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ORIGINAL PAPER

Heparan sulfate gene polymorphism in calcium oxalate nephrolithiasis

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Abstract Calcium oxalate (CaOx) nephrolithiasis has a complex pathogenic mechanism. Besides environmental factors, genetic factors also have influence on stone formation. This study represents the effects of heparan sulfate (HSPG2) gene polymorphism for determining the risk of urolithiasis. We investigated 143 CaOx stone formers with 158 healthy individuals for the *Bam*HI restriction site polymorphism located in intron 6 of the HSPG gene using the polymerase chain reaction, restriction fragments length polymorphism method. After digestion with *Bam*HI, the polymorphism was assumed to cause three genotypes according to the banding types as GG (242 bp), GT (242, 144, and 98 bp) and TT (144 and 98 bp). According to the genotype frequencies between the groups, TT genotype showed significantly increased risk for urolithiasis than TG and GG genotypes. We concluded that HSPG2 gene polymorphism might be one of the genetic factors affecting the CaOx stone formation.

Keywords Calcium oxalate · Nephrolithiasis · Gene polymorphism · HSPG2

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Introduction

Calcium oxalate (CaOx) nephrolithiasis or stone formation is a complex pathogenic mechanism in which both cellular and extracellular factors play important roles. Normal urine content has inhibitors and other molecules against crystal or nuclei formation. In normal human urine, CaOx precipitation occurs when the saturation of the urine exceeds the solubility by 7–11 times than in water because of these factors. Although supersaturation theory is one of the most accepted theories for stone formation, more authors are becoming interested in the theory stating that oxalate itself induces renal cell injury that promotes crystal attachment and retention of the crystal formation in the renal tubules to form a sufficient nidus. [1, 2] Besides ions like magnesium and citrate which are both strong inhibitors of calcium oxalate and phosphate stone formation, there are anionic molecules including glycosaminoglycans (GAGs), Tamm–Horsfall glycoprotein, other glycoproteins and urinary proteins like nephrocalcin, osteopontin and bikunin [3–5]. In the GAG group, heparan sulfate proteoglycans (HSPGs) are the main kidney GAG occurring both in cell surface (syndecan, glipican) and basement membrane (perlecan) [6].

It has been demonstrated that GAGs inhibit the growth and aggregation of CaOx crystals in vitro, and also it has been shown that stone formers had abnormal urinary GAG levels especially heparan sulfate compared to normal, non-stone forming people [7–9].

Since the genetic basis of common CaOx stone disease still remains unclear except some conditions like Bartter syndrome, Dent's disease, hypocalcemic hypercalciuria, hypophosphatemia, and familial hypomagnesemia [10], different single nucleotide polymorphisms (SNP) are under investigation for such an endemic disease as CaOx nephrolithiasis [11, 12].

In this study, we studied the heparan sulfate proteoglycan (HSPG2) gene polymorphism, which to our knowledge has not been studied before, in both nephrolithiasis patients and healthy individuals.

Materials and methods

The patients

One hundred and forty-three patients, (49 women, 94 men, aged 19–71) referred to our urology clinic for nephrolithiasis, were enrolled into the study. Stone samples were obtained either after ESWL or surgery for treatment, and all patients had stone analysis results confirming that their stone composition was calcium oxalate monohydrate or dehydrate. Also as a control group, from the same geographic area with no history or radiological finding of stone disease, 158 individuals (73 women, 85 men, aged 18–69) were studied. Informed consent was obtained from all subjects in accordance with the Helsinki Declaration 1975 (revised 2001).

Determination of the HSPG2 polymorphism

Genomic DNA was extracted from the peripheral blood leukocytes of the study subjects with an extraction kit (Heliosis, Metis Biotechnology, Turkey) according to the procedures of the manufacturer. The *Bam*HI restriction site polymorphism located in intron 6 of the HSPG gene (rs3767140) which T allele was confirmed to be a risk factor was screened using the polymerase chain reaction-restriction fragments length polymorphism (PCR-RFLP) method described by Hansen et al. [13]. Briefly, 242-bp DNA fragment containing the *Bam*HI restriction site was amplified with the primers: forward 5′-CATGTCCCATG CCCCACGTGTGCT-3′; and reverse 5′-ATTGTAGCTG TGGCAGGCAAACTC-3′. The results were evaluated with two blind observers after electrophoresed in a 2% agarose gel. *Bam*HI digestion resulted in fragments of 144 and 98 bp in the presence of the T allele, where as the PCR product containing G allele was not digested with this restriction enzyme, revealed a 242-bp fragment (Fig. 1).

Statistical analysis for comparing allele and genotype frequencies were carried out using two-sided Pearson's χ^2 and Fisher's exact tests, respectively, with SPSS software. To test for Hardy–Weinberg equilibrium, the expected genotype numbers were calculated from the allele frequencies, and deviation from the observed genotype numbers was determined using the χ^2 test; a P value below 0.05 was accepted as statistically significant.

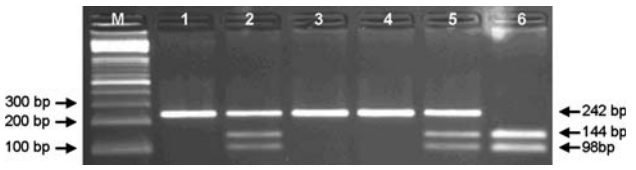

Fig. 1 Two percent agarose gel electrophoresis of *Bam*HI digestion of PCR products containing HSPG gene intron 6 polymorphism. *M* 100-bp molecular size marker, *lane 1* 242-bp PCR product. *Lanes 3–4* show GG homozygote. *Lane 2* and *lane 5* TG heterozygote, *lane 6* TT homozygote

Results

After digestion with *Bam*HI, the polymorphism was assumed to cause three genotypes according to the banding types as GG (242 bp), GT (242, 144, and 98 bp) and TT (144 and 98 bp) (Fig. 1).

