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A Cross-Sectional Study On Fetuin A Gene Polymorphism And Linkage Disequilibrium In Patients With Urinary Oxalate Stones

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Abstract

Background: Fetuin-A is a plasma glycoprotein, a potent inhibitor of calcification, the polymorphism of which is least explored in kidney stone disease. The aim of the study is to evaluate the pattern of fetuin A gene polymorphisms and their linkage disequilibrium(LD) in patients with and without urinary oxalate stones .

Methods: One Hundred subjects were recruited to the study, out of which 50 were case & 50 were controls. Analysis of the Single Nucleotide Polymorphisms (SNPs) of fetuin-A c.742C>T and c.766C>G were performed with Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR–RFLP). Linkage Disequilibrium between the SNPs was carried out using the SHEsis plus and SNP stat online.

Results: There was no significant difference in the distribution of wild and mutant alleles of Fetuin-A c.742C>T and Fetuin-A c.766C>G. The two SNPs of fetuin A showed a strong LD of D' 0.93 & R^2 is 0.77 which implies strong co-inheritance of the alleles.Haplotype CT & CG alleles showed a higher significant level p>0.0001 in patients. Among the alleles of c.742C>T & c.766C>G, the association of dominant, recessive and co-dominant alleles were insignificant. There was no significant association between the fetuin A gene polymorphisms and their expression.

Conclusion: It could be concluded from the study that there is no significant association between fetuin A gene polymorphisms and renal stone disease. LD value(D' & R^2) of both the SNPs supports co-inheritence of the alleles. However haplotypes of CT and GC of c.742C>T and c.766C>G showed a highly significant association with the kidney stone disease.

Keywords: fetuin A, alleles, polymorphism, renal stone, linkage disequilibrium

I. INTRODUCTION

Renal stone disease begins with the formation of crystals in the kidneys. The urological illness affects about 12% of the world's population, according to estimates. Urinary tract stone disease is predicted to affect at least 10% of the population in the developed countries[1]. Kidney stones are prevalent in developed countries, with a 0.5 percent to 1.9 percent annual incidence[2]. Upper and lower urinary tract stones are common in India, but the frequency varies greatly by area[3]. In comparison to other sections of the country, the southern part of the country has a lower

incidence of renal calculi[2]. It's been linked to a higher chance of kidney failure in the final stages[4].

Calcium stones are the most common kind of kidney stone, accounting for over 80% of all urinary calculi. Calcium oxalate (50%) calcium phosphate (5%),and a combination of both (45%) may account for the majority of calcium stones [5]. Calcium hydrogen phosphate, often known as hydroxyapatite, is the most common component of calcium stones [6,7]. The majority of kidney stones contain calcium oxalate in the form of calcium oxalate monohydrate (COM),calcium oxalate dihydrate (COD),or a combination of both,which accounts for more than 60% of all kidney stones [8]. The most thermodynamically stable type of stone is COM. In clinical stones,COM is seen more commonly than COD [9].

Hypercalciuria, hyperoxaluria, hypocitraturia, and hypomagnesuria are all factors that influence the production of calcium oxalate stones[10].

Urinary pH of 5-6.5 promotes calcium oxalate stone development, whereas pH greater than 7.5 promotes calcium phosphate stone formation[11,12].

Kidney stones have a complex etiology. Calcium oxalate, which forms at Randall's plaque on the renal papillary surfaces, is the most frequent type of kidney stone. Stone formation is a complicated process that involves a number of physicochemical phenomena, such as supersaturation, nucleation, growth, aggregation, and retention of urinary stone ingredients within tubular cells. An imbalance between substances that induce or inhibit urine crystallisation controls these stages[13].

Nephrolithiasis is a complicated condition with several phases and contributing variables. Identifying the chemicals and metabolic changes that influence stone formation may provide a way to intervene. Fetuin A is one of these molecules that has received the least attention. Fetuin A could be utilised as a biomarker to predict the production of calcium oxalate stones. Fetuin-A is a 45-kDa plasma glycoprotein that is released mostly by the liver[14]. It is made up of two polypeptide chains resulting from posttranscriptional cleavage. It behaves in as a negative acute phase reactant. Furthermore, fetuin-A is a powerful inhibitor of vascular calcification, which is attributed in part to the creation of the fetuin–mineral complex. It forms reversible compounds with calcium and phosphorus, increasing their solubility in the blood[15].

