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AHSG Gene In silico by Bioinformatics and Genotyping in Urinary Oxalate Stone Patients

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Abstract

The polymorphism of the plasma glycoprotein Alfa 2 HS glycoprotein (AHSG), also called as fetuin-A, a powerful inhibitor of calcification, is least studied in kidney stone disease. The purpose of the study is to select single nucleotide polymorphisms of AHSG gene by bioinformatics, compare patients with and without urinary oxalate stones in terms of the distribution of AHSG gene polymorphisms, haplotypes and associated linkage disequilibrium (LD). Bioinformatics analysis was carried out with SIFT, Provean and I –mutant tools. A total of 100 people were included in the study, of whom 50 served as case studies and the other 50 as controls. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism was used to analyze the fetuin-A c.742C>T and c.766C>G Single Nucleotide Polymorphisms (SNPs) (PCR–RFLP). SHEsis-plus and SNP-stat online were used to analyze the linkage disequilibrium between the SNPs. Bioinformatics tools could differentiate damaging and neutral polymorphisms. The distribution of wild and mutant alleles of fetuin-A c.742C>T and fetuin-A c.766C>G did not differ significantly. The two AHSG SNPs displayed a strong LD of D' 0.93 & R2 is 0.77, indicating a strong allele-to-allele correlation. Alleles of the haplotype CT and CG in patients had a greater significant level (p>0.0001). The connection of dominant, recessive, and co-dominant alleles among the alleles of c.742C>T and c.766C>G was insignificant. Bioinformatics tools were helpful in the pre-selection of SNPs. The study's findings suggest that there is no conclusive evidence linking renal stone disease with AHSG gene variants. The co-inheritance of the alleles is supported by the LD value (D' & R2) of both SNPs. However, the kidney stone disease demonstrated a significant connection with the CT and GC haplotypes of c.742C>T and c.766C>G.

Keywords: AHSG, alleles, polymorphism, renal stone, linkage disequilibrium

Full length article **Corresponding Author*, e-mail:ushachidu@yahoo.com

1. Introduction

Crystal growth in the kidneys is the first sign of renal stone disease. According to estimates, 12% of the world's population suffers from urological disease. At least 10% of people in developed nations are expected to have urinary tract stone disease [1]. With an annual incidence ranging from 0.5 percent to 1.9 percent, kidney stones are common in developed nations [2]. The occurrence of upper and lower urinary tract stones varies substantially by region in India [3]. The incidence of renal calculi is lower in the southern part of the nation compared to other regions [2]. It has been connected to an increased risk of renal failure in its advanced stages [4]. There are a number of variables that can affect the development of calcium oxalate stones, including hypercalciuria, hyperoxaluria, hypocitraturia, and hypomagnesuria [5]. A pH of 5-6.5 in the urine encourages the development of calcium oxalate stones, while a pH of more than 7.5 encourages the development of calcium phosphate stones [6,7]. These stages are controlled by an imbalance between the chemicals that promote or prevent urine crystallization [8]. Finding the chemicals and metabolic modifications that affect stone formation may enable intervention. One of these compounds that has gotten the least attention is AHSG. It may be possible to use AHSG as a biomarker to foretell the development of calcium oxalate stones.

By forming compounds with minerals in vitro, Price and Lim showed that fetuin-A suppresses the precipitation of hydroxyapatite from supersaturated calcium and phosphate solutions [9]. Additionally, it was discovered that urolithiasis patients had lower urinary fetuin-A levels than healthy participants, with a sensitivity of 97% and a specificity of 100% [10]. Aksoy et al. looked into the function of fetuin. A gene polymorphism in the pathophysiology of calcium

oxalate stone formation was studied, and the researchers came to the conclusion that this could raise the risk of calcium oxalate stone formation [11]. There is little information on gene polymorphisms and their linkage disequilibrium in nephrolithiasis.

Study Objectives:

1. Evaluate the single nucleotide polymorphisms of AHSG gene with bioinformatics tools

2. To evaluate the pattern of AHSG gene polymorphisms and their linkage disequilibrium in patients with urinary oxalate stones as compared to healthy controls.

3. To compare patients with and without urinary oxalate stones in terms of their AHSG haplotype patterns.

2. Materials and methods

Selection of SNPs by Bioinformatics In-silico analysis was carried out for the Missense mutation of Fetuin-A using SIFT, PROVEAN and I-Mutant software to detect the deleterious and tolerant variants.

