SPP1 Gene Polymorphisms Associated With Nephrolithiasis in Turkish Pediatric Patients

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Purpose: To investigate the association between SPP1 gene polymorphisms and nephrolithiasis.

Materials and Methods: A total of 65 pediatric patients and 50 healthy controls were enrolled in this study. Two known polymorphisms of the SPP1 gene, c.240T > C and c.708C > T nucleotide substitutions, both of which were also known as synonymous aminoacid polymorphisms, D80D and A236A, respectively, at SPP1 gene cDNA level, were investigated. SPP1 gene polymorphism was evaluated using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism method.

Results: In c.240T > C polymorphism, C allele frequency [Odds Ratio (OR), 2.13; 95% Confidence Interval (CI), 1.170 to 3.880; $P = .013$] and CC genotype distribution (OR, 2.946; 95% CI, 0.832 to 10.431; $P = .094$) and in c.708C > T polymorphism, T allele frequency (OR, 2.183; 95% CI, 1.197 to 3.980; $P = .011$) and TT genotype distribution (OR, 3.056; 95% CI, 0.861 to 10.839; $P = .084$) were found to be higher in the patient group.

Conclusion: SPP1 polymorphisms were found to be associated with nephrolithiasis and it may be suggested that SPP1 gene polymorphism could be a useful marker for evaluation of the early genetic risk factor in childhood nephrolithiasis.

Keywords: osteopontin, nephrolithiasis, SPP1 gene, genotype, case-control studies, polymorphism

INTRODUCTION

Kidney stone is a complex disease resulting from environmental as well as hereditary factors, and principally composes of approximately 75% calcium oxalate (CaOx) crystals, which are formed through a multi-step process. Calcium stone formation via CaOx crystals involves stages of nucleation, crystal growth, aggregation, and retention in sequence.[1] Urinary analyses of kidney stones show that they contain proteinous ingredients, and several reports have emphasized the importance of proteins in preventing nephrolithiasis.[2,3] Many macromolecules, organics, and inorganics are known to inhibit stone formation, including Tamm-Horsfall, glycosaminoglycans, Bikunin, Calgranulin, and Osteopontin.[1]

Osteopontin (OPN) is a 44-kDa negatively-charged acidic hydrophilic, multifunctional protein encoded by the SPP1 gene that is located on chromosome 4q21-25 and consists of 7 exons.[3-5] Osteopontin is mainly expressed in bone tissue, including osteoclasts as well as osteoblasts, and in other cell types, such as endothelial, smooth muscles, and epithelial cells.[4,6] Osteopontin has many crucial biological functions, including leucocyte function and recruitment, wound repair, cell survival as well as regulation of normal bone resorption and inhibition of urinary crystallization.[1,7] Urinary OPN may prevent the renal stone formation by decreasing the growth and aggregation of CaOx crystals and also inhibits the binding of CaOx crystal directly to renal tubular epithelial cells.

Quantitative and structural investigation of OPN have been studied in renal stones previously by various researchers in order to determine its genetic heritance.[1,3,7] Our aim was to investigate the SPP1 gene polymorphism distribution among pediatric patients with nephrolithiasis and to determine its association with nephrolithiasis.

MATERIALS AND METHODS

The study group consisted of 65 Turkish pediatric patients with nephrolithiasis who were followed-up in Department of Pediatric Nephrology of Celal Bayar University, Manisa, Turkey, and 50 age and gender-matched healthy subjects without a history of nephrolithiasis or a family history of urinary stone disease.

Urinary ultrasonography was also performed for the control group to demonstrate that they did not have any urinary calculi. The patients and healthy groups without any history of nephrolithiasis were selected from the same geographical area as well as race. Blood samples were obtained from both patient and control groups. Written informed consents were obtained from parents of all participants. The study was approved by the local ethics committee.

