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Evaluation of Gene Polymorphisms in Patients with Urinary Oxalate Stones: Cross-sectional Study

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Abstract

Fetuin-A, a plasma glycoprotein known for its capacity to inhibit calcification, has not been extensively studied regarding its genetic variability, particularly in relation to kidney stone disease. This study aimed to investigate the association between Fetuin-A gene polymorphisms and renal stone disease and to compare serum Fetuin-A levels between individuals with and without kidney stones.

A cross-sectional study was conducted involving 100 kidney stone patients and 100 controls. Two Single Nucleotide Polymorphisms (SNPs) of the fetuin-A gene, c.fet742 C>T (rs4917) and c.fet766 C>G (rs4918), were genotyped using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). Serum Fetuin-A levels were measured using ELISA. Statistical analysis, including Hardy-Weinberg equilibrium, chi-square tests, linkage disequilibrium, haplotype analysis, and ROC curve analysis, was performed using SPSS version 21, SNP Statonline, and Shesisplus.

No significant association was found between the genotypes of the two SNPs and kidney stone disease (χ^2=0.0833, p=0.772 and χ^2=0.339, p=0.560). Additionally, there was no significant correlation between these SNPs and serum Fetuin-A expression (χ^2=0.254, p=0.613 and χ^2=2.207, p=0.137). However, serum Fetuin-A levels were significantly lower in kidney stone patients compared to controls (p=0.018). Haplotype analysis revealed no significant differences in the distribution frequencies of the (CC, TG, CG) haplotypes of the fetuin-A SNPs between cases and controls. Strong linkage disequilibrium was observed between the two SNPs (D'=0.93, R^2=0.77), indicating a strong co-inheritance of the alleles.

To conclude, the study did not find a significant association between Fetuin-A gene polymorphisms and renal stone disease, although serum Fetuin-A levels were lower in individuals with kidney stones.

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

1. Introduction

The process of kidney stone formation is intricate and involves multiple sequential steps (Wang et al.,2021). It is a prevalent condition that affects people of various ages, genders, and ethnicities, with a higher occurrence observed between the second and fourth decades of life. Globally, the prevalence of kidney stones is increasing, with a lifetime cumulative incidence ranging from 5 to 10 percent (Rodrigues et al., 2022). This condition carries significant implications for both individuals and society, particularly in regions characterized by hot and arid climates (Wang et al.,2021). The development of calculi containing calcium is a consequence of an imbalance between factors that encourage and inhibit crystallization (Singh et al.,2022). Factors such as hypercalciuria, hyperoxaluria, hypocitraturia, and hypomagnesuria influence the formation of calcium oxalate stones. Urinary pH also plays a pivotal role, as a pH level of 5-6.5 promotes the formation of calcium oxalate stones, while a pH greater than 7.5 favors the development of calcium phosphate stones (Kishore et al 2013; Kumar 2012). Risk factors for calcium oxalate stone formation include low fluid intake, a diet rich in oxalates, and a family history of kidney stones. Kidney stone disease is influenced by a combination of genetic, dietary, and environmental factors (Singh et al.,2022). The increasing prevalence of obesity and hypertension has also been linked to the production of kidney stones (Sayer et al.,2008). Studies have demonstrated that individuals with a family history of nephrolithiasis have a 60% higher risk of developing kidney stones compared to the general population (Ramello et al., 2000).

An underexplored area in kidney stone research is fetuin-A, a glycoprotein that plays a crucial role in the formation and stabilization of calciprotein particles (CPP). These high molecular weight colloidal protein mineral complexes are responsible for transporting and eliminating mineral nanocrystals from the bloodstream. CPPs are essential regulators of calcium extracellular matrix mineralization and serve as systemic inhibitors of soft tissue and vascular calcification (Stenvinkel et al 2005; Rudloff et al., 2022). Low levels of serum fetuin-A have been associated with imbalances in mineralization and increased mortality in individuals with end-stage renal disease (Bouafi et al.,2019; Wang et al.,2018; Kumar et al.,2009; Al-Shuhaib et al.,2019). However, there is a

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paucity of studies investigating the role of fetuin-A gene polymorphisms in nephrolithiasis. Fetuin-A holds promise as a potential biomarker for predicting the development of calcium oxalate stones.