2.1. Evaluation of the Functional Impact of Coding nsSNPs Using a Sequence Homology Tool sorting intolerant from tolerant (SIFT): SIFT score varies from 0-1. Substitutions at every role with much less than a tolerance index of 0.05 have been expected as “intolerant” or “deleterious”, even as the ones more than or same to 0.05 as “tolerated”

Evaluation of the Functional Impact of Coding nsSNPs Using PolyPhen2: Polyphen2 was used to analyze the possible influence of an amino acid nsSNPs on the structure, as well as function of the protein analyzed by multiple sequencing. Three points in common have been obtained by this software; “benign”, “possibly damaging” and “damaging" are based on scores 0.0-0.15, 0.15-1.0 and 0.85-1.0 respectively.

Validating the deleterious nsSNPs through PROVEAN: The biological consequences of the observed mutations were confirmed using PROVEAN, a separation predictor between neutral and harmful amino acids, based on a threshold of -2.5, substitutions were projected. is predicted to be harmful when less than ≤ -2.5.

Evaluation of the Functional Impact of Coding nsSNPs Using I mutant 3.0: This tool is used to predict the changes in a protein's stabilit y following a single point mutation. I-Mutant 3.0 predictions are performed either starting from the protein structure or, more importantly, from the protein sequence. I Mutant 3.0 showed <0, which implies decreased stability.

An observational cross-sectional study was carried out between June 2020 and March 2022. Patients who visited the Department of Urology at the Justice K. S. Hegde Charitable Hospital in Mangalore, Karnataka, India had kidney stone disease, as determined by ultrasonography were recruited. Additionally, blood samples were studied in the Molecular Genetics and Central Research Laboratory wings of the KS Hegde Medical Academy. Samples of kidney stones were taken either following surgery or extracorporeal shock wave lithotripsy for therapy. Biochemical techniques were used to examine the calculi. The study only included patients with calcium oxalate stones. The investigation was started after receiving approval from CEC Ref, NU/CEC/2020/0289 Nitte (Deemed to be University). Patients who met all of the aforementioned criteria were considered cases; By means of convenient selection, 50 patients in the age range of 18 to 65 years, of either sex, with kidney stones that were proven to be COM or COD were selected. Patients who agreed to participate in the trial with their consent were included. Patients who met even one of the following requirements were disqualified; Patients with cysteine/uric acid stones identified with qualitative testing and People with primary hyperparathyroidism who have been examined. Participants who met all of the following requirements were included as controls; Fifty healthy volunteers (18–65 years old, either gender) without urinary stones, as determined by ultrasonography, Healthy individuals willing to take part in the study and Subjects with the history of urinary stone or family history of urinary stone and Gout were excluded.

2.2. Laboratory Analysis

Genotyping: 5 ml of blood were drawn and placed in EDTA vacutainers to study gene polymorphisms. The salting out approach was used to isolate DNA[12]. The spectrophotometer (ratio of OD260/OD280) was used to verify the quantity and purity of the DNA. Gene genotyping was validated by PCR-RFLP. The fetuin-A c.742C>T and c.766C>G single nucleotide polymorphisms were examined using the PCR-RFLP method. Molecular grade water (Himedia) and Taq DNA Polymerase Master Mix RED (1.5Mm MgCl2 Concentration, NH4+ buffer system, dNTPs, and the front of red tracking dye runs at 300-1000bp on 0.5-1.5% agarose gel) were used to create PCR mixtures of volume 25 l. (Ampliqon IIII) 0.5 l of forward and reverse primer (Sigma-Aldrich) and 1 l of DNA (300–500 ng/ml) are required. The fetuin-A 742C>T polymorphism was amplified using Primer 3Plus utilizing the forward and reverse oligonucleotide primers 5'-CCTCCCACAAGCAGAAAC-3' and 5'-TGATGATTC-CGCATACCC-3', respectively. MiniAmp + Thermal cycler was used for amplification (Thermofischer Scientific). The PCR procedure was carried out in 35 cycles, with the first denaturation taking place at 95 °C for 5 minutes, the second at 94 °C for 1 minute, the third at 58 °C for 1 minute, the fourth at 72 °C for 1 minute, and the fifth and final extension taking place at 72 °C for 5 minutes. The PCR Product was examined using a Gel DocTM EZ imager from Bio-Rad and a Mini-PROTEAN Tetra Cell from Bio-Rad, USA, along with 0.5 g/ml of ethidium bromide and DM012-R500 50 bp DNA Ladder. Gene Direx, Inc.'s ready for usage in the TAE Buffer (1X). A 3% agarose gel electrophoresis was used to separate the PCR products after they had been digested with 0.5 l NlaIII (NEB) restriction endonucleases overnight at 37°C. The digested products were then visible using ethidium bromide. The oligonucleotide primers forward 5'-GTCAC-CCCTCCTTGTAAC-3' and reverse 5'-CCCCAATGAGAC-CACA-3' were used to