Molecular Analysis

Molecular analysis was carried out on genomic DNA extracted from ethylenediaminetetraacetic acid (EDTA) anticoagulated venous blood using QiAamp DNA Blood Mini kit (QIAGEN GmbH, Hilden, Germany) according to manufacturer's. c.708C > T polymorphism in the 7th exon of SPP1 gene was genotyped by polymerase chain reaction (PCR) and endonuclease digestion.[5] Polymerase chain reaction amplification (life technology) was conducted using a gradient thermal cycler device in a 25 mL reaction mixture in PCR strip tubes containing 100 ng genomic DNA solution. Platinum Taq Enhancer 2.0 mmol $MgCl_2$, 50 mmol/L each of the dGTP, dATP, dTTP, and dCTP (Promega), 5 pmol each of forward and reverse primers, and 1.0 U Platinum Taq polymerase (Invitrogen Co, Paisley, UK). The sequences of the forward and reverse primers were used; 5'-TACCCTGATGCTACAGACGAGG-3' and 5'-CTGACTATCAATCACATCGGAATG-3', respectively. The cycling conditions comprised a denaturation step at 95 ºC for 10 min, followed by 35 amplification cycles at 95 ºC for 30 sec, 59 ºC for 30 sec, and 72 ºC for 45 sec, and a final extension at 72 ºC for 7 min. The 252 bp PCR products for SPP1 gene were analyzed on a 2.0% agarose gel prestained with ethidium bromide. Genotyping was performed using the AluI (New England Biolabs, Beverly, MA, USA) restriction enzyme as manufactured. Briefly, 10 mkl of PCR product was mixed with 5U AluI and appropriately buffered and incubated at 37 ºC during a 16-h period.

The fragments of 147 + 61 + 44 bp for the TT genotype, 147 + 105 bp for the CT genotype, and four fragments of 105 + 61 + 44 + 42 bp for the CC genotype were separated on a 3% metaphor agarose gel (FMC BioProducts, BioConcept, Allschwill, Switzerland) stained with ethidium bromide, and visualized under ultraviolet light (Figure 1).

DNA Sequencing Method of the SPP1 Gene

Four entire coding exons of SPP1 (OPN) gene (NM_000582) were amplified by PCR using flanking intronic primers (NCBI Reference Sequence: NC_000004.11). All synthetic oligonucleotide primers were synthesized and purchased from Invitrogen (Invitrogen, Paisley, UK) as the HPLC purification grade.

Primer details were:

Exon 4: 5-forward-TTTGTGATCATTTTGTAATGTGG; 5'-reverse-TACGTTTCTTGCACCTCTCG

Exon 5: 5-forward-TTTTCCATTCATCCCTACATTTC; 5'-reverse-ATGGCCTGAGTGTGGCTATC

Exon 6 : 5-forward-CTAATGTGCTATAAAGGCTAA-GGG; 5'-reverse-TGCAAACTGTGGTTTCCTAGAC

Exon 7: 5-forward-CCATATTCCCATCCCTAGCC; 5'-reverse-CAGACTCAAATAGATACACATTCAACC

Polymerase chain reaction amplification was carried out on Corbett Palm-Cycler gradient thermal cycler (Corbett Life Science, Australia) in a 25 µL reaction mixture in 0.2 mL thin-wall PCR strip tubes (Axygen Scientific, Inc., CA) containing 1 µL genomic DNA solution, 1.0 U Platinium TAQ with Enhancer Buffer (Invitrogen Ltd. Paisley, UK), 50 µmol/L each of the dGTP, dATp, dTTP, and dCTP (Promega, Madison,WI), 5 pmol each forward and reverse primers. The cycling conditions comprised of a hot start at 95 ºC for 10 min, followed by 35 amplification cycles at 95 ºC for 45 sec, 58 to 60 ºC (gradient program) for 45 sec, 72 ºC for 45 sec, and a final extension at 72 ºC for 7 min. Before cycle sequencing reactions, the amplified PCR products were purified through enzymatic method using Exo-SAP PCR purification kit (Amersham Life Scinces) according to the appropriate protocol. Cycle sequencing PCR was performed using BigDye Terminator v.3.1 kit as manufacturer's (PE Applied Biosystems, Foster City, CA). Cycle sequencing PCR products after purification with BigDyeXT Terminator kit (PE Applied Biosystems, Foster City, CA) were analyzed by ABI 3130×l Genetic Analyzer. DNA sequencing was performed in both directions, initiated from the forward, and the reverse primers was used in the initial PCR reaction. The primers design was similar to the one used for PCR amplification. For sequence evaluation, the SeqScape 2.0 sequencing analysis software was employed and SPP1 gene NCBI mRNA series (NM_000582.2) was used as reference to determine the nucleotide substitution (Figures 2 to 5). The reflection of this nucleotide substitution to protein structure could be achieved using NCBI protein series reference (NP_000573.1) for evaluation.

