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ORIGINAL ARTICLE

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Vascular endothelial growth factor gene polymorphism is associated with calcium oxalate stone disease

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Abstract Growth factor-related genes regulate cell growth, differentiation and apoptosis in the kidney in response to cellular injury. One of the theories of stone formation is that cellular injury, and thus growth factors, play a role. We therefore investigated the association between growth factor genes and calcium oxalate stone disease. The most frequently seen polymorphism of the vascular endothelial growth factor (VEGF) gene is *Bst* U I C/T, which is located upstream at the −460th nucleotide. Other growth factor-related gene polymorphisms include the cytochrome P450c17α enzyme (CYP17) gene *MspA* I C/T polymorphism at the 5′-UTR promoter region, the epidermal growth factor receptor (EGFR) gene *Bsr* I polymorphism (A to T) at position 2,073, and the insulin-like growth factor-2 (IGF-2) gene *Apa* I A/G at exon 9. All four polymorphisms were used as genetic markers in this study in the search for an association between stone disease and growth factor related genes. A normal control group of 230 healthy people, and 230 patients with calcium oxalate stone, were examined. The polymorphism was seen following polymerase chain reaction based restriction analysis. The result revealed a significant difference between normal individuals and stone patients ($P = 0.0003$, Fisher's exact test) in the distribution of the VEGF gene polymorphism as well as an odds ratio of 1.30 (95% confidence interval = 0.993–1.715) per copy of the "T" allele. Whereas, the IGF-2, EGFR and CYP17 gene polymorphisms did not reveal a significant association with stone disease. We conclude that the VEGF gene *Bst* U I polymorphism is a suitable genetic marker of urolithiasis.

Keywords VEGF · CYP17 · IGF-2 · EGFR gene polymorphism · Urolithiasis · Single nucleotide polymorphisms (SNPs) · Hormone therapy

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Introduction

Calcium oxalate stone is the most commonly seen form of urolithiasis. It is a common, painful, and costly medical condition. The causes of calcium oxalate stone are heterogenous, possibly involving both genetic and environmental factors. It has been reported that an unknown sequence of events caused by cellular injury is involved in stone formation [9]. Growth factors regulate cell growth, differentiation, and apoptosis in response to environmental injuries. However, few studies on the association between variable growth factor-related genes and the formation of calcium urolithiasis have been made. Recently, researchers have begun to use single nucleotide polymorphisms (SNPs) to identify the genes associated with calcium oxalate stone disease [2, 16].

In this study, we chose four growth factor related genes, vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR), insulin-like growth factor-2 (IGF-2), and the CYP17 gene to investigate SNPs related to urolithiasis. These four genetic polymorphisms are well-known and have been reported to be associated with other diseases [1, 12, 19, 22]. VEGF is a cytokine with angiogenic properties which plays an important role in neovascularization [23]. Jackson et al., using immunohistochemical staining and

polymerase chain reaction, found the widespread expression of VEGF in prostate tissue [12]. EGFR is a tyrosine kinase receptor which plays an important role in the control of cell growth and differentiation [19, 25]. IGF-2 is believed to play a role in fetal growth and development [8]. The insulin like growth factor system performs an important role in regulating the processes of proliferation and apoptosis in cells [22]. The CYP17 gene encodes the cytochrome p450c17α enzyme that mediates both sex steroid hormones [17]. Sex hormones are considered to be involved in the pathogenesis of stone disease because they stimulate cell growth, and the prevalence of stone disease is predominantly male specific [21]. Our aim was to determine whether or not the polymorphisms of these growth factor-related genes are associated with stone disease.

Patients and methods

Patient selection

A total of 230 patients (175 males and 55 females) between the ages of 26 and 78 years (average age: 44.7 ± 11.7), with at least two episodes of recurrent calcium oxalate stone regardless family history [15], were enrolled in this study. Serial blood and urine biochemistry tests were undertaken to exclude possible hypercalcemia, hyperuricemia, and hyperuricosuria. Patients who showed symptoms of urinary tract infections during the period of stone treatment were excluded. Stone composition was verified by infrared spectroscopy and revealed either calcium oxalate monohydrate, dihydrate, or a combination of the two.