2. Objectives

This study encompasses a comprehensive investigation into various aspects of Fetuin A and its potential role in urinary kidney stone disease. It commences with the application of bioinformatics methods to pinpoint specific SNPs within the Fetuin A gene that might be associated with urinary kidney stone disease. Subsequently, it delves into the exploration of the connection between these gene polymorphisms and the presence or absence of urinary oxalate stones, along with a comparative analysis of serum Fetuin A levels in individuals afflicted by these stones. The research also scrutinizes the relationship between Fetuin A gene variations and the serum levels of Fetuin A in patients dealing with urinary oxalate stones. In addition, it assesses the correlation between the genetic variations and the metabolic profile of urinary stone disease. Lastly, the study delves into the examination of Linkage Disequilibrium patterns of specific SNPs in the Fetuin A gene, specifically c.742C>T and c.766C>G, in patients both with and without urinary kidney stone disease. This multifaceted approach aims to shed light on the complex interplay between genetic factors, biochemical markers, and urinary stone disease, providing valuable insights into potential diagnostic and therapeutic avenues.

3. Materials and Methods

This cross-sectional study was designed to investigate the relationship between Fetuin-A gene polymorphisms and kidney stone disease. The research was conducted at the Central Research Laboratory of KSHEMA in collaboration with the Department of Urology at Justice K S Hegde Charitable Hospital, Mangalore, Karnataka, India.

3.1. Selection of SNPs by Bioinformatics

In-silico analysis was utilized to identify Single Nucleotide Polymorphisms (SNPs) in the Fetuin-A gene that might be associated with kidney stone disease. Four bioinformatics tools—Sorting Intolerant from Tolerant (SIFT), PolyPhen2, PROVEAN, and I-Mutant 3.0—were employed to evaluate the structural impact, potential detrimental effects, and stability changes of the identified non-synonymous SNPs (nsSNPs).

3.2. Genotyping and Metabolic Parameters Assessment

Inclusion Criteria:

- Cases: Individuals aged 18-65 years with a confirmed diagnosis of kidney stones via ultrasonography and a normal glomerular filtration rate (GFR).
- Controls: Individuals aged 18-65 years without a history of kidney stones, confirmed to have a normal GFR.

Exclusion Criteria:

- Cases: Patients with specific types of stones (e.g., uric acid stones), primary hyperparathyroidism, or any condition that might affect serum calcium and phosphorus levels.
- Controls: Individuals with a family history of urinary stones or gout, and those with abnormal metabolic profiles that could predispose them to kidney stones.

Recruitment Process and Sample Size Calculation:

Patients were recruited from the outpatient department of Urology at Justice K S Hegde Charitable Hospital. The sample size was calculated based on a pilot study, aiming for a power of 80% and a significance level of 0.05, which determined the need for 100 cases and 100 controls. The study was conducted over 36 months, from January 2020 to December 2023.

Ethics Approval and Informed Consent: The study received approval from the Institutional Ethics Committee (Approval No. CEC/NDU/IEC/2020). Written informed consent was obtained from all participants before enrollment, ensuring compliance with ethical standards.

3.3. Laboratory Investigations

A blood sample of three ml was drawn into EDTA vacutainers to analyze gene polymorphisms.

DNA Isolation

DNA was extracted from the blood collected in the EDTA tube using a modified protocol based on Miller et al.'s method. The isolated DNA was quantified using a Nano-drop spectrophotometer at 260nm. The purity of the DNA sample was determined by calculating the ratio of OD260 to OD280. The quantified DNA was then carefully sealed and stored at -20°C until further analysis**.**

Amplification and Genotyping of the gene polymorphism

Genotyping of the genes was confirmed by PCR-RFLP.

Fetuin-A Genotyping:

PCR-RFLP analysis was employed to assess the fetuin-A c.742C>T and c.766C>G single nucleotide polymorphisms.

A forward and reverse oligonucleotide primers were used to amplify the fetuin-A 742C>T polymorphism 5’-CCTCCCACAAGCAGAAAC-3’ & 5’-TGATGATTCCGCATACCC-3’designed respectively using Primer 3Plus.The PCR product was digested overnight at 37°C with NlaIII restriction enzyme then visualized using gel electrophoresis with the Gel DocTM EZ imager (Bio-Rad).

