

A Preliminary Study on Single Nucleotide Polymorphisms (SNPs) in SPP1 Gene with Higher Threat of Urolithiasis in Victims from West Bengal, India

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Urolithiasis is a painful experience associated with hematuria, damage to kidney tissue and renal failure. It is a multi-factorial disorder while in India, its prevalence is rapidly increasing imposing a large burden for both healthcare and economy globally. In this article, we aimed to evaluate the association between genetic defects in SPP1 gene and urolithiasis from East Indian patients. 75 urolithiasis patients were recruited from SSKM Hospital & Institute of Post Graduate Medical Education & Research (IPGME&R), Kolkata, India while 75 healthy controls were recruited from the same community. SNPs based areas of SPP1 gene were analyzed by direct sequencing to identify genetic defects. We identified 3 polymorphisms one synonymous and two 3'UTR variants rs1126616: p.Ala250Ala, rs1126772: 7315 a>g, rs9138: 7471 a>c in SPP1 gene in study individuals. Genotype and allele frequency analysis of these SNPs revealed that, rs9138 SNP was significantly associated with urolithiasis risk in East Indian patients. To our knowledge this is the first study reporting the role of the gene with urolithiasis in the population of West Bengal, India.

Keywords. Kidney tissue, Renal failure, Human genetics, Eastern part of India

Short Title: Association of SPP1 gene with urolithiasis in East Indian study group

1. Introduction

In dearth attempt in understanding pathogenic mechanisms of urolithiasis causes a crucial barrier to its early detection and treatment. In spite of being a major issue, fragmented studies have conducted towards renal stones in northern Indian region¹⁻², while our published data on genetic association of renal stones in calcium homeostasis³ is believed to be the first published report in East Indian population. In the realm of renal stones, secreted-phosphoprotein 1(SPP1) is reported to be an important modulator⁴⁻⁵, whereas rs9138 (7471 a>c) of SPP1 gene is established to be highly polymorphic⁶⁻⁹. Thus, the rationale of our present study is to analyse association of SPP1 gene variations with urolithiasis in West Bengal, eastern part of India study group population which potentially believed to be the first ever report herein.

Urolithiasis a common painful ailment which forms in kidneys when normal substances of urine become too concentrated¹⁰. Based on our recent work on genetic association of renal stones in calcium homeostasis pathway, we reported calcification procedure in which genotype frequency of rs9138 polymorphism was considerably connected with the risk of renal stone formation ($p=0.001$)³. To continue the discussion further in the realm of genetically natural inhibitor, references can be made where SPP1 acts as a natural inhibitor of abnormal calcification in the kidneys including crystallization, crystal retention and crystal congregation¹¹⁻¹³. To investigate the connection between SPP1 with West Bengal, India population, a preliminary study of 75 patients having renal stone with age and sex matched 75 healthy individuals is being conducted. Three variants of SPP1 gene reported (rs1126616: p.Ala250Ala, rs1126772: 7315 a>g, rs9138: 7471 a>c) here in our preliminary study. As a future scope, we are continuing our work to establish the correlation between SPP1 gene variants and urolithiasis in larger sample size to understand better of useful early genetic markers.

2. Materials and Methods

2.1 Study participants

The pilot study has been executed with 75 urolithiasis patients from SSKM Hospital & Institute of Post Graduate Medical Education & Research (IPGME&R), Kolkata, India. Age and sex complimentary healthy subjects without a history of renal stone or ancestral histories were taken as controls from the same community. The clinical characters of these patients containing serum creatinine, serum calcium and urinary calcium excretions are shown in Table 1. Patients having any hormonal or other disorders besides urolithiasis with abnormal clinical parameters were excluded. The study design was endorsed by IPGME&R ethics committee, Kolkata, India with participant's written consent.