A control group was drawn up of 230 healthy volunteers over the age of 40 who had no history of familial stone disease or cancer. Renal ultrasonography and routine tests of urinary microscopic hematuria were made in order to exclude individuals who may have had renal calcification. There were 183 males and 47 females in the control group (age range: 40–73years, average age: 61.9 ± 12.7 years). Informed consent was obtained from both groups participating in the study. The genomic DNA was prepared from peripheral blood by use of a DNA Extractor WB kit (Wako, Japan).

Polymerase chain reaction

Polymerase chain reactions (PCRs) for the genes were carried out to a total volume of 50 μl, containing genomic DNA; 2–6 pmole of each primer; 1× Taq polymerase buffer (1.5 mM $MgCl_2$); and 0.25 units of AmpliTaq DNA polymerase (Perkin Elmer, Foster City, Calif., USA). The primers for the VEGF gene were: forward 5′-TGTGCGTGTGGGGTTGAGCG-3′ and backward 5′-TACGTGCGGACAGGGCCTGA-3′ according to the report by Watson et al. [23]. PCR amplification was performed in a programmable thermal cycler GeneAmp PCR System 2400 (Perkin Elmer).

The 175 bp PCR product of the VEGF gene was mixed with 2 units of *Bst*U I (Takara, Japan) and the reaction buffer, according to the manufacturer's instructions. The restriction site was located −460 bp upstream of exon I, (C to T); transcription site "C" was a cutable site. Two fragments measuring 155 bp and 20 bp were present if the product was digestible. The uncutable band was 175 bp on the gel. The polymorphisms were categorized as "CC" homozygote (cutable), "TT" homozygote (uncutable) and "CT" heterozygote.

The polymorphism in exon 9 of the IGF-2 gene was chosen because it is digestible by *Apa* I restriction endonuclease. The primer for IGF-2 was 5′-CTTGGACTTTGAGTCAAATTGGC-3′ and 5′-GCGGTACGAGCGACGT GCCCAC-3′ [22]. The PCR product of 126 bp was mixed with 2 units *Apa* I (New England Biolabs, Beverly, USA). If the product was digestible, two unequal lengths of double-strand restriction fragments of 61 bp and 65 bp were present (CC homozygote). The polymorphism was categorized into CC homozygote (digestible), TT homozygote (indigestible), and C/T heterozygote.

PCR for the EGFR gene polymorphism was designed for a product of 195 bp. The primers used for PCR differed from Shintani et al. (they used cDNA) [19] and were designed as: 5′-ATATATGCCAAAGAA GTAG-3′ and 5′-TGATCAGGACAGAGGACAG-3′. After reaction with *Bsr* I, the product was excised into fragments of 136 bp and 59 bp if the restriction site was present (A to T). Otherwise, a 195 bp fragment was present on 3% agarose gel electrophoresis.

The primer for the CYP17 gene 5′-UTR was 5′-CCACAA GGCAATGAGATAACA-3′ and 5′-AGGGTAAGCAGCAAGAGAGC-3′, which differed from that reported by Habuchi et al. [10]. The PCR product of 169 bp was mixed with 2 units *MspA* I (New England Biolabs) and the reaction buffer, according to the manufacturer's instructions. Two fragments of 102 bp and 67 bp were present if the product was digestible. The polymorphism was categorized into CC homozygote (digestible), TT homozygote (indigestible) and C/T heterozygote.

We analyzed the data by comparing the allelic frequency distribution of the polymorphisms between both groups. The software used for the calculation was the SPSS system. When an assumption of the χ^2-test was violated (i.e. when 1 cell had an expected count of < 1, or $> 20\%$ of the cells had an expected count of < 5), Fisher's exact test was used. A P value of < 0.05 was considered statistically significant.