For Analysis of fetuin-A 766C>G polymorphism was performed with the oligonucleotide primers forward 5’-GTCACCCCTCCTTGTAAC-3’ and reverse 5’-CCCCAATGAGACC ACA-3’ for PCR .The PCR product was digested overnight at 37°C with SacI restriction enzyme and digested products were separated on 3% agarose gel.

Biochemical parameters, including serum calcium, phosphorus, uric acid, creatinine, albumin, and Fetuin-A levels, were measured using standardized laboratory techniques.

3.4. Analysis of Linkage Disequilibrium of SNPs and Haplotypes

Linkage disequilibrium (LD) analysis between the SNPs and haplotype analysis was carried out using ShesisPlus and SNPstatonline. This analysis provided

insights into the genetic relationships and co-inheritance patterns of the selected SNPs.

3.5. Statistical Analysis

Statistical analysis was conducted using SPSS version 23. The χ^2 test was used to assess the association between genetic polymorphisms and nephrolithiasis. Metabolic parameters were compared between cases and controls using the Mann-Whitney U test. Additionally, chi-square analysis was applied to evaluate the relationship between gene polymorphisms and metabolic parameters.

4. 3. Results

In the NCBI Reference Protein isoform 2 Sequence (NP_001613.2) of the FETUIN A gene, SIFT analysis was performed, identifying a total of 274 coding variants, encompassing the entire spectrum of variations. Among these, approximately 97% (268 coding variants) were subject to prediction, with 61% (165 variants) classified as "tolerated" and 39% (103 variants) as "harmful." Specifically, the dataset comprised 97% non-synonymous variations (268) and 3% synonymous variations (6). Notably, the analysis identified 97% (256 variants) as novel coding variants. For comprehensive details, please refer to Table 1.

Out of the 274 missense mutations, two specific Single Nucleotide Polymorphisms (SNPs), namely rs4918 (S256N) and rs4917 (M248T), were selected. These SNPs, while having a SIFT score of 0.58 classifying them as "tolerated," were of particular interest due to their association with kidney stone diseases. It is worth mentioning that rs4918 (S256N) is a novel variation. In addition to SIFT, the analysis of these two SNPs was further assessed through Polyphen 2, Provean, and I-Mutant analyses, with the results detailed in Table 1.

Table 1 . Analysis of Fetuin A c.742C>T & c.766C>G Polymorphisms

SNP	Amino Acid Change	SIFT Prediction	SIFT Score	PolyPhen 2 Score	PROVEAN	PROVEAN Score	I-Mutant 3.0 Stability
rs4918 (novel)	S256N	Tolerated	0.58	0.833	Deleterious	-4.994	-0.03 Kcal/mol
rs4917	M248T	Tolerated	1.00	0.00	Neutral	-0.670	-0.70 Kcal/mol

In the study comparing cases (n=100) and controls (n=100), the median age for cases was 46 years with an interquartile range (IQR) of 37-55, whereas the median age for controls was 37 years with an IQR of 26-48. The difference in age between the two groups was statistically significant with a p-value of 0.0011, indicating that cases were significantly older than controls. In terms of sex distribution, 62% of cases were male and 38% were female, while the controls had a higher percentage of males at 78% and a lower percentage of females at 22%.

The study found no statistically significant association between the polymorphism of fetuin-A c.742C>T and individuals prone to developing kidney stones. This is evident from the chi-square value of 0.0833 and the corresponding p-value of 0.772 (Odds Ratio (OR)=1.087, 95% Confidence Interval (CI)=1.914-0.617, as presented in Table 2). Furthermore, there were no noteworthy distinctions observed in the ratio of wild-type and mutant allele variants between the group of individuals with kidney stones and the control group.