Table 1. Clinical variables of urolithiasis patients and controls

Variables	Cases; n=75	Controls; n=75	p-value
Age (years)	39.32±10.19	38.63±10.13	0.0477
Sex: Male/Female	51/24	49/26	0.862
BMI (kg/m ²)	22.10±1.19	22.20±1.34	nonsignificant
Serum calcium (mg/dl)	9.39±0.19	9.44±0.17	nonsignificant
Urinary calcium (mmol/24 h)	7.53±0.52	5.12±0.52	<0.001
Serum creatinine (mg/dl)	1.23±0.64	0.721±0.10	<0.001

* p-values given are averages ± standard deviation

2.2 Genotyping

Blood specimens were drawn from the urolithiasis patients and healthy subjects in ethylenediaminetetraacetic acid (EDTA) anticoagulated vial. Genomic DNA was extracted using the isolation kit (QIAamp Blood Kit, QIAGEN, Germany). SNPs based areas were

amplified by PCR using a gradient thermal cycler due to their reported association. Amplification was taken in a tube of 25 μ l volume carrying 100ng of DNA, 0.5 μ l of designed primer (10 μ M), 0.5 μ l of dNTP mix (10 mM; Invitrogen, USA), 1 μ l magnesium chloride (50 mM), 2.5 μ l PCR buffer (10x) and enzyme Taq DNA Polymerase of 1.5 units (5 units/ μ l; Invitrogen). The primers were formulated using software IDT and primer3.

Primer details were:

P1: 5'- Forward TACCATATTCCCATCCCTAGCC

P1: 5'- Reverse GGAGTTTCCATGAAGCCACAA

P2: 5'- Forward CCAAAGTCAGCCGTGAAT

P2: 5'- Reverse AAACATCACACCGTACCC

The cycling conditions that we followed is as herein: (i) preliminary denaturation was conducted at 95°C for 3min trailed by 42 cycles for 30s, (ii) following denaturation was annealing procedure conducted at 58°C-64°C for 45s, extension at 72°C for 45s, and (iii) finally it was extension undergone at 72°C for 5min. The Cycle sequencing PCR products were fulfilled using the Big dye terminator kit v 3.1 (Applied Biosystems, Foster City, USA) on an ABI prism DNA sequencer (Model 3700; Applied Biosystems, Foster City, USA). Finally, sequencing was performed in both forward and reverse directions and sequences were aligned in between case and control individuals using ClustalW program.

2.3 Statistical methods

Comparison of mean values of continuous self-determining variables (age, serum creatinine, calcium) between cases and controls were done using t-test. The genotype and allele frequencies in cases were compared with control subjects using the chi-square test. Chi-squared

or Fisher exact test were taken when desirable for examining the association of each SNP. Chi-square tests were used to determine whether individual SNP are in Hardy–Weinberg equilibrium ($p < 0.001$). Mann-Whitney U test was done to investigate nonparametric variables. Association analyses for dominant, recessive and co-dominant effect of each of the polymorphisms were measured using Graphpad InStat software (SanDiego, CA).

3. Results and Discussion

Our study has been conducted with total 75 urolithiasis patients including 51(68%) male and 24(32%) female participants. Mean age with standard deviation (SD) for the case group was 39.32 ± 10.19 years. Our study population is unbiased in terms of age and sex. Males have higher frequency of manifestation of stones than female participants (2:1). Compared to control individuals, there was no significant difference documented about BMI at the time of sampling. In our study serum creatinine, serum calcium and urinary calcium excretion in both cases and controls were evaluated. The analysis released that levels of serum creatinine [(case- 1.23 ± 0.64 ; control- 0.721 ± 0.10); $p < 0.0001$] and urinary calcium excretion [(case- 7.53 ± 0.52 ; control- 5.12 ± 0.52); $p < 0.0001$] were significantly increased in case than in control participants.

Association of genetic polymorphisms of SPP1 gene and risk of urolithiasis was evaluated in our study. From this genetic study we identified 3 polymorphisms one synonymous and two 3'UTR variants rs1126616: p.Ala250Ala, rs1126772: 7315 a>g, rs9138: 7471 a>c in SPP1 gene in study individuals. The frequencies (allele and genotype) of these polymorphisms are shown in Table 2 and 3.

The risk allele frequency of rs1126616 (T) were 38% and 28% for cases and controls respectively (OR = 1.58; 95% CI= 0.97–2.56; $p = 0.086$). G allele of rs1126772 had 2.94 times

increased risk (OR= 2.94; 95% CI=1.56–5.55; p=0.001) in urolithiasis patients. The allele frequencies of rs1126616 and rs1126772 in the present study were quiet similar compared to European and Asians (NCBI Hapmap project). Towards the end, allele frequencies of rs9138 (OR= 2.24; 95% CI=1.30–3.87; p-0.005) were dissimilar compared to East Asians (A: 29%, C: 71%) but similar to South Asians populations (NCBI Hapmap project) (Table 2).

