was designated as "aa" (fig. 1 1). With TaqI fragment there were also three scenarios: TT homozygote (only one 745 bp band present), Tt heteozygote (three bands with length of 745 bp, 495 bp, 254 bp, respectively), and tt homozygote (two bands with the length 495 bp and 254 bp). The results of electrophoresis were shown in fig. 1.

FokI has three genotypes: FF, Ff and ff which are similar to the above-mentioned scenarios. The results of electrophoresis are showed in fig. 2.

2.2 Frequency of VDR Genotypes

The frequency of VDR genotypes of control group, group of calcium oxalate calculus with normal urine calcium and group of hypercalciuric calculus are listed in table 1. The distribution of VDR polymorphous genotypes of ApaI and TaqI had no significant difference between the last two groups and control group.

There was significant difference in FokI gene polymorphism of VDR between group of calcium oxalate calculus and control group and between group of hypercalciuric calculus and control group. But no significant difference was found between the two experimental groups. Table 2 showed that groups of calcium oxalate calculus and hypercalciuria calculus had high frequency of genotype ff.

2.3 The Relationship Between VDR Genotypes and Urine Calcium

The 24-h urine calcium of subjects with different genotypes is showed in table 3. There was a similar trend in the 3 groups, as shown by table 3. Urine calcium of genotype ff was significantly higher than that of genotype FF in the 3 groups (t-test, $P<0.05$).

Table 1 Distribution of VDR gene polymorphism of ApaI and TaqI

Groups	ApaI*			TaqI*		
	AA	Aa	aa	TT	Tt	tt
Control	11 (13 %)	38 (48 %)	31 (39 %)	33 (41 %)	36 (45 %)	11 (14 %)
Calcium oxalate calculus	32 (21 %)	69 (46 %)	49 (33 %)	52 (35 %)	74 (49 %)	24 (16 %)
Hypercalciuric calculus	9 (25 %)	16 (44 %)	11 (31 %)	12 (33 %)	19 (53 %)	5 (14 %)

* No significant difference in distribution of genotypes was found among the three groups

Table 2 Distribution of FokI gene polymorphism of VDR

Groups	Types			P
	FF	Ff	ff	
Control	17 (21 %)	44 (55 %)	19 (24 %)	
Calcium oxalate calculus	27 (18 %)	64 (43 %)	59 (39 %)	<0.01
Hypercalciuric calculus	5 (14 %)	13 (36 %)	18 (50 %)	<0.05*

* No significant difference of distribution of genotypes was found between the two experimental groups

Table 3 The 24-h urine calcium of different genotypes (mmol/L)

Groups	FF	Ff	ff
Control	0.031±0.003	0.048±0.004	0.051±0.004
Calcium oxalate calculus	0.032±0.003	0.047±0.003	0.074±0.004
Hypercalciuric calculus	0.108±0.014	0.107±0.009	0.135±0.011

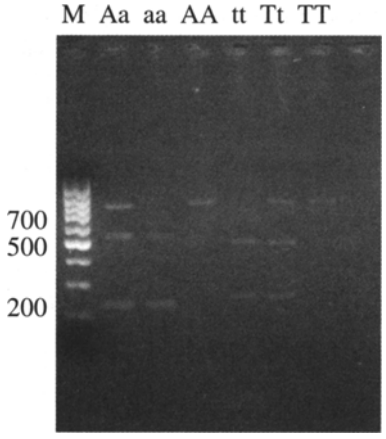

Fig. 1 Electrophoregram of VDR-PCR products digested by Apa1 enzyme and Taq1 enzyme