Statistical Analysis

All statistical analyses were performed with SPSS software (the Statistical Package for the Social Sciences, Version 11.0, SPSS Inc, Chicago, Illinois, USA) using the Chi-Square and Fisher's exact tests. Univariate odds ratios (OR) and their corresponding 95% confidence intervals (CIs) for different genotypes and their alleles were calculated using logistic regression models. The Hardy-Weinberg equilibrium test was done for each polymorphism.

RESULTS

The study population consisted of 37 (56.9%) males and 28 (43.1%) females with the male-to-female ratio of 1.37. The mean age of the patient group was 84.4 months (range, 4 months to 17.5 years) and the mean age at diagnosis was 67.2 months (range, 3 to 210 months). Twenty-one (32.3%) children were younger than 12 months of age at diagnosis. Follow-up duration was 13 months (range, 1 to 45 months).

Family history of nephrolithiasis was found in 46 (70.8%) patients. Seventeen (26.2%) patients were born from consanguineous marriages. In 15 (23.0%) patients, stones were bilateral, and 29 (44.0%) patients had multiple stones. Only 8 patients had stone analysis. Calcium oxalate was the most common compound. During the follow-up period, 4 (6.1%)

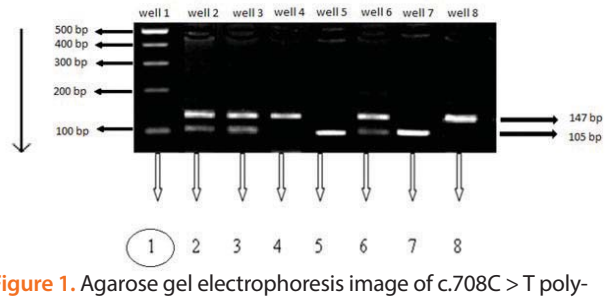

Figure 1. Agarose gel electrophoresis image of c.708C > T polymorphism.
Well 1 was 100 bp DNA ladder
CT genotype was indicated in wells 2, 3, and 6.
TT genotype was indicated in wells 4 and 8.
CC genotype was indicated in wells 5 and 7.

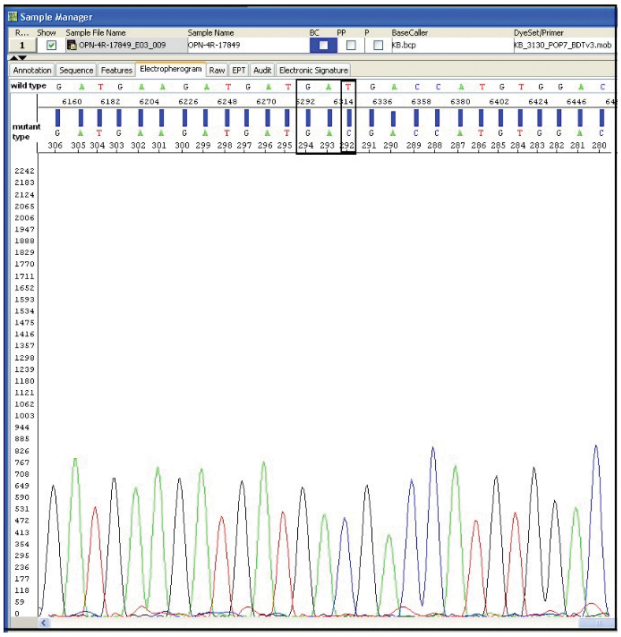

Figure 2. Electropherogram for c.240T > C homozygous nucleotide substitution in exon 6 (D80D) of SPP1 gene.