Table 2.Association of Fetuin-A Gene Polymorphisms (c.742C>T and c.766C>G) with Renal Stone Disease

Fetuin-A SNP	Genotype	Cases (n=100)	Controls (n=100)	Chi-square Value	p-value	OR (95% CI)
c.742C>T	Wild Type (CC)	59	61	0.0833	0.772	1.087 (0.617-1.914)
	Mutant (CT+TT)	41	39			
c.766C>G	Wild Type (CC)	60	64	0.3396	0.560	0.843 (0.476-1.495)
	Mutant (CG+GG)	40	36			

Similarly, the study did not reveal a significant association between the presence of the Fetuin-A c.766C>G polymorphism and the formation of kidney stones. The chi-square value of 0.3396 and the corresponding p-value of 0.560, along with an Odds Ratio (OR) of 0.843 and a 95% Confidence Interval (CI) ranging from 1.495 to 0.476 (as provided in the results displayed in Table 2), all indicate that there is no significant connection between this polymorphism and kidney stone formation.

Furthermore, among the control group, a higher percentage of individuals (64%) possessed the wild-type allele (CC) compared to the cases (60%). Conversely, cases had a higher percentage (40%) of the mutant allele (CG+GG) compared to the controls (36%), as outlined in Table 2.

To assess the diagnostic capability of fetuin-A in predicting the presence of renal stone disease, a Receiver Operating Characteristic (ROC) curve was constructed. Ultrasound-based stone detection was employed as the gold standard for this evaluation.

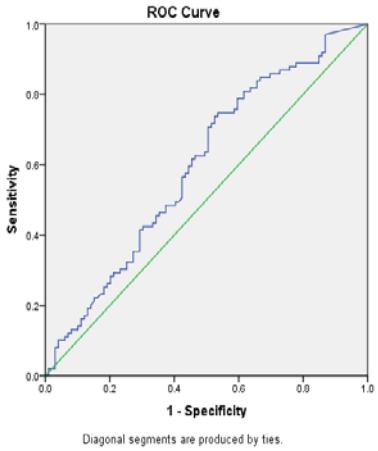

Figure 1. ROC for serum fetuin A as a diagnostic marker for kidney stone disease

The ROC curve on the plot was positioned to the left of the diagonal line (Figure 1), yet it did not reach either the upper or the left border. The ROC analysis suggests that Fetuin-A might not be an ideal marker for the diagnosis of renal stone disease, as indicated by an Area Under the Curve (AUC) value of 0.598. The sensitivity was calculated at 61.6%, and the specificity at 54.5%, using a cut-off value of 75.3.

Tables 3 presents the results of an investigation into the potential associations between specific genetic variations (Fetuin A c.742C>T and Fetuin A c.766C>G) and the serum levels of Fetuin A in individuals with urinary oxalate stones. The data is categorized into two groups based on serum levels, with a cutoff point of 75.339 as the dividing criterion. For the Fetuin A c.742C>T variation, the findings show no significant correlation with either serum group, as indicated by the chi-square values, degrees of freedom, and p-values. The odds ratios with their respective 95% confidence intervals reinforce the lack of substantial association. Similarly, the analysis for the Fetuin A c.766C>G variation yielded no significant relationships within either serum subgroup, as demonstrated by the corresponding statistical values. These results suggest that these specific genetic variations may not be significant contributors to variations in serum levels of Fetuin A among individuals with urinary oxalate stones. The findings underscore the complex and multifaceted nature of the interplay between genetic factors and serum biomarkers in this context.

Table 3. Association of Fetuin-A Gene Polymorphisms (c.742C>T and c.766C>G) with Serum Levels of Fetuin-A in Patients with Urinary Oxalate Stones

Fetuin-A SNP	Genotype	Serum Fetuin-A Levels	Chi-square Value, df	p-value	OR (95% CI)
c.742C>T	Wild Type (CC)	<75.339: 24	X^2=0.254, df=1	0.613	1.238 (0.539-2.84)
		>75.339: 36			
	Mutant (CT+TT)	<75.339: 14			
		>75.339: 26			
c.766C>G	Wild Type (CC)	<75.339: 24	X^2=2.207, df=1	0.137	1.894 (0.891-4.42)
		>75.339: 36			
	Mutant (CG+GG)	<75.339: 14			
		>75.339: 26			

The association between different allele models (co-dominant, dominant, recessive, and over dominant) of c.766C>G with kidney stone disease (KSD) did not demonstrate statistical significance (p=0.68, p=0.66, p=0.4, and p=0.88, respectively) (Table 8). In the co-dominant model (adjusted, the "G/G" genotype), there was a 2.10-fold increase in the risk of kidney stone disease, but this association was not statistically significant. Table 4 presents a comprehensive analysis of different models assessing the association between the Fetuin A c.766C>G polymorphism and a specific condition, possibly kidney stones. These models explore various genetic variations and their impact on the risk of the condition, with comparisons between control and case groups. Additionally, they provide odds ratios (OR) and 95% confidence intervals (CI), p-values, and information criteria (AIC and BIC) to evaluate the fit of each model.