The capacity of serum to suppress calcium–phosphate product precipitation was greatly decreased in dialysis patients with low fetuin-A levels, and it was shown that dialysis patients with low fetuin-A levels had coronary or other calcification loci. Furthermore, restoration of these patients serum with pure fetuin-A to attain physiological quantities restored the patients' defective precipitation inhibition[16]. In vitro z, Price and Lim demonstrated that fetuin-A prevents the precipitation of hydroxyapatite from supersaturated calcium and phosphate solutions by creating complexes with minerals[17]. Urinary fetuin-A levels were also found to be lower in individuals with urolithiasis compared to healthy participants, with a sensitivity of 97 percent and a specificity of 100 percent in urolithiasis [18].

Aksoy et al. investigated the role of fetuin A gene polymorphism in the pathogenesis of calcium oxalate gene polymorphism in the pathogenesis of calcium oxalate stone formation and concluded that this may increase the risk of calcium oxalate stone formation[19].However, research on the role of a fetuin A gene polymorphism in nephrolithiasis is limited.

Assessing the patterns of LD has become an important issue in both evolutionary biology and medical genetics since the rapid accumulation of densely spaced DNA sequence variation data in several organisms. LD is an event where two alleles at different loci are genetically linked and show non-random association in same chromosome within a given population. LD is influenced by many factors including selection, the rate of genetic recombination, mutation rate, genetic drift, the system of mating, population structure and genetic linkage.

Rationale

Kidney stones have been associated with increased risk of chronic kidney disease, end stage renal failure, cardiovascular diseases, diabetes and hypertension. Environmental and genetic factors are among the factors that contribute to the production of oxalate stones. The goal of our research is to determine the involvement of genetic variables in the production of oxalate stones. A greater understanding of genetic factors could aid in the early detection or prediction of oxalate stones. Fetuin A gene polymorphism could be used as a marker for urinary stone disease prediction. To the best of our knowledge, there are just a few research that have focused on gene polymorphismsof fetuin A and linkage disequilibrium with oxalate stone development.

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

A. 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 nsSNP 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 prot ein's stability 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

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 Fetuin A 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

Objectives

1. To evaluate the pattern of fetuin A gene polymorphisms in patients with urinary oxalate stones as compared to healthy controls.
2. To assess the linkage disequilibrium of fetuin A SNPs in patients with and without urinary oxalate stones.
3. To analyze the haplotype patterns of fetuin A in patients with and without urinary oxalate stones.

II. EXPERIMENTAL DESIGN AND METHODS

Study design and setting: In June 2020-March 2022,a Observational cross-sectional study was conducted. A patient with Kidney stone disease confirmed by ultrasonography who visited the

Department of Urology,Justice K S Hegde charitable Hospital,Mangalore,Karnataka,India. And blood sample were examined at the KS Hegde Medical Academy's Central Research Laboratory and Molecular Genetics wing.

Study size: The study size was calculated using $\mathbf{n = 2[Z_{1-\alpha/2}+Z_{\beta}/d]^2 \times S^2}$formula and hospital prevalence rate of renal calculi cases.

Kidney stone samples were obtained either after extracorporeal shock wave lithotripsy or surgery for treatment. Calculi were analyzed using chemical methods. Only patients with calcium oxalate stones were included in the study.

Ethical consideration

The study was initiated after obtaining approval on 15/6/2020 from CEC Ref, NU/ CEC/ 2020/ 0289 Nitte (Deemed to be University) and eligible patients were given information on the study procedure, goal and role in the study and written patient informed consent was obtained from all the participants in their respective native languages.

Cases:

Inclusion Criteria: Patients fulfilling all below mentioned criteria were included as cases

1. Fifty patients in the Age group of 18-65yrs of either sex with the COM or COD kidney stone confirmed by qualitative analysis were recruited by convenient sampling.
2. Patients who were willing to give their consent to be a part of this study.

Exclusion Criteria: Patient having any one of the following criteria were excluded:

1. Patients with uric acid / cysteine stones diagnosed by qualitative tests
2. Patients with primary hyperparathyroidism diagnosed by investigation.

Controls:

Inclusion Criteria: Participants fulfilling all below mentioned criteria were included as controls

1. Fifty Healthy subjects of either gender,in the age group of 18-65 yrs,without urinary stones as confirmed by ultrasonography
2. Healthy individuals willing to take part in the study

Exclusion Criteria: Subjects with the history of urinary stone or family history ofurinary stone and Gout.