The frequencies of genotypes and alleles of the HSPG2 gene for stone formers and control group are presented in Tables 1 and 2. The genotype frequencies of the *Bam*HI G/T polymorphism in the patient group were in Hardy–Weinberg equilibrium. According to the genotype frequencies between the stone formers and control group, TT genotype showed significantly increased risk for urolithiasis than TG and GG genotypes ($P = 0.048$). Furthermore, TT genotype was found to be statistically significant against GT + GG genotypes in the patients and controls ($P = 0.034$). The allelic distribution of T and G alleles were not statistically significant between the two groups ($P = 0.676$).

Discussion

After exposing to oxalate ions and calcium oxalate crystals of renal epithelial cells, there is an increase in production of several urinary macromolecules such as Tamm–Horsfall protein, osteopontin, bikunin, α1-microglobulin, heparan sulfate and matrix Gla protein which all modulate nucleation, growth, aggregation and retention of these crystals in the kidneys. [14]. Recent studies indicate that a crystal–cell interaction is an important step during microlith formation [15]. Although the extracellular events were the focus of stone researches before, currently cellular events are under

Table 1 Genotypic frequencies of HSPG2 gene polymorphism

Genotypes	Urolithiasis group ($n = 143$)	Control ($n = 158$)
GG	85 (59.44%)	85 (53.80%)
TG	54 (37.76%)	73 (46.20%)
TT	4 (2.80%)	0

p1 (GG/TT) = 0.04800, *p2* (GG + GT/TT) = 0.03431

Table 2 Allelic frequency distribution of HSPG2 gene between groups

Alleles	Urolithiasis group ($n = 143$)	Control ($n = 158$)	P value
G	224 (78.32%)	243 (76.90%)	0.676
T	62 (21.68%)	73 (23.10%)	

investigation. Oxalate secreted in urine as an end product of metabolism, can induce renal cell injury promoting crystal attachment and later retention within collecting system of tubules [16]. In most of the studies, urinary glycosaminoglycans containing either heparan (~80%), dermatan (~20%) or chondroitin (~1–2%) sulfate have roles in inhibiting calcium oxalate crystallization [17].

We now know that heparan sulfate is a family of cell surface proteins like syndecan and glypican and basement membrane proteoglycan like perlecan [6] Former studies revealed the importance of HSPG in urolithiasis generally without these subgroups [18]. Syndecan-1 was first HSPG studied and showed evidence as a protector for calcium oxalate nephrolithiasis [19–21], based on rat kidney or cell line experiments. In these studies, although the up regulation of heparan sulfate was shown in CaOx nephrolithiasis, genetic basis for this alteration still remains unclear. In this study, we decided to study with perlecan which is a protein from the same HSPG family and also had a known genetic alteration as a single gene polymorphism. Studies confirming that HSPG2 (Perlecan) was found not only in glomeruli, but also in the tubular basement membrane and peritubular capillaries on human kidney cortex as well as in tubuli located in the papillary area of normal rat kidneys make this study more significant because of these areas' known importance in nephrolithiasis [22, 23].

In a multifactorial disease like urolithiasis, every possible cause must be a matter of investigation. Today especially, the genetic basis of these alterations like single gene nucleotide polymorphisms (SNP) are the interest of researchers in genetic research for polygenic diseases like urolithiasis. For urinary stone disease, a considerable amount of SNPs were observed including urokinase gene 5′-UTR, vitamin D receptor, osteopontin gene, matrix GLA protein, and many others. HSPG2 SNP has been studied in diseases like Alzheimer's disease, prostate cancer, glomerular diseases and chondrodystrophic myotonia which perlecan was shown to play an important role in basement membrane and extracellular matrix structures [24–27].

In this study, we indicated a single gene polymorphism in the structure of heparan sulfate proteoglycans to be important in the etiology of urolithiasis. Our results revealed that TT genotype may be a risk factor for the development of CaOx stones. People with TT homozygous genotype could be accepted to be predisposed to CaOx stone formation with respect to people with GG (wild type) or TG heterozygous genotypes.

Further studies are needed to find out the mechanisms which the changes in heparan sulfate proteoglycans cause or facilitate the urolithiasis formation. Although the etiology remains unclear, there are hypothesis for syndecan-1 which the pathology may be creation of a charge barrier against COM crystal attachment or progression of urothelial damage. Like these, HSPG2 gene polymorphism may be also responsible for the change in the charge or the polarity of the molecule to be a nidus for crystal formation or it can have a decreased inhibitory effect in the urine. Missense mutations and conformational changes about the three dimensional structure of the protein can cause alterations in quality or quantity of it. These hypothetical presumptions need to be evaluated with larger and detailed studies. Detailed and direct evidences could be obtained from experimental models. Also other known metabolic factors like hypercalciuria or hyperoxaluria, and environmental factors like dietary habits must be investigated in relation with these genetic polymorphisms.

In conclusion, to our knowledge, this is the first study to explore the distribution and the possible role of the HSPG2 polymorphism in nephrolithiasis patients. We believe that this significance of gene polymorphism will encourage researchers to carry out detailed molecular studies to clarify the underlying mechanism. Until then, early genetic analysis of people especially living in endemic places or families for polymorphisms like HSPG2, may give clinicians a notice for precautions before the beginning or the follow-up of the disease.

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