Table 2. Allele frequencies of SPP1 gene polymorphisms of urolithiasis patients and controls

SNP	Allelomorph	Allele Frequencies		OR(95% CI)	p-value
		Cases ; n=75	Controls ; n=75		
rs1126616:c>t	C	93(0.62)	108(0.72)	Ref	0.086
	T	57(0.38)	42(0.28)	1.58(0.97-2.56)	
rs1126772:a>g	A	111(0.74)	134(0.89)	Ref	0.001
	G	39(0.26)	16(0.11)	2.94(1.56-5.55)	
rs9138:a>c	A	102(0.68)	124(0.83)	Ref	0.005
	C	48(0.32)	26(0.17)	2.24(1.30-3.87)	

*p-values for chi squared test. OR, odds ratio; CI, confidence interval

Among these three SNPs, two (rs1126772 and rs9138) showed significant association with urolithiasis in our study population. Odds ratio under a dominant model showed a strong association for rs1126772 (OR=2.67, 95% CI=1.28-5.54; p=0.013). In contrast, rs1126616 showed no association with urolithiasis which overlaid the necessity for future study with larger population (OR=1.38, 95% CI= 0.73-2.62; p=0.414). We observed a strong association of 3'UTRvariant rs9138: 7471 a>c in our study group. 3 fold increased risk of genotype AC was associated with for urolithiasis patients compared with the wild type genotype AA(OR=3.25, 95% CI=1.64-6.44;p=0.001). Odds ratio under a dominant model showed a strong association for rs9138 (OR=3.33, 95% CI=1.71-6.49; p-0.001).Our result positively explained that risk to develop urolithiasis is increased with the predominance of carrying C allele or CC genotype in our population (Table 3).

Table 3. Genotype frequencies of SPP1 gene polymorphisms of urolithiasis patients and controls

SNP	Genotype	Cases/ Controls	OR(95% CI)	p-value
rs1126616:c>t	CC	33/39	Ref	
	CT	27/30	CC vs CT: 1.06(0.53-2.13)	0.997
	TT	15/6	CC vs TT: 2.95(1.03-8.48)	0.069
			CC vs CT+TT: 1.38(0.73-2.62)	0.414
rs1126772:a>g	AA	45/60	Ref	
	AG	21/14	AA vs AG: 2.00(0.92-4.36)	0.118
	GG	9/1	AA vs GG: 12(1.47-98.18)	0.012
			AA vs AG+GG: 2.67(1.28-5.54)	0.013
rs9138:a>c	AA	30/51	Ref	
	AC	42/22	AA vs AC: 3.25(1.64-6.44)	0.001
	CC	5/2	AA vs CC: 4.25(0.78-23.28)	0.167
			AA vs AC+CC: 3.33(1.71-6.49)	0.001

* p-values for chi squared test.

SPP1 as a regulator of mineralization is highly related with calcification process especially in the matrix of renal stones¹⁴⁻¹⁵. It was reported that the concentrations of SPP1 was dropping in urine in stone formers¹⁶. Genetic association studies with SPP1 gene have been studied by various researchers in various populations either with urolithiasis or various malignant disorders or inflammation, leukocyte recruitment, cell survival and wound healing which paved away for our present study further¹⁷⁻¹⁹. In an earlier published report it was elucidated that SNPs in SPP1 gene poses nontrivial influence in patients with recurrent urolithiasis²⁰. These relevant earlier results demonstrate a podium for further investigation of SPP1 significance in urolithiasis. In this realm, our preliminary result on Genotype and allele frequency analysis demonstrates that, rs9138 in exon 7 is expressively associated with urolithiasis while serum creatinine and 24 hours urinary calcium excretion revealed substantial difference between study groups ($p < 0.001$). Together these findings strongly indicate, a proper power calculation with larger sample size should be used for future research direction with a more robust correlation between SPP1 and urolithiasis therein.

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Author Contributions

Conceived and designed the experiments: MD. Performed the experiments: MG. Analysed the data: MG, HB. Contributed reagents/materials/analysis tools: MD. Wrote the paper: MG, HB. Gave patients samples: DKP.

Conflict of Interest

We declare that there is no conflict of interest among the authors.

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