Results

Representative PCR-based restriction analyses are shown in Figs. 1, 2 and 3. The frequencies of the genotypes in the stone group and control group of the VEGF gene are shown in Table 1. There was a significant difference in the distribution of the VEGF gene C/T polymorphism between controls and stone patients ($P < 0.001$, Fisher's exact test). The allele distribution in the control group was: "T", 58.0% and "C", 42.0%, whereas in the stone group it was: "T", 64.6% and "C", 35.4%. The odds ratio (OR) per copy of the T allele for the risk of stone disease was 1.30 (95% confidence interval = 0.993–1.715) ($P < 0.05$).

For the distribution of the CYP17 gene C/T polymorphism there was no significant difference between the healthy control group and the stone patient group

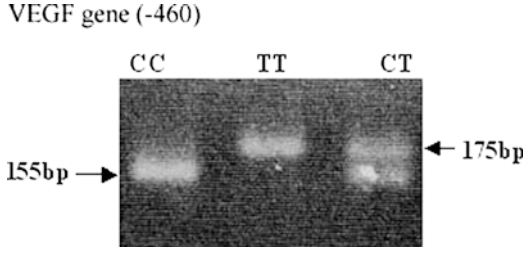

Fig. 1 PCR-base restriction analysis of vascular endothelial growth factor gene −460 T/C I polymorphism shown on 3% agarose electrophoresis. The polymorphic region was amplified by PCR resulting in a digestible fragment in *lane 1* (*CC* homozygote), an indigestible fragment in *lane 2* (*TT* homozygote) and a heterozygote (*CT*) in *lane 3*

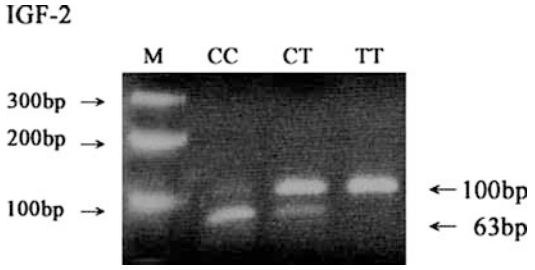

Fig. 2 PCR-base restriction analysis of insulin-like growth factor II gene 3′-UTR *Pml* I polymorphism shown on 3% agarose electrophoresis. The polymorphic region was amplified by PCR resulting in a digestible fragment in *lane 2* (*CC* homozygote), a heterozygote (*CT*) in *lane 3* and an indigestible fragment in *lane 4* (*TT* homozygote). *M*: marker (*lane 1*), 100-bp ladder

(Table 1, $P = 0.263$). The allele frequency of the C/T polymorphism was 61.9%/38.1 % in normal individuals and 58.6%/41.4% in the stone patient group, respectively. There was no statistical difference in the distribution of allele frequencies between normal individuals and stone patients ($P = 0.312$, χ^2-test).

The distribution of the IGF-2 gene C/T polymorphism was compared and no significant difference between the healthy control group and the stone patient group was found (Table 1, $P = 0.584$). The results showed that there is no association between EGFR gene A/T polymorphism and stone disease (Table 1, $P = 0.180$).

Discussion

The distribution of the VEGF gene in the C/T polymorphism differed between stone patients and the control group, indicating that the VEGF gene C/T polymorphism can serve as a candidate genetic marker for screening calcium stone disease. The "T" allele of the VEGF gene was associated with a relatively high risk of stone disease. Whereas the data revealed that the CYP17, IGF-2, and EGFR gene polymorphisms are not candidate genetic markers.

VEGF, a homodimeric glycoprotein with a relative molecular mass of 45kDa, plays an important role in neovascularization and is a potent inducer of endothelial cell growth [5]. Hypoxia, androgen, interleukin-1, and tumor necrosis factor are reported to regulate or at least correlate with VEGF in the enhancement of neovascularization [6, 13], indicating that it is activated by upstream signaling [3]. Published reports indicate that VEGF might combine with upstream signals to enhance epithelial cell growth and vascular permeability. Therefore, the pathogenesis of stone disease occur via a VEGF-related signal transduction pathway.