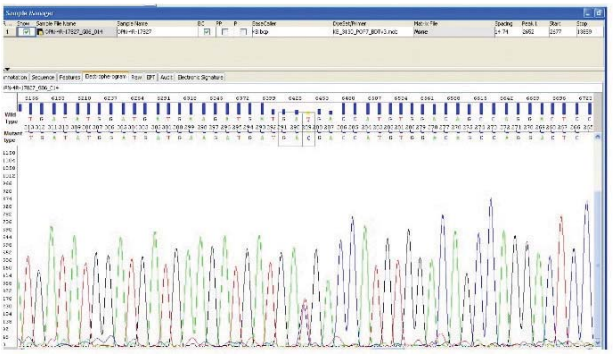

Figure 3. Electropherogram for c.240T > C homozygous nucleotide substitution in exon 6 (D80D) of SPP1 gene.

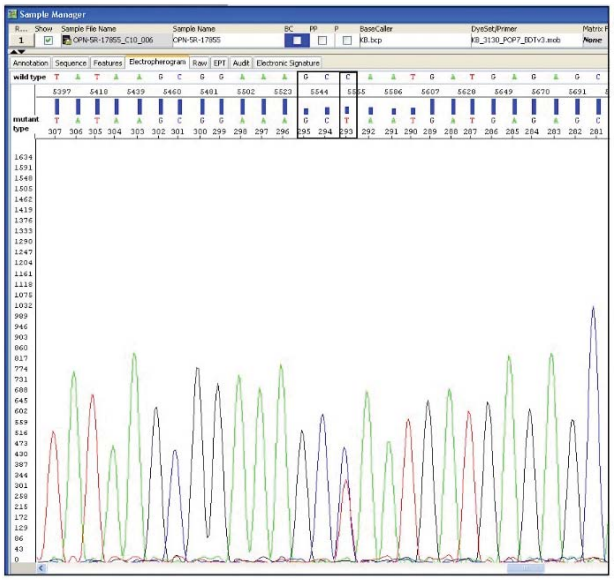

Figure 4. Electropherogram for c.708C > T homozygous nucleotide substitution in exon 7 (A236A) of SPP1 gene.

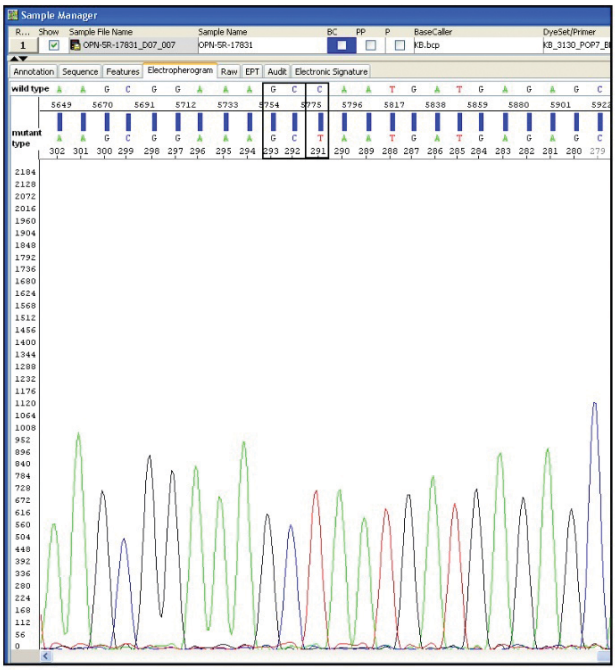

Figure 5. Electropherogram for c.708C > T homozygous nucleotide substitution in exon 7 (A236A) of SPP1 gene.

patients had recurrence.

Genotype distribution and allele frequency of c.240T > C polymorphism was analyzed in each group. In patient group, C allele frequency was higher than in control group (OR, 2.13; 95% CI, 1.170 to 3.880; P = .013). Regarding genotype distribution, the TC genotype (OR, 2.448; 95% CI, 1.066 to 5.622; P = .035) and the CC genotype (OR, 2.946; 95% CI, 0.832 to 10.431; P = .094) were higher in the patient group. Genotype distribution and allele frequencies of c.240T > C polymorphism are shown in Table 1.

Genotype distribution and allele frequency of c.708C > T polymorphism was analyzed in each group. In patient group, T allele frequency was higher than in control group (OR, 2.183; 95% CI, 1.197 to 3.980; P = .011). Regarding

Table 1. Genotype distribution and allele frequencies of c.240T > C polymorphism in patients and controls.