analyse the fetuin-A 766C>G polymorphism. The PCR procedure was carried out in the following steps: initial denaturation at 95 °C for 5, 35 cycles denaturation at 94 °C for 1, annealing at 56 °C for 1, extension at 72 °C for 1, and a final extension at 72 °C for 5. The SacI restriction enzyme was used to digest the PCR product overnight at 37°C, and the digested products were sorted on a 3% agarose gel. The serum's FETUINA gene expression was measured using an ELISA kit (Fine Test, Wuhan Fine Biotech Co.,Ltd.). Biochemical parameters like serum calcium, phosphorus and creatinine were estimated by semiautomated chemistry analyzer. Estimated Glomerular Filtration Rate (eGFR) was calculated using Modification of Diet in Renal Disease (MDRD) formula.

2.3. Statistical analysis

The statistical analysis was carried out utilizing SPSS version 23. In order to determine if observed and anticipated alleles are in equilibrium, the Hardy Weinberg equilibrium was calculated. The chi square test was used to examine the relationship between the genes and stone creation. Web-based Shesis-Plus (http://shesisplus.bio-x.cn/SHEsis.html) software was used for the LD analysis, and SNP-stat (https://snpstats.net/start.htm) was used for the online haplotype analysis.

3. Results and Discussions

Out of 809 number coding variants (100%), 777 coding variants are predicted (96%), 457 were tolerated (58%), 320 were damaging (42%), 784 were non synonymous (96%), 24 were synonymous (4%) and 758 of them were Novel. SIFT analysis of AHSG c.742C>T & c.766C>G (amino acid change S256N & M248T) was found to be tolerated with a sift score 0.58 & 1, these were analyzed by Polyphen2 tool with a score 0.83 suggesting damaging & 0.00 suggesting benign, Provean tools values found to be <-2.5 suggesting deleterious. I mutant suite 3.0 was used to predict the effects of single point mutation of these two SNPs. DDG value of these SNPs showed values <0 implying decreased stability. Demographic data of the cases and controls showed that they were age matched [no significant difference in age (controls 33.7±10.47 vs cases 36±13.87) (p=0.17)]. The Hardy Weinberg equilibrium study of the two AHSG SNPs revealed that there is no significant difference between the anticipated and observed alleles. The distribution of wild and mutant types of alleles of Fetuin-A c.742C>T and Fetuin-A c.766C>G (tables 1 and 2) did not significantly differ. D' and r2 were calculated to determine the degree of LD in paired combinations of SNPs. LD between the c.742C>T and c.766C>G AHSG haplotypes was discovered utilizing the SHEsis-plus computer platform. The analysis excluded haplotypes with frequencies of less than 0.03 from consideration. LD looked at the analysis of combined genotyping data between two SNPs for patients and controls. Two LD plots were produced. The two SNPs of fetuin-A showed a strong LD of 0.93 (Figure 3A), as suggested by high the D' values. The R2 value of 0.77 (Figure. 3B) supports the co-inheritance of the above alleles. If the D’ and R2 values are closer to one, it suggests a strong co-inheritance of the alleles. Values that are closer to zero or equal to zero indicate that the alleles are only weakly inherited together. The SNPs of AHSG were found to have higher D' values, indicating their co-inheritance. A ‘highly significant association was observed between kidney stone disease and Haplotypes C, T, G, C SNPs (p<0.0001) (table 3). The CT allele was significantly associated with kidney stone disease (Chi-squared=6.185, p=0.028). Other gene interactions were statistically significant as shown by binary analysis (table 4).