The Codominant model scrutinizes the C/C and C/G genotypes individually, revealing no significant differences between cases and controls for these genotypes. The Dominant model groups C/G and G/G genotypes, yielding similar results. The Recessive model also indicates no substantial distinctions between groups, with the G/G genotype not significantly associated with the condition. The Overdominant model pairs C/C and G/G genotypes but does not show significant associations. Finally, the Log-Additive model, assuming a log-additive relationship between genotypes, also fails to establish a significant connection between the genetic variation and the condition.

Collectively, these models suggest that the Fetuin A c.766C>G polymorphism may not be significantly linked to the condition under investigation, as none of the models reveal a statistically significant relationship. The AIC and BIC values further support the notion that these models may not provide a strong fit to the data, emphasizing the complexity of genetic factors in the context of the condition being studied.

Similarly, the association between different allele models (co-dominant, dominant, recessive, and over dominant) of c.742C>T with kidney stone disease (KSD) also showed no significant results (p=0.22, p=0.77, p=0.09, and p=0.56, respectively) (Table 4).

Table 4. Different Models of Fetuin-A c.766C>G and c.742C>T Polymorphisms

Model	Genotype	Control	Case	OR (95% CI)	P-value
Codominant	c.766C>G				0.68
	C/C	63 (63%)	60 (60%)	1.00	
	C/G	35 (35%)	36 (36%)	1.08 (0.60-1.94)	
	G/G	2 (2%)	4 (4%)	2.10 (0.37-11.89)	
Dominant	c.766C>G				0.66
	C/C	63 (63%)	60 (60%)	1.00	
	C/G-G/G	37 (37%)	40 (40%)	1.14 (0.64-2.01)	
Recessive	c.766C>G				0.4
	C/C-C/G	98 (98%)	96 (96%)	1.00	
	G/G	2 (2%)	4 (4%)	2.04 (0.37-11.41)	
Overdominant	c.766C>G				0.88
	C/C-G/G	65 (65%)	64 (64%)	1.00	
	C/G	35 (35%)	36 (36%)	1.04 (0.59-1.86)	
Log-additive	c.766C>G	---	---	1.18 (0.71-1.96)	0.52
Codominant	c.742C>T				0.22
	C/C	59 (59%)	61 (61%)	1.00	
	C/T	41 (41%)	37 (37%)	0.87 (0.49-1.54)	
	T/T	0 (0%)	2 (2%)	NA (0.00-NA)	
Dominant	c.742C>T				0.77
	C/C	59 (59%)	61 (61%)	1.00	
	C/T-T/T	41 (41%)	39 (39%)	0.92 (0.52-1.62)	
Recessive	c.742C>T				0.095
	C/C-C/T	100 (100%)	98 (98%)	1.00	
	T/T	0 (0%)	2 (2%)	NA (0.00-NA)	
Overdominant	c.742C>T				0.56
	C/C-T/T	59 (59%)	63 (63%)	1.00	
	C/T	41 (41%)	37 (37%)	0.85 (0.48-1.49)	
Log-additive	c.742C>T	---	---	1.00 (0.58-1.72)	1.00

Table 4 provides a comprehensive overview of different models used to investigate the relationship between the Fetuin A c.742C>T polymorphisms and a specific condition, potentially kidney stones. These models offer a detailed analysis of the genetic variations and their potential impact on the risk of the condition. Each model presents odds ratios (OR) with their respective 95% confidence intervals (CI), p-values, and information criteria (AIC and BIC) to assess the adequacy of the model fit.

The Codominant model scrutinizes the C/C, C/T, and T/T genotypes individually, indicating no significant differences between cases and controls for these genotypes. The Dominant model groups C/T and T/T genotypes together, yielding similar results. The Recessive model presents no significant distinctions between the two groups, with the T/T genotype not significantly associated with the condition. The Overdominant model pairs C/C and T/T genotypes together but does not establish significant associations. The Log-Additive model, assuming a log-additive relationship between genotypes, also fails to indicate a substantial connection between the genetic variation and the condition.