III.LABORATORY INVESTIGATIONS:

Gene Analysis

Blood specimen of 5ml was collected in EDTA vacutainers for assessing gene polymorphisms.DNA isolation was done by the salting out method[20].

The quantification and purity of DNA was checked by the spectrophotometer (ratio of OD260 / OD280). DNA concentration was calculated using the following formula:

Concentration (µg/ml) of DNA in original solution= Absorbance x 100 x 50 µg/ml.

Amplification and Genotyping of the gene polymorphism: Genotyping of the genes was confirmed by PCR-RFLP

Fetuin-A Genotyping:

The PCR-RFLP analysis was used to evaluate fetuin-A c.742C>T and c.766C>G single nucleotide polymorphisms. PCR mixes of volume 25 µl were set up, containing 11.5 µl of molecular grade water(Himedia) and 12.5 µl of Taq DNA Polymerase Master Mix RED(1.5Mm $MgCl_2$ Concentration,NH_4^+ buffer system, dNTPs ,and the front of red tracking dye runs at 300-1000bp on 0.5-1.5% agarose gel) (Ampliqon IIII) 0.5 µl Forward and reverse primer(Sigma-Aldrich) and 1µl of DNA (1 µl of DNA at the concentrations of 300-500ng/ml).

A forward and reverse oligonucleotide primers were used to amplify the fetuin-A 742C>T polymorphism 5'-CCTCCCACAAGCAGAAA C-3' and 5'-TGATGATTC-CGCATACCC-3'respectively using Primer 3Plus.

Amplification was performed in MiniAmp plus Thermal cycler (Thermofischer Scientific)The PCR Process was performed in 35 cycles under initial denaturation at 95°C for 5 minutes denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 5minutes. The PCR Product was checked by gel

electrophoresis system(Gel Doc™ EZ imager Biorad)

Using Gel Blotting setup(Mini-PROTEAN Tetra Cell, Bio-Rad, USA) ,containing 0.5 µg/ml ethidium bromide and DM012-R500 50 bp DNA Ladder Ready to use(Gene Direx, Inc.) in TAE Buffer(1X).

PCR products was digested with 0.5 µl NlaIII (NEB) restriction endonucleases overnight at 37°C,and the digested products were separated by 3% agarose gel electrophoresis and visualized using ethidium bromide.

For Analysis of fetuin-A 766C>G polymorphism was performed with the oligonucleotide primers forward 5'-GTCAC-CCCTCCTTGTAAC-3' and reverse 5'-CCCCAATGAGAC-CACA-3'. The PCR condition was conducted in the following steps: initial denaturation at 95°C for 5 minutes,35 cycles denaturation at 94°C for 1 minute, annealing at 56°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 5 minutes. The PCR product was digested overnight at 37°C with SacI restriction enzyme and digested products were separated on 3% agarose gel.

Fetuin A gene expression was assayed in the serum by ELISA Kit(Fine Test, Wuhan Fine Biotech Co., Ltd.) Procedure: plate was washed twice before adding standard, 1ml of sample was added(diluted at least ½ with sample dilution Buffer) & incubated for 90 minutes at 30°C,aspirated and plates were washed twice. Biotin-labeled antibody working solution (0.1ml) was added to each well and incubated for 60 minutes at 37°C,aspirated and plates were washed thrice. Streptavidin conjugate (SABC) working solution(0.1ml) was added into each well and incubated for 30 mins at 37°C,aspirated plates were and washed 5 times, TMB substrate solution (0.9ml) was added and incubated for 10-20 minutes at 37°C and stop solution (0.5ml)was added. Reading was taken at 450nm immediately using Multimode Microplate & Fluorescence Intensity Bottom Reader(TecanSpark, Bioscreen, Switzerland).

Biochemical parameters like serum calcium, phosphorus and creatinine were estimated by semi automated chemistry analyser. Estimated Glomerular Filtration Rate (eGFR) was calculated using Modification of Diet in Renal Disease (MDRD) formula.

IV. STATISTICAL ANALYSIS:

Statistical analysis was done using SPSS version 23 software.Hardy Weinberg equilibrium was calculated to find out whether observed and expected alleles are in equilibrium.Association of the genes with stone formation and their expression (serum fetuin A levels) was checked by chi square test. Comparison of biochemical parameters was carried out using Unpaired t test or Mann Whitney U test for parametric and non parametric parameters respectively.