The SNPs of the fetuin gene are analysed using the linkage disequilibrium map. The pairwise linkage disequilibrium relationship between the two SNPs is represented by the gene, LD R2 values. R2 values between 0 and 1 suggest bright coloured squares, whereas R2 = 0 indicates low linkage disequilibrium (darkest coloured square) (lightest coloured squares). However, both D' and R2 are taken into account when forecasting the co-inheritance of the alleles. Using the SNP Stat online tool in a pairwise fashion between the SNPs, the haplotype connection with kidney stone disease was assessed. With the exception of the CT allele, the relationships had a p>0.05 significance level. The fetuin-A c.742C>T haplotypes did, however, differ in a statistically significant way (p=0.028). The SNPs using Shesis-plus and SNP Stat online analysis allowed us to determine that the fetuin-A alleles were in equilibrium. High D' and R2 values supported its co-inheritance. The gene's haplotypes and the condition of kidney stones were not significantly correlated. A reliable screening marker for fetuin-A, which may be utilized to ascertain the risk of renal stone disease, was shown to exist between the haplotypes. c.766C>genotypic G's distributions of CC, CG, and GG showed no discernible difference between patients and controls (p=0.620). Also, not statistically significant (p=0.640) were the frequency distributions of CC, CT, and TT of the c.742C>T mutation. In addition, even after combining the variant TT and CT genotypes (i.e., CT+TT) and assuming a mutant recessive genetic model, the relationship for the allele c.742C>T remained negligible (Chi-squared=0.164; p=0.685). (Chi-squared=0.029, p=0.866) The distribution of C and T allelic frequencies between patients and controls was not statistically significant. For the alleles of c.766C>G, a comparable mutant recessive model was created, although the correlation for CG+GG was not significant (p=0.685). The allelic frequencies of C and G between patients and controls did not differ statistically significantly (p=0.728). When compared to the overall haplotype association, which was determined to be p=0.018, the haplotypes CT and CG were found to be significantly associated with kidney stone disease (p0.0001). Fetuin-A is one of the numerous non-collagenous proteins involved in osteogenesis and is known to have a strong affinity for calcium ions [13,14]. It has been demonstrated that fetuin-A prevents calcium phosphate crystals from growing and forming [15-17]. By generating a fetuin-mineral complex, hydroxyapatite crystals appear to be absorbed by fetuin-A, preventing its deposition and inhibiting non-bone calcification [18-20]. Fetuin-A has been proposed as a predictor of poor prognosis in persons with acute atherosclerosis and patients receiving hemodialysis.

Table 1. Distribution of genotypes and allele frequencies of Fetuin-A c742C>T

AHSG (c.742 C>T) Genotype	Control(n=50) %	Case (n=50)%	OR(95% CI, p value)
CC CT TT	27(54%) 22(44%) 1(1%)	30(60%) 18(36%) 2(4%)	x^2=0.891, df=2 ,p=0.640
CC CT+TT	27(56%) 23(44%) x^2=0.367,df=1,p=0.545	30(60%) 20(40%)	OR=0.962(0.480-1.926,p=0.912)
Alleles C T	76(77%) 24(24%) x^2=0.029,df=1,p=0.70	78(78%) 22(22%)	OR=0.893(0.5-1.597,p=0.702)

Table 2: Distribution of genotypes and allele frequencies of Fetuin-A 766C > G

AHSG (c.766 C>G) Genotype	Control (%)	Case (%)	P value
CC CG GG	32(64%) 17(32%) 1(4%)	30(60%) 18(36%) 2(4%)	x^2=0.426, df=2,p=0.808
CC CG+GG	32(64%) 18(36%) x^2=0.170, df= 1,p=0.680	30(60%) 20(40%)	OR=0.844(0.376-1.894, p=0.680)
Alleles C G	81(80%) 19(20%) x^2=0.276, df= 1,p=0.599	78(78%) 22(22%)	OR=0.832(0.418-1.655, p=0.599)

RFLP patterns of allelic distribution is depicted in figures 1 and 2.