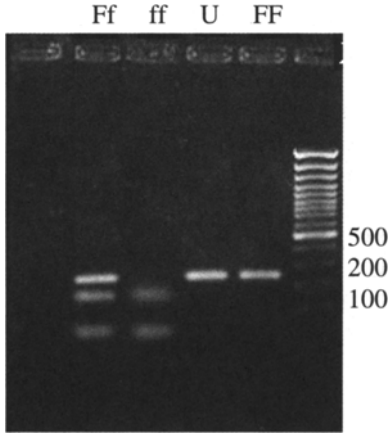

Fig. 2 Electrophoregram of VDR-PCR products digested by Fok1 enzyme

3 DISCUSSION

VDR is a nuclear receptor mediating biological effect of 1, 25 $(OH)_2D_3$. Signal molecule 1, 25 $(OH)_2D_3$ combines with VDR in nucleus of target cells to form hormone-receptor compound, which bonds with special DNA sequence of target gene to regulate expression of structure gene. VDR-1, 25 $(OH)_2D_3$ compound would increase expression of calcium-binding proteins in small intestine epithelial cells and parathyroid hormone receptors in parathyroid gland cells, regulate self-synthesis of 1, 25$(OH)_2D_3$ in cutaneous keratinocyte and renal cells. Therefore, VDR is a ligand-dependent nuclear transcription factor. VDR play important roles in maintaining the equilibrium of metabolism of calcium and phosphorus and regulating cell proliferation and differentiation. Current data has confirmed that VDR gene polymorphism is related to osteoporosis, hyperparathyroidism and rickets. The polymorphism appears frequently on sites of Apa1, Bsm1, Fok1 and Taq1.

The mechanism of calcium calculus's formation is very complex, which is still obscure now. Hypercalciuria is an important risk factor related to the formation of calcium calculus. Vitamin D and its receptor VDR are biological molecules to regulate metabolism of calcium in the organism. So the formation of the calcium calculus may be closely related to Vitamin D and VDR. Excessive intake of Vitamin D will lead to disturbance of calcium metabolism in body and formation of calculus as has been proved. In recent years, the relation between urinary calculus and VDR begins to draw the attention of researchers. Heilberg, Chen, Ruggiero, Jackman *et al* studied polymorphism of VDR gene Bsm1[10—12] Taq1[13] but failed to show any correlation between them. Recently Chen[14] studied the promoter Fok1 polymorphism of VDR gene and suggested an obvious correlation between polymorphism of allelomorph Fok1 and calcium oxalate calculus. He recommended further studies in larger population.

The site Fok1 lies in the promoter of VDR gene. The mutation of bases may affect its transcription activity. Ingles *et al* found the correlation between polymorphism of allelomorph Fok1 in VDR gene and the cancer of colon[15]. Lim *et al* found the correlation between polymorphism of Fok1 and polymorphism of estrogen's receptor in Korean women[16]. Choi *et al* found lower bone mineral density of lumbar spine in Korean women after menopause in genotype ff than in genotype FF[17]. Chiu *et al* reported the relation between polymorphism of Fok1 and sensibility of insulin in patients with diabetes[18]. All these proved indirectly that polymorphism of allelomorph Fok1 in VDR gene may affect transcription of its target gene. The correlation between polymorphism of Fok1 in VDR gene and calcium oxalate calculus as well as hypercalciuria has not been well studied.

This study examined the polymorphism of three sites Apa1, Taq1 and Fok1 of VDR gene in patients with calcium oxalate calculus. No significant difference in the frequency of genotypes of sites Apa1 and Taq1 was found among healthy people, patients with calcium oxalate calculus and patients with hypercalciuria. The difference in the frequency of genotypes of sites Fok1 between healthy people and patients with calcium oxalate calculus or between healthy people

and patients with hypercalciuria was significant. But there was no significant difference between patients with calcium oxalate calculus and patients with hypercalciuria. It is suggested that the polymorphism of FokI of VDR gene is closely correlated with calcium oxalate calculus. Our results were consitent with those reported by Chen[14]. Moreover, this study also found that the content of urine calcium in patients with genotype ff was much higher than genotype FF in each group. Genotype ff is obviously correlated with excretion of urine calcium. So it is possible that genotype ff may well become the genetic marker of calcium oxalate calculus and idiopathic hypercalciuria.

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