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Osteocalcin gene *Hind* III polymorphism is not correlated with calcium oxalate stone disease

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Abstract The formation of urinary stones is presumed to be associated with polymorphism of the osteocalcin gene. The most frequently seen polymorphism is the *Hind* III type located at the promoter region. This polymorphism has been used as a genetic marker in the search for a correlation between urolithiasis and normal subjects. In our study, a normal control group of 105 healthy people and 102 patients with calcium oxalate stones were examined. The polymorphism was seen following polymerase chain reaction-based restriction analysis. The results revealed no significant differences between normal individuals and stone patients ($P = 0.978$), and distribution of the TT homozygote in the control group (42.9%) was similar to that in the patient group (42.2%). Further categorization of the stone patients into normocalciuric and hypercalciuric groups also revealed no statistical differences from controls. We conclude that *Hind* III polymorphism of the osteocalcin gene is not a suitable genetic marker of urinary stone disease. Further searches for other polymorphisms on this gene correlated with stone disease are suggested.

Key words Osteocalcin gene polymorphism · Urolithiasis · Single nucleotide polymorphisms

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Introduction

Urolithiasis is a multifactorial disease commonly seen in urological patients. The cause of calcium oxalate stones is heterogeneous, possibly involving both genetic and environmental factors. Although the genetic causes have been studied extensively, no chromosomal mapping has been achieved in stone patients with idiopathic hypercalciuria [2]. The only conclusive comment, made by Resnick and Coe, is that urolithiasis is a polygenic defect and partially penetrative [5, 14]. Recently, single nucleotide polymorphisms (SNPs) were used for mapping the disease gene [11], and polymorphism of the vitamin D receptor (VDR) gene was reported to be associated with stone disease [6], making it therefore possible to search for the genes responsible for stone disease.

Osteocalcin is a vitamin K-dependent protein gene located at chromosome 1q25-q31 (also known as bone Gla protein, or BGP), which has been used as a genetic marker for the prediction of bone mass density in menopausal Japanese women [3]. Because osteocalcin biosynthesis is connected to bone formation and calcium metabolism and is regulated by vitamin D and parathyroid hormone [10, 22], it was proposed as an excellent marker for studying bone metabolism. Osteocalcin is also related to bone resorption and may change blood levels of calcium ions [15]. Therefore, osteocalcin might correlate with stone disease. A novel polymorphism – *Hind* III – is located at the promoter region of the osteocalcin gene's 198th nucleotide upstream from exon 1. This polymorphism with one base change (C to T) was used by Dohi et al. as a genetic marker for the correlation of bone mineral density in postmenopausal Japanese women [3]. There are no ethnic data for osteocalcin gene polymorphism among healthy people and stone patients in Taiwan. The following study was performed to determine whether this polymorphism is associated with calcium oxalate stone disease in that population.

Patients and methods

Patient selection

A total of 102 patients (76 males and 26 females ranging in age from 23 to 76, average 44.6 years) with two or more calcium oxalate stones were enrolled in this study. Among them, 19 males and 11 females had hypercalciuria as defined by 24-h urine calcium concentrations higher than 300 mg in males or 250 mg in females on a random diet. The remaining patients revealed normal urine calcium levels and were categorized as normocalciuric (57 males and 15 females). Serial blood and urine biochemistry tests were undertaken to exclude possible hypercalcemia, hyperuricemia, and hyperuricosuria. Patients showing symptoms of urinary tract infections during the stone treatment period were excluded. A control group was drawn up of 105 healthy volunteers over the age of 40 (60 males and 45 females from 40 to 87 years of age, average 52.7 years) with no history of familial stone disease or signs of renal calcification following renal ultrasonography tests. Routine tests were made for urinary microscopic hematuria to exclude those who may have had such conditions. Informed consent was obtained from all members of both groups. Stone composition was verified by infrared spectroscopy revealing calcium oxalate monohydrate or dihydrate or a combination of the two. The genomic DNA was prepared from peripheral blood using a genomic DNA isolation kit (Blossom, Taipei, Taiwan).