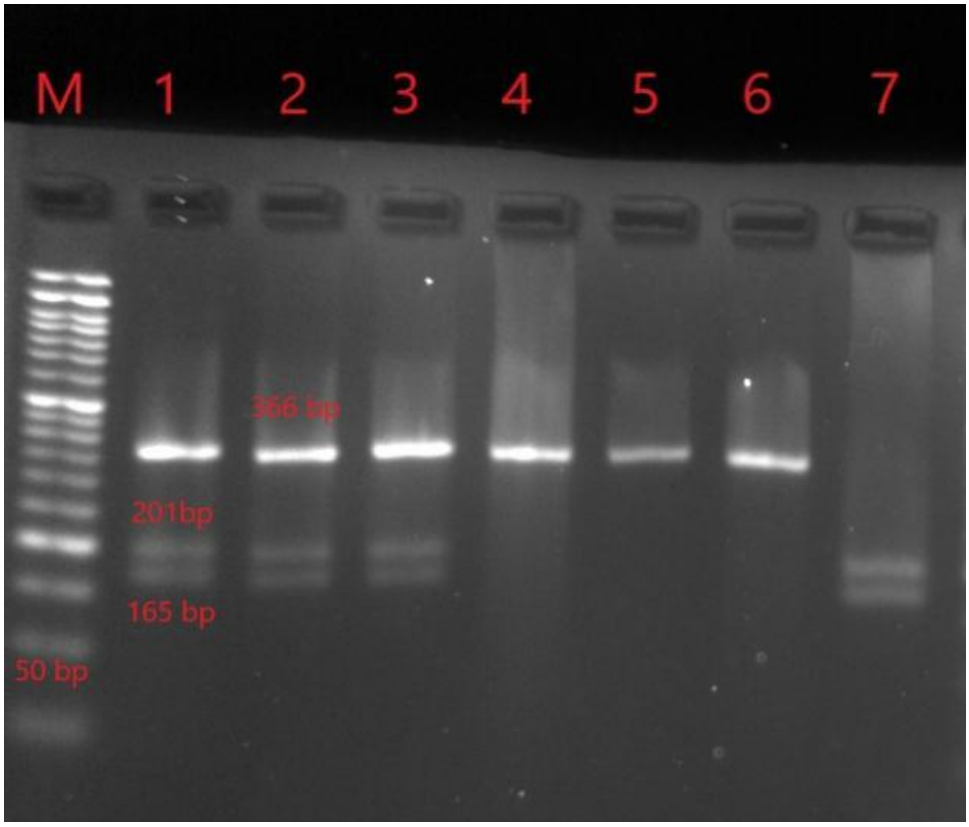

Figure 1. PCR RFLP analysis c.742C>T polymorphism in the of AHSG.Lane M: 50 bp marker: Lanes 1,2,3: CT alleles ;Lanes 4,5,6: CC alleles ; Lanes 7:TT alleles