LD analysis was done by using web based ShesisPlus (http://shesisplus.bio-x.cn/SHEsis.html) software and haplotype analysis by SNPstat online (https://snpstats.net/start.htm).

V. RESULTS

Demographic data of the cases and controls showed that they were age matched [no significant difference in age (p=0.17)](table 1).A significantly higher creatinine and lower eGFR(p=0.046 and p<0.0001) were noted in cases. Difference in the serum calcium and phosphate levels were insignificant.(table 1).

Table 1:*Demographic and biochemical parameters*

Parameters	Control group (n=50)	Case group(n=50)	P value
Age(Yrs)	33.7±10.47	36±13.87	0.17

Gender(Male/Female)	34(68%)/16(32%)	18(36%)/32(64%)	>0.0009
Creatinine(mg/dL) Median(range)	0.81(0.66-0.98)	1(0.7-1.41)	**0.046***
Calcium(mg/dL) (mean±SD)	9.3±1.84	8.45±3.81	0.146
Phosphorus(mg/dL) Median(range)	5.4(4.5-6.3)	4.8(4.2-5.7)	0.181
eGFR Median(range)	122.9(96.6-208.1)	78.2(53.8-105)	**<0.0001*****
Fetuin A(ng/ml) (mean±SD)	40.09±14.56	37.54±16.53	0.43

* **P<0.05 significant**

Hardy Weinberg equilibrium analysis showed that there is no significant deviation of between the expected and observed alleles of both the SNPs of fetuin A. There was no significant difference in the distribution of wild and mutant type of alleles of Fetuin-A c.742C>T and Fetuin-A c.766C>G(tables 2 and 3).

Table 2. *Genotype distribution and alleles frequencies of Fetuin-A c742C>T*

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

Table 3: *Genotype distribution and alleles frequencies Fetuin-A 766C > G*

Fetuin A (c.766 C>G) Genotype	Control (%)	Case (%)	P value
CC	32(64%)	30(60%)	

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

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

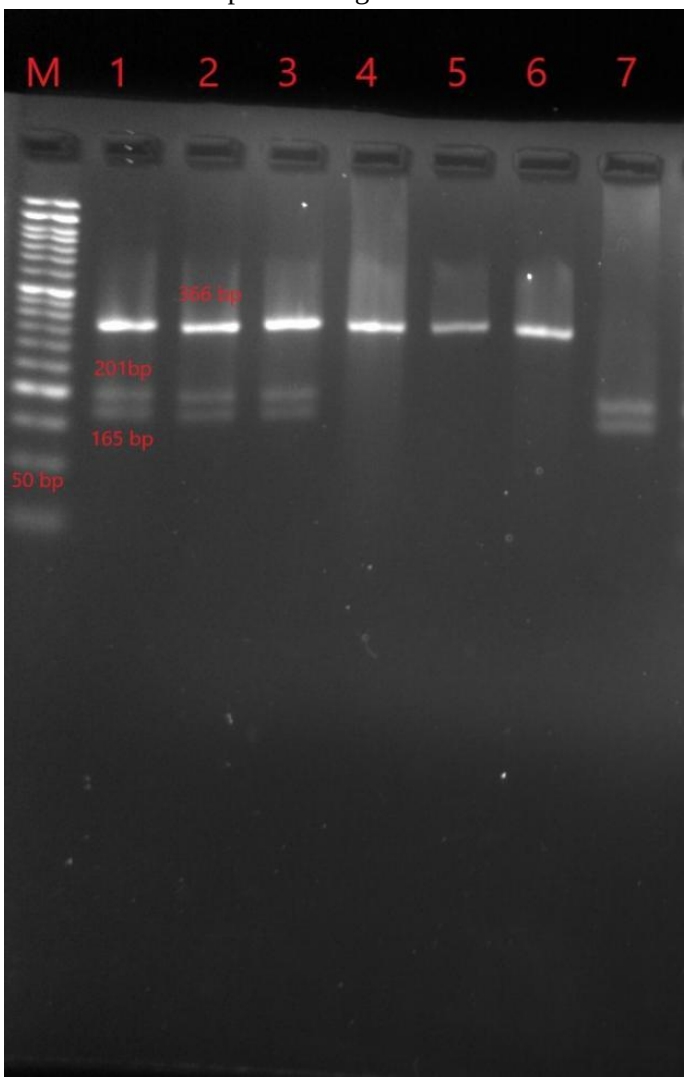

Fig 1: *PCR RFLP analysis c.742C>T polymorphism in the of Fetuin A.Lane M: 50 bp marker: Lanes 1,2,3: CT alleles ;Lanes 4,5,6: CC alleles ; Lanes 7:TT alleles*