	Patient	Control			
Genotype	**n (%)**	**n (%)**	**Odds Ratio**	**95% Confidence Interval**	***P***
CC	10 (15.4)	4 (8)	2.946	0.832 to 10.431	.094
TC	27 (41.5)	13 (26)	2.448	1.066 to 5.622	.035*
TT	28 (43.1)	33 (66)	1 (ref)		
Total	55 (100.0)	50 (100.0)			
Allele					
C	47 (36.2)	21 (21.0)	2.130	1.170 to 3.880	.013*
T	83 (63.8)	79 (79.0)	1 (ref)		
Total	130 (100.0)	100 (100.0)			

*$P < .05$

Table 2. Genotype distribution and allele frequencies of c.708C > T polymorphism in patients and controls.

	Patient	Control			
Genotype	**n (%)**	**n (%)**	**Odds Ratio**	**95% Confidence Interval**	***P***
TT	10 (15.6)	4 (8)	3.056	0.861 to 10.839	.084
CT	27 (42.2)	13 (26)	2.538	1.102 to 5.848	.029*
CC	27 (42.2)	33 (66)	1 (ref)		
Total	55 (100.0)	50 (100.0)			
Allele					
T	47 (36.7)	21 (21.0)	2.183	1.197 to 3.980	.011*
C	81 (63.3)	79 (79.0)	1 (ref)		
Total	130 (100.0)	100 (100.0)			

*$P < .05$

genotype distribution, the CT genotype (OR, 2.538; 95% CI, 1.102 to 5.848; $P = .029$) and the TT genotype (OR, 3.056; 95% CI, 0.861 to 10.839; $P = .084$) were higher in the patient group. Genotype distribution and allele frequencies of c.708C > T polymorphism are shown in Table 2.

In case-only analysis of patients with nephrolithiasis, c.240T > C and c.708C > T polymorphisms were found not to be associated with urinary metabolic risk factors, gender, early age at diagnosis, positive family history, consanguinity, stone recurrence, and bilateral or multiple stones (Table 3).

DISCUSSION

Nephrolithiasis is a commonly known disease threatening the human kind for many years. There are not many studies investigating the genetic role of OPN in renal stones. Some researchers have been emphasized the importance of the relation between OPN and crystal formation. Limited number of studies have been conducted on OPN gene structure for familial and recurrent renal stones.[7]

Single nucleotide polymorphisms (SNP) of the human OPN gene has been reported to be associated with many diseases.[5,8-11] Especially, Gao and colleagues have investigated 61 polymorphisms and evaluated four haplotypes among these polymorphisms. Two of these haplotypes have been identified as risk factors for nephrolithiasis. They have tried to shed a light to understand the mechanism of how the OPN gene can change the structure of OPN molecule.[7]

In this study, we have investigated the difference in SPP1 gene polymorphisms between patients with nephrolithiasis

Table 3. Genotype distribution and allele frequencies of c.240T > C and c.708C > T polymorphisms and urinary metabolic risk factors.

	Hypercalciuria	Hyperoxaluria	Hypocitraturia	Hyperuricosuria	Cystinuria	Hypomagnesuria
n (%)	15 (23.1)	14 (21.5)	23 (35.4)	16 (24.6)	9 (13.8)	10 (15.4)
240T > C						
TT	6 (40.0)	5 (35.7)	9 (39.1)	6 (37.5)	5 (55.6)	5 (50.0)
TC	6 (40.0)	2 (14.3)	8 (34.8)	6 (37.5)	3 (33.3)	4 (40.0)
CC	3 (20.0)	7 (50.0)	6 (26.1)	4 (25.0)	1 (11.1)	1 (10.0)
P	0.8	0.7	0.2	0.4	0.7	0.8
T	18 (60.0)	17 (60.7)	26 (56.5)	18 (56.3)	13 (72.2)	14 (70.0)
C	12 (40.0)	11 (39.3)	20 (43.5)	14 (43.8)	5 (27.8)	6 (30.0)
P	0.6	0.6	0.1	0.3	0.4	0.5
708C > T						
CC	6 (40)	5 (35.7)	9 (39.1)	6 (37.5)	4 (50.0)	5 (50)
CT	6 (40)	2 (14.3)	8 (34.8)	6 (37.5)	3 (37.5)	4 (40)
TT	3 (20)	7 (50.0)	6 (26.1)	4 (25)	1 (12.5)	1 (10)
P	0.8	0.7	0.2	0.4	0.7	0.8
C	18 (60.0)	17 (60.7)	26 (56.5)	18 (56.3)	13 (72.2)	14 (70.0)
T	12 (40.0)	11 (39.3)	20 (43.5)	14 (43.8)	5 (27.8)	6 (30.0)
P	0.6	0.7	0.2	0.3	0.6	0.4