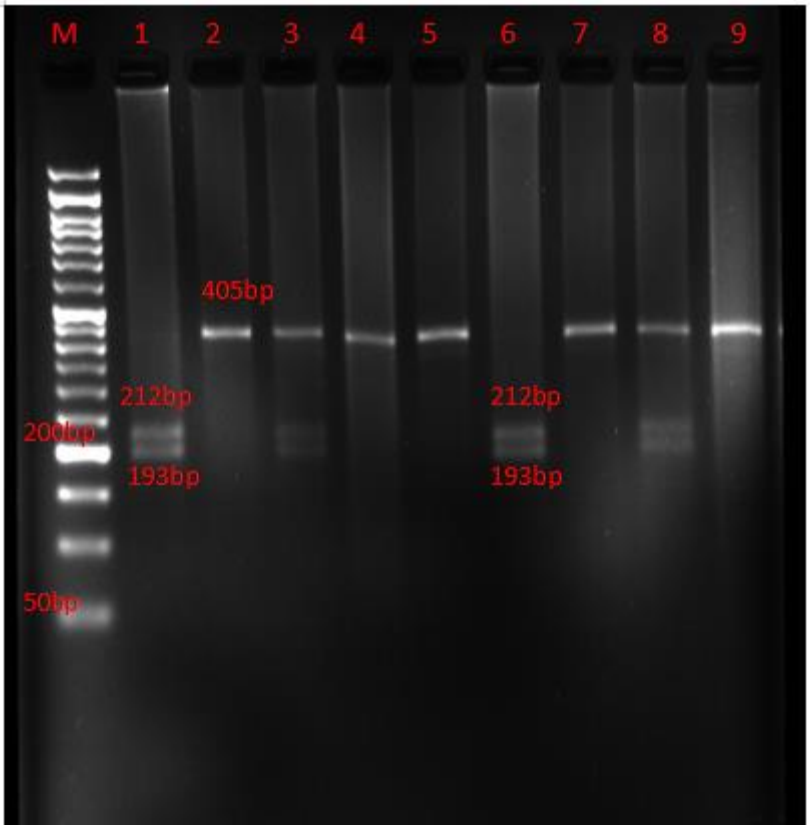

Figure 2. PCR RFLP analysis c.766C>G polymorphism in the of AHSG. Lane M: 50 bp marker: Lanes 1,6: GG alleles; Lanes 2,4,5,7,9: CC alleles; Lanes 3,8: CG alleles

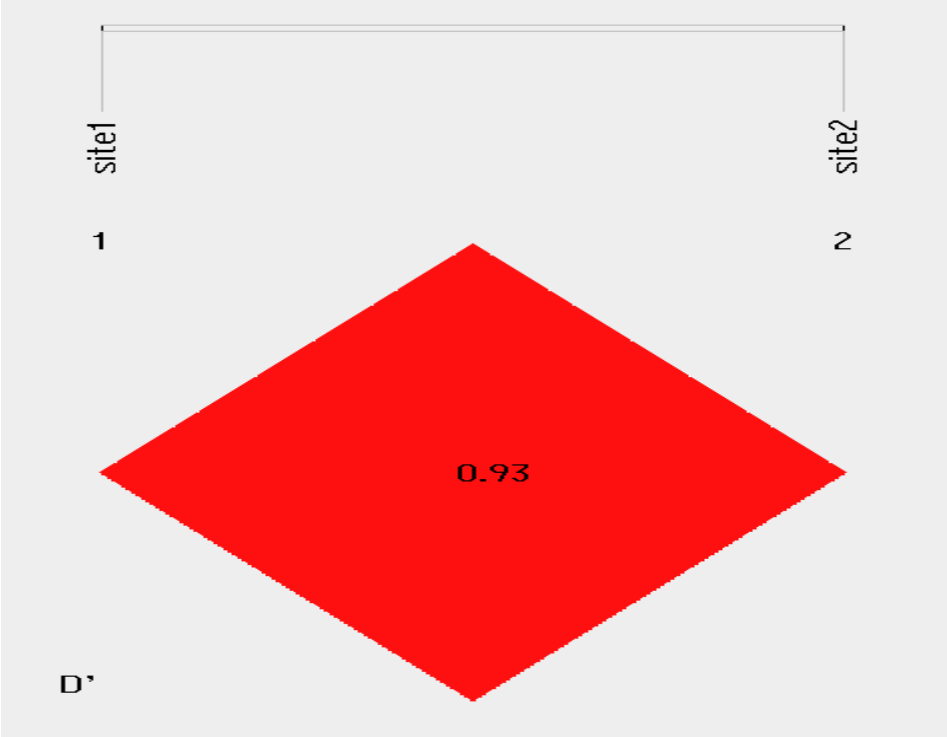

Figure 3A. Haplotype block (D') of two sites, AHSG c742C>T (rs4917) & AHSG c766C>G (rs4918) SNPs