Polymerase chain reaction

Polymerase chain reactions (PCRs) for polymorphism in the osteocalcin gene promoter region were carried out to a total volume of 50 μl containing genomic DNA, 2–6 pmol of each primer, 1X 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 osteocalcin were forward 5′-CCGCAGCTCCCAACCACAATAAGCT-3′ and backward 5′-CAATAGGGCGAGGAGT-3′ according to the report of Dohi et al. [3]. The PCR amplification was performed in the GeneAmp PCR System 2400 programmable thermal cycler (Perkin Elmer). The cycling conditions for *Hind* III were set as follows: one cycle at 94 °C for 8 min, 30 cycles at 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 60 s, and one final cycle of extension at 72 °C for 10 min.

The PCR product of 253 bp was mixed with 2 IU of *Hind* III (Takara, Tokyo, Japan) and the reaction buffer according to the manufacturer's instructions. The restriction site was designed to be located 198 bp upstream of exon I, near the promoter box (C298 T), −152 transcription site T to form a cuttable site. Two fragments measuring 232 bp and 21 bp will be present if the product is excisable. The uncuttable band shows up as a 253-bp length on the gel. The reaction was then incubated for 2 h at 37 °C. Ten microliters of the products were loaded into a 3% agarose gel containing ethidium bromide for electrophoresis. The polymorphism was categorized as cuttable or uncuttable homozygotes or heterozygotes. For statistical analysis of the allelic frequency distribution in this polymorphism, the two groups were compared using the chi-squared test. When the assumption of this test was violated (i.e., when one cell had an expected count of <1 or $>20\%$ of the cells had expected counts of <5), Fisher's exact test was performed. P values of <0.05 were considered statistically significant.

Results

The PCR analysis revealed cuttable homozygotes (TT), uncuttable homozygotes (CC) and heterozygotes (TC) (Fig. 1). Frequencies of the genotype in the stone and control groups are shown in Table 1. The distributions of polymorphism in the normocalciuria and hypercalciuria groups are also listed. Neither the hypercalciuria nor the normocalciuria stone patient group showed significant differences when compared with controls. Age distribution between the normocalciuria and hypercalciuria groups in each gender also revealed no statistical differences.

The distribution in the control group was: TT 42.9%, TC 45.7%, and CC 11.4%. The frequency of the T allele in the patient group (42.2%) was similar to that in the control group. The allelic distribution of TT/CC polymorphism at the promoter region of the osteocalcin gene was 0.657 for T alleles and 0.343 for C alleles in normal healthy subjects and stone patients. Using the chi-squared test, the distribution of osteocalcin *Hind* III polymorphism was compared, showing no significant differences between healthy controls and stone patients ($P = 0.978$). We further subdivided the groups by gender, which revealed no significant differences between male and female patients in the distribution of *Hind* III polymorphism ($P = 0.355$) (Tables 2, 3).

Discussion

Unlike single gene diseases, complex or multifactorial diseases such as urolithiasis may result from the interaction of environmental factors and involve multiple

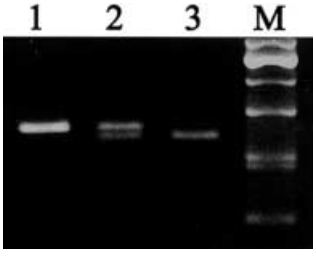

Fig. 1 PCR base restriction analysis of osteocalcin gene *Hind* III polymorphism shown on 3% agarose electrophoresis. The polymorphic region was amplified by PCR, resulting in a cuttable fragment at lane 1 (232 bp and 21 bp), an uncuttable fragment at lane 2 (253 bp), and a heterozygous form at lane 3. The 21-bp band was obscure during the electrophoresis process. *M* marker (*lane 4*), 100-*bp* ladder

Table 1 Chi-squared test results for distribution of osteocalcin gene *Hind* III polymorphism between healthy control subjects and calcium oxalate stone patients

	TT (*n*)	TT (%)	TC (*n*)	TC (%)	CC (*n*)	CC (%)	Total (*n*)	χ^2	*P*
Controls	45	42.9	48	45.7	12	11.4	105		
Stone patients	43	42.2	48	47.1	11	10.8	102	0.045	0.978
Normocalciuria	28	38.9	36	50.0	8	11.1	72	0.332	0.847
Hypercalciuria	15	50.0	12	40.0	3	10.0	30	0.482	0.786

Table 2 Fisher's exact test analysis of distribution of osteocalcin gene *Hind* III polymorphism between healthy controls and female calcium oxalate stone patients