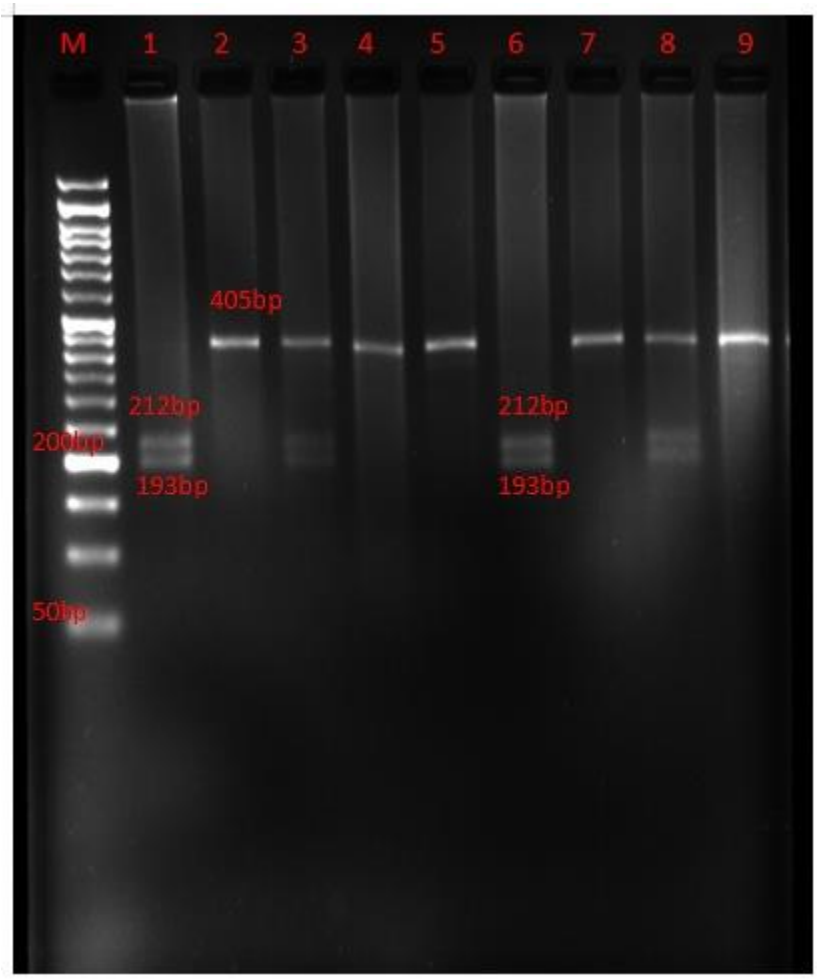

Fig 2 : *PCR RFLP analysis c.766C>G polymorphism in the of Fetuin A.Lane M: 50 bp marker: Lanes 1,6: GG alleles ;Lanes 2,4,5,7,9: CC alleles ; Lanes 3,8 :CG alleles*

To assess the extent of LD in pairwise combinations of SNPs,D' and r^2were calculated. LD between haplotypes of fetuin A c.742C>T andc.766C>G,were determined using the SHEsisplus program platform. Haplotypes with frequencies of less than 0.03 were omitted from the analysis.

Analysis of combined genotype data between two SNPs was investigated by LD for cases and controls. Two plots of LD were generated. The two SNPs of fetuin-A showed a strong LD of 0.93 (Figure 3A),as suggested by high the D' values. The R^2 value of 0.77 (Figure. 3B) supports the co-inheritance of the above alleles. If the D' and R^2 values are closer to one,it suggests a strong co-inheritance of the alleles. If the values are closer or equal to zero,it suggests that the alleles are weakly co-inherited.Higher D' values were noted for the SNPs of fetuin A suggesting their co-inheritance.