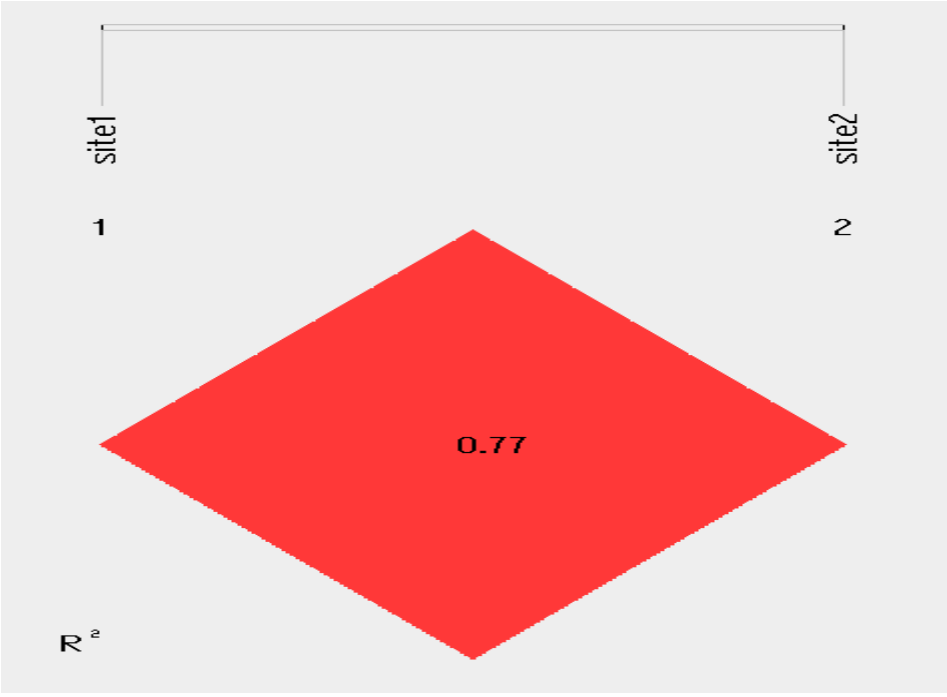

Figure 3B. Haplotype block (R^2) of two sites, AHSG c742C>T(rs4917) & AHSG c766C>G (rs4918) SNPs

Table 3. Association of single haplotypes of AHSG gene with kidney stone disease

Fetuin-A c.766C>G	Fetuin-A c.742C>T	Frequency Case	Frequency control	OR(95% CI)	P Value
C	C	0.7857	0.7536	1.00	-
G	T	0.2143	0.1752	0.86(0.40-1.85)	0.7
C	T	-	0.0503	36.57(36.18-36.96)	<0.0001
G	C	-	0.0209	15.58(15.55-15.61)	<0.0001

Table 4. Association of haplotypes of AHSG gene with gene with one disease

Haplotype	Case	Control	Chi^2	Fisher's p	Pearson's p	OR(95% CI)
CC	78	75	0.25	0.738	0.616	1.181(0.614-2.274)
GT	22	17	0.796	0.475	0.372	1.377(0.68-2.785)
CT	0	6	6.185	0.028	0.012	-

Rats' heart, lungs, kidneys, and skin have all shown extensive calcification in response to low serum fetuin-A levels [21]. Additionally, it stops the calcification of vascular smooth muscle cells [20]. According to Cai et al., this protein may be important in the malfunction of mineral deposition or other mechanisms that lead to defective mineralization [22]. It may also be involved in preventing bone mineralization on the outer surface. On the other hand, it was shown by Umekawa and Nishio that the fetuin-A protein is insufficient to stop the development of hydroxyapatite crystals [23,24]. However, a strong linkage disequilibrium between these SNPs suggests that co-inheritance of these two alleles have a significant association (haplotype association) with kidney stone disease. Our results indicate that there is no significant association between c.742C>T and c.766C>G with renal stone disease. Therefore, a disease condition may not be caused by a single mutation but rather by an allelic/gene-gene interaction. AHSG interacts with ions to increase their solubility, which helps to restrict mineralization to a lesser extent. However, at saturation or close to saturation concentration, this inhibition is abolished, and fetuin precipitates as mineralo-protein complexes. It is possible for these apatite nuclei to grow and crystallize. The presence of the haplotypes fetuin-A c.766C>G allele G and c.742C>T, allele C (GC) and fetuin-A c.766C>G allele C and c.742C>T, allele T (CT) increases the risk of kidney calcification by 1.5 and 3.6-fold, respectively, as compared to the wildtype genotype (CC). The study by Mohammadi-Noori et al. demonstrated that the presence of fetuin-A haplotypes c.766C>G, allele G, c.742C>T, allele C, and c.766C>G, allele T, in comparison to wildtype (CC) genotype, increases the risk of calcification of the heart valves and coronary artery by 1.78 and 2.38-fold, respectively [25].

4. Conclusions

The allelic frequencies of c.742C>T and c.766C>G amongst the participants with and without renal stones did not differ significantly. High D' values indicated a substantial LD of 0.93 between the two AHSG SNPs. The above alleles' co-inheritance is supported by the R2 value of 0.77. Kidney stone disease demonstrated an extremely significant correlation with the CT and GC haplotypes of c.742C>T and c.766C>G.

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Conflicts of interest: None

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