	TT (*n*)	TT (%)	TC (*n*)	TC (%)	CC (*n*)	CC (%)	Total (*n*)
Controls	20	44.4	22	48.9	3	6.7	45
Stone patients ($P = 0.925$)	13	50.0	12	46.2	1	3.8	26

Table 3 Distribution of the osteocalcin gene *Hind* III polymorphism between healthy controls and male calcium oxalate stone patients. Chi-squared test, degree of variance 2

	TT (*n*)	TT (%)	TC (*n*)	TC (%)	CC (*n*)	CC (%)	Total (*n*)	χ^2
Controls	25	41.7	26	43.3	9	15.0	60	
Stone patients ($P = 0.886$)	30	39.5	36	47.4	10	13.1	76	0.241

genes, many of which have relatively minor effects but some of which cause major disease [16]. Association studies comparing genetic maker frequencies in nonrelative disease patients and control groups often implicate a candidate gene in the etiology of a complex disease [4]. Occurring on an average of 1 per 1000 base pairs and providing resources to map complex genetic traits, SNPs are the most abundant form of human genetic variation [1] and have gained popularity in recent years as the genetic markers of choice for studying complex genetic traits [1]. Studying them provides new ways of identifying genes associated with disease. However, a very large set of genetic markers is required to determine the genotypes of a large population. A set of markers evenly distributed throughout the genome is needed to perform an association study. Therefore, a subset of SNPs is functionally important in the study of complex disease traits.

There have been reports that osteocalcin is related to calcium stone disease. The measurement of serum osteocalcin was proposed as a means for the differential diagnosis of hypercalciuria and calcium stone disease [17]. In clinical study of the osteocalcin response to a calcium-restricted diet, Strohmaier et al. suggested that diet restriction treatment for hypercalciuric stone patients should depend on the negative responsiveness of osteocalcin to a restricted calcium diet [15]. Furthermore, osteocalcin has been identified as a major stone matrix protein [12, 21]. Because the organic matrix of renal calculi has long been considered to influence the crystal growth that occurs in these pathological mineral deposits [7], it was proposed that during urolithiasis, secretion of osteocalcin and the subsequent incorporation of these proteins into the kidney stone matrix may influence the nucleation, growth process, aggregation, and/or tubular adhesion of renal calculi in mammalian kidneys [12]. Because osteocalcin may involve the formation of urolithiasis, it was chosen as an SNP marker.

There are three noncollagenous matrix proteins (osteonectin, osteopontin, and osteocalcin) in osteogenic and chondrogenic development. The expression of mRNA in these genes has been studied, revealing that osteogenic differentiation in bone development is characterized by the sequential expression of these three genes and suggesting that they are expressed differentially and specifically in association with extracellular matrix mineralization [18]. Calcium oxalate urolithiasis has been extensively studied using osteopontin [8, 9, 19]. Among stone patients, a high frequency of GCC-GCT mutations in Ala-250 at exon 7 was recently observed [20]. Therefore, examining the inheritance of Ala-250 within a family could be a method for identifying the predisposing hereditary factors of stone patients.

The present study used a method of PCR-based restriction analysis to approach the SNPs. It might be suitable as a method for studying disease genes in urolithiasis, defining patients at risk of the disease, assisting the exact prognosis of the patients, and possibly selecting appropriate therapies based on specific genetic variations [13]. The likelihood that these regions will contain a candidate gene is growing with the accumulation of mapped genes. Such candidate genes might provide further analysis of tissue expression or clinical presentations in a variety of groups. However, there are few expression sequence library data about stone disease. Therefore, proteome analysis as "reverse genetics" such as two-dimensional electrophoresis and mass spectrometry could be important methods for scanning candidate genes involved in urolithiasis.

Although *Hind* III polymorphism of the osteocalcin gene was shown to influence bone metabolism in the Japanese population [3], there seems to be no association between *Hind* III polymorphism and the risk of calcium oxalate stone disease. This might be caused by ethnic factors or inadequate choice of the polymorphism itself. Other polymorphisms located on this gene could be associated with stone disease. Nevertheless, our results indicate that osteocalcin gene *Hind* III polymorphism is not a suitable genetic marker in screening for the causes of calcium oxalate stone. While using data from Taiwanese patients for a preliminary report, it is suggested that further studies of this form of polymorphism be made.

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