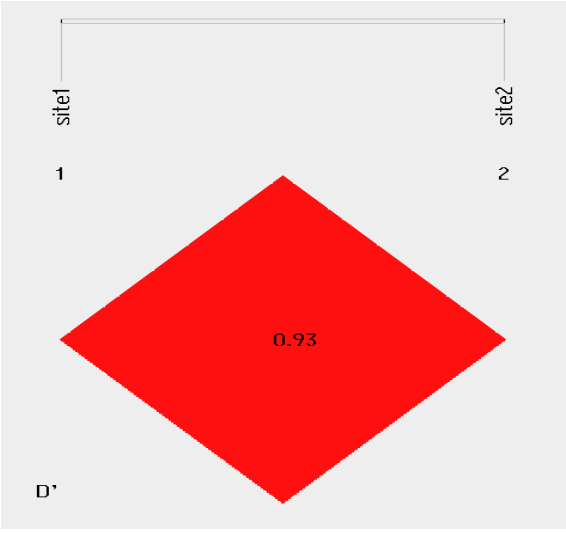

Fig3A:*Haplotype block (D')of two sites, fetuin A* ***c742C>T(*** *rs4917) & fetuin A* ***c766C>G*** *(rs4918) SNPs*

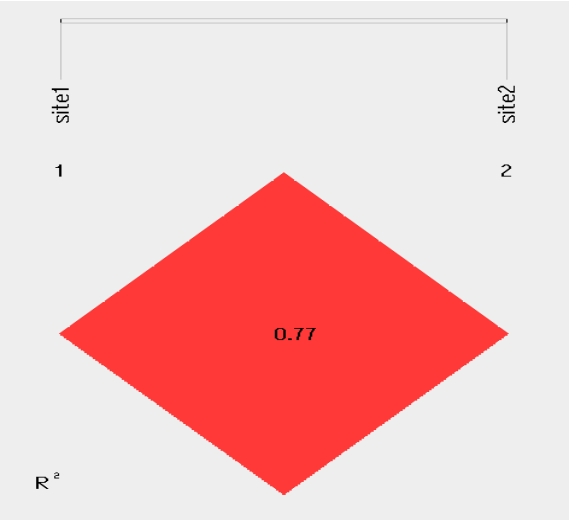

Fig3B:*Haplotype block (R^2)of two sites, fetuin A* ***c742C>T(*** *rs4917) & fetuin A* ***c766C>G*** ***(****rs4918) SNPs*

A 'highly significant association was observed between kidney stone disease and Haplotypes C,T,G,C SNPs (p<0.0001) (table 4).

Table 4: *Association of single haplotypesof fetuin A 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

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 5).

Table 5:*Association of haplotypesof fetuin A gene with kidney stone 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	-

The linkage disequilibrium map is used to analyze the SNPs of the fetuin-A gene, LD R^2 values represent a pairwise linkage disequilibrium relationship between the two SNPs. R^2=1 indicate high linkage disequilibrium (darkest colored square); R^2 values between 0 & 1 indicate light colored squares; R^2 = 0 indicate low linkage disequilibrium (lightest colored squares). However, while predicting the co-inheritance of the alleles, both D' and R^2 are taken into consideration.

Haplotype association with kidney stone disease was evaluated using the SNP Stat online tool in a pairwise manner between the SNPs. The

associations were insignificant with p>0.05 (table 5) except for CT allele. However, there was a statistically significant difference between the haplotypes of fetuin-A c.742C>T (p=0.028). After analyzing the SNPs using Shesisplus and SNP Stat online, it could be concluded that the alleles of fetuin-A were in equilibrium. Their co-inheritance was confirmed by high D' and R^2 values. There was no association significance between the haplotypes of the gene and the kidney stone disease. There was a significant LD between the haplotypes of fetuin-A, whichmay be used as a reliable screening marker to determine the risk of renal stone disease.

Genotypic distributions of CC,CG and GG of c.766C>G showed no significant difference (p=0.620) between cases and controls. Similarly, frequency distributions of CC,CT and TT of c.742C>T were also not statistically significant (p=0.640).

A study by Hulya *et al*. reported there was a significant difference in the genotypic frequencies of fetuin-A c.766C>G between the control and patient groups (p=*0*.003) in kidney stone disease [15]. There was no statistical significance distribution for fetuin-A c.742C>T polymorphism in cases and controls (p=0.77). Both the polymorphisms of fetuin-A were insignificantly associated with kidney stone disease (p>0.05). The associations of dominant, recessive and co-dominant alleles of c.766C>G with kidney stone disease (KSD) were insignificant (p=0.88,p=0.53,p=0.82 respectively),with the odds ratio of 0.94,0.47 and 0.47 times with 95% CI (0.42-2.1),(0.04-5.36) and (0.04-5.44) respectively(table 6).