Collectively, these models suggest that the Fetuin A c.742C>T polymorphism may not be significantly linked to the condition under investigation, as none of the models reveal a statistically significant relationship. The AIC and BIC values further support the notion that these models may not provide a strong fit to the data, underscoring the complexity of genetic factors in the context of the condition being studied.

The current study employed haplotype analysis to estimate haplotype frequencies in a random sample set and resolve ambiguous haplotypes. The analysis platform can

automatically generate results for both individual haplotypes and the entire dataset, independently estimating haplotype frequencies in control and case groups. In case-control studies, low-frequency haplotypes may be combined to yield a single outcome (SHI 2005).

The haplotyping results for the two SNPs are presented in the table. The presence of haplotypes fetuin-A c.742C>T, Fetuin-A c.766C>G (GT), fetuin-A c.742C>T, Fetuin-A c.766C>G (GC), and Haplotypes CC were compared, revealing that the haplotypes fetuin-A c.742C>T, Fetuin-A c.766C>G (GT), and fetuin-A c.742C>T, Fetuin-A c.766C>G (GC) increase the risk of kidney stone disease by 1.14, 1.00, and 0.98, respectively (Table 10).

Table 5 presents the results of haplotype analysis for the Fetuin A gene, specifically considering the combinations of the Fetuin-A c.766C>G and Fetuin-A c.742C>T alleles. The table displays the frequency of each haplotype, along with the odds ratio (OR) and 95% confidence interval (CI) for their potential association with a specific condition, likely kidney stone disease (KSD). Additionally, it reports the global haplotype association p-value.

The analysis reveals that none of the identified haplotypes, including CC, TG, CG, and CT, show a statistically significant association with KSD. The most prevalent haplotype, CC (0.7639 frequency), serves as the reference, with an OR of 1.00. The remaining haplotypes, TG, CG, and CT, do not exhibit significant associations with KSD, as indicated by their respective ORs and p-values. The global haplotype association p-value is 0.44, further underscoring the lack of a significant relationship between these haplotypes and the condition.

Table 5. Haplotype Analysis of Fetuin-A Gene

Haplotype	Case (freq)	Control (freq)	Chi²	Fisher's p	Pearson's p	OR (95% CI)	Frequency	Global haplotype association p-value
CC	149 (0.745)	154 (0.77)	0.34	0.64	0.559	0.872 (0.552-1.37)	0.7639	0.44
TG	43 (0.215)	32 (0.16)	1.985	0.199	0.158	1.437 (0.866-2.386)	0.1764	
CG	6 (0.03)	6 (0.03)	0	1	1	1.00 (0.316-3.15)	0.0311	
CT	---	---	---	---	---	0.37 (0.09-1.47)	0.0286	

Table 5 further examines the association between these haplotypes in case and control groups. It provides the frequency of each haplotype in both groups, along with chi-square, Fisher's p, Pearson's p, and OR values. The analysis confirms that none of the haplotypes, including CC, TG, and CG, display a significant association with KSD when assessed between the case and control groups.

In summary, the haplotype analysis for Fetuin A gene variations does not reveal any significant associations with kidney stone disease. The data suggests that these specific haplotypes, including CC, TG, and CG, do not play a significant role in influencing the risk of developing KSD. The global haplotype association p-value reinforces the absence of a substantial association between these genetic combinations and the condition.

Linkage disequilibrium (LD) was assessed by calculating Lewontin's D' (|D'|) and R2 for each pair of genetic markers. These metrics are commonly utilized in LD analysis, block detection, and SNP tagging studies. They range from 0 to 1, with D' indicating complete LD or correlation between alleles and R2 being more frequently used as it considers allele frequency. D', on the other hand, is independent of allele frequency and only signifies whether alleles are inherited together (Perrera 2022).

In the present study, the calculated D' value was 0.84, indicating that when the rare allele is present, it tends to be inherited on the same haplotype as the common allele (Fig. 2). Meanwhile, the R2 value was 0.7, which suggests that one allele is rare, and the other allele is common (Fig. 3).