and normal subjects. Two SNPs in SPP1 gene were found to be associated with nephrolithiasis. As was previously stated in literature, although both nucleotide substitutions take place in the SPPI gene coding region, this does not cause change in aminoacid and is identified as synonymous mutations.

Synonymous substitutions between nucleotides in genes produce structurally identical OPN. Difference may be present in the sequence of transcription factor binding sites and synthesis of the transcription factors suggesting that a change in transcription step may generate differences in the amount of mRNA and protein.[12]

In recent quantitative studies, reduced excretion of OPN was thought to be due to reduced synthesis of OPN or its incorporation into growing stones with normal amount of synthesis.[13] In a rat model, OPN and its mRNA were enhanced in rats in which urinary stone formation was induced with oxalate precursor.[14] Furthermore, in an urolithiasis model, OPN mRNA was observed to enhance in ethylene glycol-administered rats.[3]

Yamate and colleagues investigated the difference in OPN DNA between normal subjects and patients with urolithiasis. Nucleotide substitution of GCC to GCT, encoding synonymous Ala-250, was reported to be higher in patients with urolithiasis. Based on the difference in gene frequency between groups, examination of Ala-250 inheritance was suggested to be a diagnostic method for patients with urolithiasis and a predisposing hereditary factor.[12] Gao and coworkers reported that an SNP at position 9401 was determined to be seen more frequently in patients with renal stones. Hence, they have speculated on the relationship between OPN sequence variants and the risk of nephrolithiasis. Due to these results, they have stated that there is an association between OPN and calcium stones and it might be a candidate genetic marker for evaluating the genetic risk of renal stone disease[15] In our study, we have found no association between urinary metabolic risk factors and OPN polymorphisms.

There are certain factors indicating that urolithiasis may be related to genetic predisposition, such as family history of urolithiasis, higher incidence of recurrences, and early onset of the disease in such patients, and drag the clinical

focus to genetic factors.[16,17] However, we have not found any association between family history and early presentation of nephrolithiasis and OPN polymorphisms.

In a study from Turkey performed on adult patients with nephrolithiasis, the association of A236A SNP in 7th exon as well as -593T > A polymorphism in the promoter region with nephrolithiasis was investigated. In this study, the importance of this polymorphism in the promoter region of the SPP1 gene is emphasized.[18]

Protein coding exon region of the SPP1 gene was investigated in the present study. We have determined that the SNP is located on this region. It was aimed to associate the synonymous aminoacid mutation distributions of the more common SNP's D80D and A236A with phenotypic properties. We have also confirmed new polymorphisms in SPP1 gene (unpublished data).

The study was performed on a low number of patients and therefore it may have insufficient statistical power. This may be a limitation of the present study. We did not find a statistically significant relationship between CC genotype and nephrolithiasis (Table 1) or between TT genotype and nephrolithiasis (Table 2). This may be due to the low number of study sample. The power for the comparison of CC genotype between patients and controls was 21.4% and that was also found 22.3% for the comparison of TT genotype between patients and controls. Therefore, this should be studied in greater number of subjects.[19]

CONCLUSION

To the best of our knowledge, this study is the first in literature to investigate the relationship between childhood nephrolithiasis and OPN. SPP1 polymorphisms were found to be associated with nephrolithiasis and we suggest that OPN may be a useful marker evaluating the early genetic risk of childhood nephrolithiasis. Although SNPs were determined as synonymous, it is still necessary to measure the expression of OPN mRNA in urine and blood to further clarify this association. We believe, in the future, further studies on different races and ethnic groups are required to verify our findings and clarify the relationship between childhood nephrolithiasis and OPN.

CONFLICT OF INTEREST

None declared.

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