According to the theories of fixed particles and cellular injury in the formation of calcium oxalate stone [7, 9, 14, 18, 20], hyper-oxaluria and chronic oxalosis cause non-lethal injury to tubular epithelial cells which results in the production of cytokines, osteopontin and

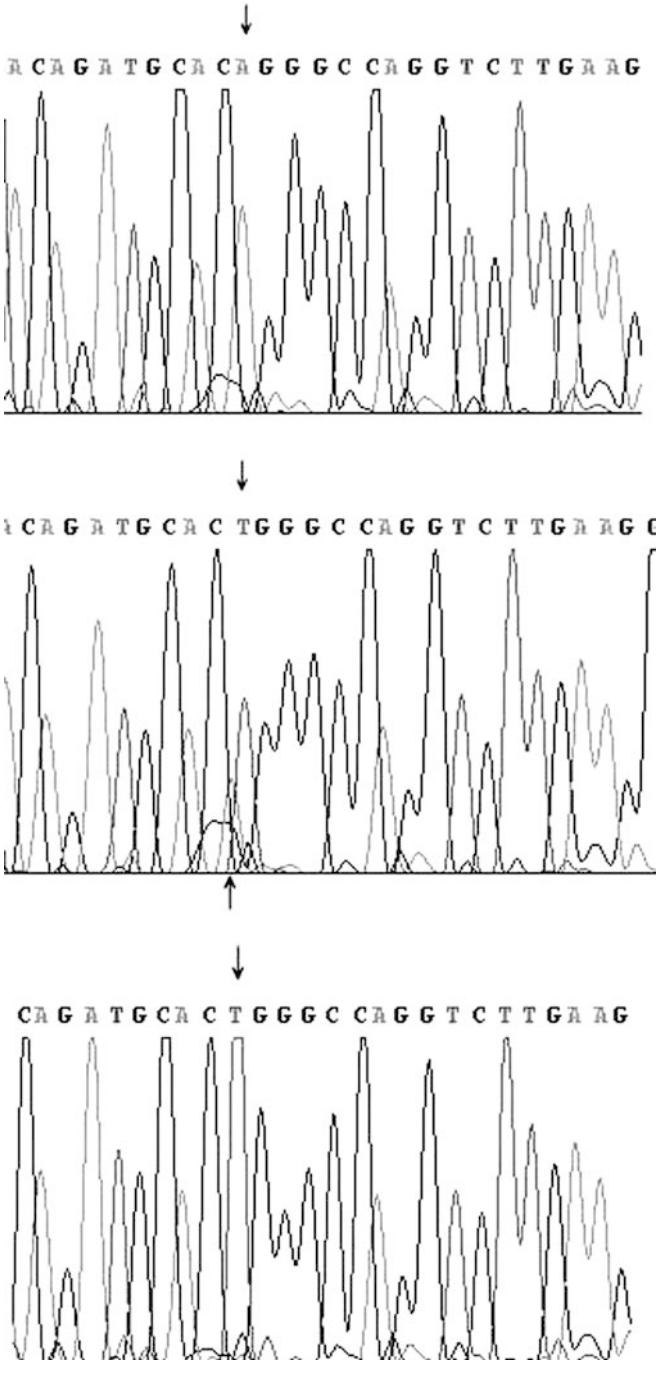

Fig. 3 The sequencing displays different sequences of epidermal growth factor gene *Bsr* I polymorphism. *Upper*: *AA* homozygote (*arrow*), *middle*: *AT* heterozygote (*upper arrow*, at *lower arrow* of middle figure indicates a double signal of *AT* nucleotide), and *lower*: *TT* homozygote (*arrow*)

other inflammatory proteins [4]. Damaged epithelial cells caused by the formation of calcium oxalate crystals are excreted in the urine, phagocytosed, endocytosed, undergo cell division, and finally apoptosis. All of the above events contribute to stone formation. The process of retained calcium oxalate crystals in renal tubules is necessary for stone formation. Renal crystal deposition