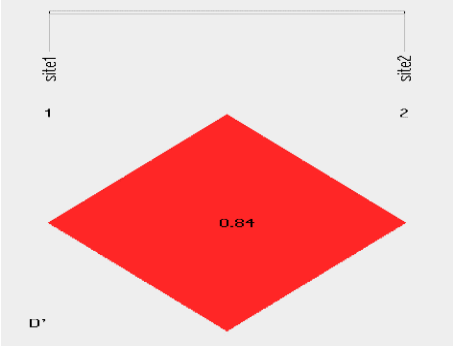

Figure 2. Depicting D' of LD

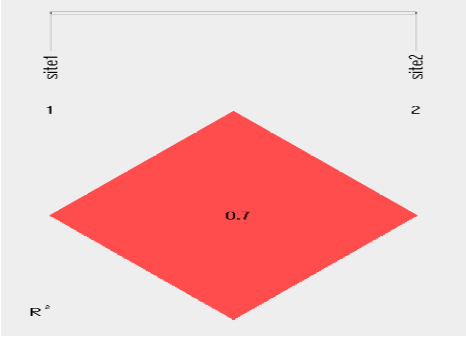

Figure 3. Depicting R^2 of LD

Table 6 displays the demographic characteristics of both cases and controls. In comparison to the controls, the median levels of calcium were significantly higher in cases, with values of 10 (8.95-11.35) versus 9.97 (8.83-12.39). Similarly, the median levels of creatinine were also significantly higher in cases, with values of 1 (0.83-1.27) versus 0.8 (0.56-0.96).

On the other hand, the serum levels of phosphorous, uric acid, and Fetuin-A were significantly lower in patients compared to the controls. The median levels of phosphorous were 5.35 (4.7-6.2) in cases versus 6.11 (4.8-7.3) in controls. The median levels of uric acid were 4.23 (2.15-5.77) in cases versus 5.13 (4.3-6.14) in controls. Lastly, the median levels of Fetuin-A were 74.09 (59-83) in cases versus 77.83 (70-86) in controls.

Table 6.Biochemical parameters in calcium oxalate stone disease

	Case (n=100) Median(interquartile range)	Controls (n=100) Median(interquartile range)	P value
Blood Biochemistry			
Uric acid(mg/dL)	4.23(2.15-5.77)	5.13(4.3-6.14)	<0.0001***
Creatinine(mg/dL)	1(0.83-1.27)	0.8(0.56-0.96)	<0.0001***
eGFR(mg/dL)			
Phosphorous(mg/dL)	5.35(4.7-6.2)	6.11(4.8-7.3)	0.0071**
Calcium(mg/dL)	10(8.95-11.35)	9.97(8.83-12.39)	0.739
Albumin(g/dL)	4.31(2.76-4.9)	4.02(3.13-4.64)	0.226
Fetuin-A(ng/ml)	74.09(59-83)	77.83(70-86)	0.018*

Table 7 present a comparison of biochemical parameters between dominant and recessive alleles of fetuin-A c.742C>T and fetuin-A c.766C>G genotypes within both case and control groups.

In the case of the CT+TT genotype for fetuin-A c.742C>T, the analysis revealed that serum uric acid levels were significantly lower in cases (3.9 mg/dL with a range of 2.3-5.7) compared to controls (5.3 mg/dL with a range of 4.2-6.2), with a statistically significant p-value of 0.0017. This suggests that individuals with the CT+TT genotype may exhibit reduced uric acid levels in cases of the condition, likely kidney stone disease. Additionally, the serum calcium levels in patients with the CT+TT genotype were significantly higher than those in the control group.

These findings indicate a potential link between the CT+TT genotype of fetuin-A c.742C>T and altered uric acid and calcium levels, which may have implications in the context of kidney stone disease. Further investigation is needed to understand the underlying mechanisms and clinical relevance of these observed differences in biochemical parameters.