Table 6:*Association of CG genotype of Fetuin-A c.766C>G SNP with kidney stone Disease*

Model	**Genotype**	**Ca=Ca**	**Ca=Co**	**OR (95% CI)**	**P-value**	**AIC**	**BIC**
Codominant	C/C	30 (61.2%)	32 (62.8%)	1.00	0.82	144.2	152
	C/G	17 (34.7%)	18 (35.3%)	0.99 (0.43-2.27)			
	G/G	2 (4.1%)	1 (2%)	0.47 (0.04-5.44)			
Dominant	C/C	30 (61.2%)	32 (62.8%)	1.00	0.88	142.6	147.8
	C/G-G/G	19 (38.8%)	19 (37.2%)	0.94 (0.42-2.10)			
Recessive	C/C-C/G	47 (95.9%)	50 (98%)	1.00	0.53	142.2	147.4
	G/G	2 (4.1%)	1 (2%)	0.47 (0.04-5.36)			
Overdominant	C/C-G/G	32 (65.3%)	33 (64.7%)	1.00	0.95	142.6	147.8
	C/G	17 (34.7%)	18 (35.3%)	1.03 (0.45-2.34)			
Log-additive	---	---	---	0.89 (0.43-1.81)	0.74	142.5	147.7

The association of dominant, recessive and co-dominant alleles of c.742C>T with KSD were insignificant (p=0.52,p=0.089,p=0.16 resp) with the odds ratio of 1.3,0.0 and 1.45 times with 95% CI (0.58-2.88),(0-0) and (0.64-3.26) respectively.

Simultaneously, when we combined the variant TT genotype with the CT genotype (i.e.,CT+TT),assuming a mutant recessive genetic model, the association was still insignificant (Chi-squared=0.164; p=0.685) for the allele c.742C>T. Distribution of C and T allelic frequencies between cases and controls was statistically insignificant (Chi-squared=0.029,p=0.866). A similar mutant recessive model was constructed for the alleles of c.766C>G, for CG+GG, the association was insignificant (p=0.685). There was no statistically significant difference in the allelic frequencies of C and G between cases and controls (p=0.728) (table 7).

Table 7:*Association of CT genotype of Fetuin-A c.742C>T SNP with kidney stone disease*

Model	**Genotype**	**Ca=Ca**	**Ca=Co**	**OR (95% CI)**	**P-value**	**AIC**	**BIC**
Codominant	C/C	30 (61.2%)	28 (54.9%)	1.00	0.16	140.9	148.7
	C/T	17 (34.7%)	23 (45.1%)	1.45 (0.64-3.26)			
	T/T	2 (4.1%)	0 (0%)	0.00 (0.00-NA)			
Dominant	C/C	30 (61.2%)	28 (54.9%)	1.00	0.88	142.6	147.4
	C/T-T/T	19 (38.8%)	23 (45.1%)	1.30 (0.58-2.88)			
Recessive	C/C-C/T	47 (95.9%)	51 (100%)	1.00	0.53	142.2	144.9
	T/T	2 (4.1%)	0 (0%)	0.00 (0.00-NA)			
Overdominant	C/C-T/T	32 (65.3%)	28 (54.9%)	1.00	0.95	142.6	146.7
	C/T	17 (34.7%)	23 (45.1%)	1.55 (0.69-3.46)			
Log-additive	---	---	---	1.08 (0.52-2.25)	0.83	147.8	148.7

Haplotypes of CT and CG were found to bear a highly significant with kidney stone disease, p<0.0001,as compared to the global haplotype association p=0.018(table 8)

Table 8:*Association of haplotypes of C G & C T allele of c.766C>G and c,742C>T with kidney stone disease*

Fetuin-A c.766C>G	Fetuin-A c.742C>T	**Freq**	**OR (95% CI)**	P Value
C	C	0.7697	1.00	
G	T	0.1947	0.86 (0.40 - 1.85)	0.7
C	T	0.0253	3.68636542857 (3686365428.18 - 3686365428.96)	<0.0001
G	C	0.0103	159568069758 (1595680697.55 - 1595680697.61)	<0.0001