Table 1 Fisher's exact test for the distribution of the vascular endothelial growth factor (VEGF) gene *Bst* U I polymorphism between the healthy control subjects and the stone patient.Distributions of the CYP17 gene 5′ C/T polymorphism, IGF-2 gene *Apa* I polymorphism, and EGFR gene *Bsr* I polymorphism between the healthy control subjects and the stone patients were compared using the χ^2-test. The *P* value represents the result of the stone patient group compared with the control group

VEGF gene	TT	TC	CC	Total	χ^2	*P*-value
Control	38 (16.6%)	192 (83.4%)	0 (0%)	230 (100.0%)		
Stone patient	70 (30.4%)	157 (68.3%)	3 (1.3%)	230 (100.0%)		0.0003
CYP17 gene	CC	T/C	TT	Total		
Control	75 (32.7%)	135 (58.7%)	20 (8.6%)	230 (100.0%)		
Stone patient	71 (30.8%)	128 (55.7%)	31 (13.5%)	230 (100.0%)	2.669	0.263
IGF-2gene	AA	AG	GG	Total		
Control	67 (29.1%)	123 (53.5%)	40 (17.4%)	230 (100.0%)		
Stone patient	75 (32.6%)	112 (48.7%)	43 (18.7%)	230 (100.0%)	1.074	0.584
	AA	AG	GG	Total		
Control	73 (31.7%)	124 (53.9%)	33 (14.4%)	230 (100.0%)		
Stone patient	57 (24.7%)	130 (56.5%)	43 (18.8%)	230 (100.0%)	3.427	0.180

starts when the crystals and crystal aggregates are retained. The interstitial matrix becomes enlarged and edematous due to cell proliferation stimulated by chemokines and cell adhesion molecules. This indicates that crystal deposition in the kidney causes an inflammatory reaction.

Although we did not find an association between the IGF-2 gene and stone disease, we still believe that this gene plays some kind of role in stone pathogenesis. Winter et al. found that lower levels of IGF-2 in some patients with prostate cancer were associated with an increased risk of cancer formation [24]. Also, Gnanapragasam et al. suggested that the action of anti-androgen is mediated partly by an ability to suppress IGF-2 expression [8]. It is also known that androgen is associated with stone disease [21]. Therefore, other related factors, such as androgen receptors, should be taken into consideration when studying the association between the IGF-2 gene and stone disease.

The CYP17 gene encodes a member of the cytochrome P450 superfamily of enzymes which localizes in the endoplasmic reticulum [1]. Cytochrome P450 proteins are monooxygenases which catalyze many reactions involved in drug metabolism and the synthesis of cholesterol, steroids and other lipids. The CYP17 encoded protein has both 17-alpha-hydroxylase and 17,20-lyase activities and is a key enzyme in the steroidogenic pathway that produces progestins, mineralocorticocoids, glucocorticoids, androgens, and estrogens [17]. Our data are compatible with those of Huang et al. which showed that the C allele (which is equal to the A2 allele in their study) was found in 35.8% of Taiwan female breast cancer patients compared to 30.8% found in our stone patients group [11]. They also reported that breast cancer patients with the CT genotype has a 14-fold higher incidence rate of breast cancer than other types [11], revealing a strong association between the CYP17 gene C/T polymorphism and breast cancer. Although the CYP17 gene seems related to androgen, we did not find any difference in this polymorphism between stone patients and normal individuals. The distribution of the "C" allele in normal individuals also differed between our study and that by Huang et al.: 32.7% and 22.2%, respectively. Even after adjusting for gender, OR analysis did not show any significant difference between stone patients and normal controls. Therefore, our study revealed no evidence that the CYP17 5′-UTR C/T polymorphism is associated with stone disease.

In conclusion, the VEGF gene polymorphism is associated with stone disease and is a candidate genetic marker of urolithiasis.

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