Global haplotype association p-value: 0.018

Serum fetuin-A levels were assayed as a marker of fetuin-A gene expression. In the present study, serum fetuin-A levels was insignificantly low in cases as compared to controls.(table 1).Association between Fetuin A gene polymorphism and serum fetuin-A levels showed an insignificant difference(Chi-squared=1.75,p=0.18 with OR 2.29 (95% CI 0.66-7.95). SNPs of fetuin-A did not bear any significant association with eGFR in cases (Chi-squared=1.389,p=0.23,OR=2.04 (95% CI=0.61 - 6.75) for both the SNPs). Odd's ratio confirmed that the heterozygous dominant CC genotype was at higher risk of KSD with low fetuin-A levels and showed low eGFR values. The negative correlation between fetuin-A levels and eGFR (R=-0.2581 p=0.07) provides substantial evidence for the renal protective role of fetuin-A.

VI. DISCUSSION

Fetuin-A is thought to have a great affinity for calcium ions, making it one of the abundant non-collagenous proteins present in osteogenesis [21,22]. Fetuin-A has been shown to hinder the formation and growth of calcium phosphate crystals [23-25]. Fetuin-A appears to be absorbed by hydroxyapatite crystals [26],prevent its sedimentation, and inhibit non-bone calcification by forming fetuin-mineral complex [27,28]. Fetuin-A has been suggested as a predictor of poor prognosis in patients with hemodialysis and subjects with acute atherosclerosis [16]. Low serum fetuin-A levels have been linked to widespread calcification in various organs in rats, including the heart, lungs, kidney and skin [29]. It also prevents vascular smooth muscle cells from calcifying [28]. Cai *et al*. suggested that this protein is involved in preventing bone mineralization on the exterior surface, its importance perhaps seen in mineral deposition dysfunction or other mechanisms acting towards impaired mineralization [30].

On the contrary, Umekawa and Nishio demonstrated that fetuin-A protein is inadequate to prevent the growth of hydroxyapatite crystals [31,32].

Our results show that there is no significant association between c.742C>T and c.766C>G with renal stone disease, however a strong linkage disequilibrium between these SNPs suggest that co-inheritence of these two alleles have a significant association (haplotype association)with the kidney stone disease. So it may not a single mutation, but it may be an allelic/gene gene interaction may influence a disease condition.

Fetuin-A acts as an inhibitor of calcium phosphate mineral (apatite) precipitation by formation of complexes with Ca and P. Low urinary levels of fetuin have been found in patients with documented urolithiasis and those with fetuin-A gene polymorphism are at a higher risk of CaOx nephrolithiasis. In addition to

nephrolithiasis fetuin has been linked to other calcification related pathologies in the body. The patients on dialysis have low serum fetuin-A levels and they are more prone to coronary or other calcifications. Fetuin-A has been isolated from kidney stones and calcified vascular loci.Apatite nucleation is suppressed by the presence of fetuin-A, which is eventually overcome by the excess calcium or phosphate present in bodily fluids, which precipitates mineral-protein complexes.Fetuin play a smaller part in the process of inhibiting mineralization by interacting with ions to make them more soluble.However, this inhibition is overcome at saturation or near saturation concentration, and fetuin precipitates as mineralo-protein complexes.These apatite nuclei have the capacity to enlarge and crystallise[33].

In the present study,When compared to wildtype genotype (CC), 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.This finding is supported by the study by Mohammadi-Noori et al demostrated that the presence of haplotypes fetuin-A c.766C>G, allele G andfetuin-A c.742C>T, allele C (GC) and haplotypes fetuin-A c.766C>G, allele C and c.742C>T, allele T (CT) in comparison with wildtype(CC)genotype increases the risk of calcifcation of the heart valves and coronary artery by 1.78 and 2.38-fold, respectively[34].

VII. CONCLUSION

It can be concluded that there was no significant difference in the allelic frequencies of c.742C>T and c.766C>G between the subjects with and without the renal stones.The two SNPs of fetuin A showed a strong LD of 0.93 as suggested by high D' values. R^2 value of 0.77 support the co-inheritance of the above alleles.Haplotypes of CT and GC of c.742C>T and c.766C>G showed a highly significant association with the kidney stone disease.There was no association between the fetuin A gene polymorphisms and their expression(fetuin A levels).However the negative correlation between fetuin A levels and eGFR confirms the renoprotective role of fetuin A.

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