Patients with the CG+GG genotype exhibited some notable differences in biochemical parameters when compared to the control group. Specifically, individuals with the CG+GG genotype had significantly lower levels of uric acid (4 mg/dL with a range of 2.3-5.2) compared to the control group (5.3 mg/dL with a range of 4.3-6), with a p-value of 0.0013. Furthermore, they displayed lower phosphorous levels (5.1 mg/dL with a range of 4.4-6.2) compared to the control group (6 mg/dL with a range of 5.4-7.6). Additionally, patients with the CG+GG genotype had lower serum Fetuin-A levels (70.2 mg/dL with a range of 54.8-81) compared to the control group (77.9 mg/dL with a range of 68.6-86), with a p-value of 0.009. In contrast, they exhibited higher creatinine levels (1 mg/dL with a range of 0.89-1.44) compared to the control group (0.7 mg/dL with a range of 0.5-0.95), with a p-value of less than 0.0001. These findings suggest that the CG+GG genotype may be associated with alterations in these biochemical parameters, potentially related to kidney stone disease.

Table 7. Comparison of biochemical parameters based on fetuin-A genotypes

Biochemical Parameter	Genotype	Case Median (IQR)	Control Median (IQR)	p-value
Fetuin-A c.742C>T				
Age (years)	CC	46 (34-55)	37 (25-50)	0.0322*
	CT+TT	47 (37-57)	38 (26-47)	0.0061**
Uric Acid (mg/dL)	CC	4.2 (1.8-5.8)	4.9 (4.3-6.1)	0.0036**
	CT+TT	3.9 (2.3-5.7)	5.3 (4.2-6.2)	0.0017**
Creatinine (mg/dL)	CC	0.8 (0.6-1.2)	0.8 (0.6-0.9)	0.0014**
	CT+TT	1.0 (0.8-1.4)	0.7 (0.5-0.9)	<0.0001***
Phosphorous (mg/dL)	CC	5.4 (4.8-6.19)	6.3 (4.8-7.5)	0.029
	CT+TT	5.1 (4.4-6.2)	5.7 (4.9-6.8)	0.12
Calcium (mg/dL)	CC	10.7 (9-13)	10 (9-11.4)	0.12
	CT+TT	9.7 (8.6-10.9)	9.3 (8.4-10.4)	0.39
Albumin (g/dL)	CC	4.2 (2.9-4.9)	3.9 (3.1-4.6)	0.33
	CT+TT	4.4 (2.2-4.8)	4.0 (2.9-4.6)	0.4
Fetuin-A (ng/ml)	CC	77.55 (55.3-81.6)	77.86 (70.11-87.8)	0.5
	CT+TT	74.4 (66.8-86.2)	77.69 (70-83)	0.66
Fetuin-A c.766C>G				
Age (years)	CC	36 (26-47)	46 (33-55)	0.005**
	CG+GG	44 (37-54)	39 (26-52)	0.12
Uric Acid (mg/dL)	CC	4.9 (4.3-6.2)	4.2 (1.9-5.8)	0.003**
	CG+GG	4.0 (2.3-5.2)	5.3 (4.3-6.0)	0.0013**
Creatinine (mg/dL)	CC	0.81 (0.6-0.98)	0.96 (0.8-1.2)	0.0007***
	CG+GG	1.0 (0.89-1.44)	0.7 (0.5-0.95)	<0.0001***
Phosphorous (mg/dL)	CC	5.5 (4.7-6.2)	6.2 (4.7-7.3)	0.159
	CG+GG	5.1 (4.4-6.2)	6.0 (5.4-7.6)	0.0045**
Calcium (mg/dL)	CC	10 (8.9-11.3)	10.7 (9-13)	0.086
	CG+GG	9.7 (8.5-10.5)	9.9 (8.9-11)	0.26
Albumin (g/dL)	CC	4.3 (3-4.9)	3.9 (3.2-4.6)	0.099
	CG+GG	2.1 (4.1-4.8)	4.0 (3-4.6)	0.99
Fetuin-A (ng/ml)	CC	77.3 (69.1-87)	78.1 (72.4-83.1)	0.727
	CG+GG	70.2 (54.8-81)	77.9 (68.6-86)	0.009**

For patients with the CT+TT genotype of fetuin-A c.742C>T, they had significantly lower calcium levels compared to those with the CC genotype. However, for patients with the CG+GG genotype of fetuin-A c.766C>G, no significant differences were observed between cases and controls in terms of calcium levels. These differences in calcium levels might be indicative of specific effects associated with these genotypes, highlighting their potential significance in the context of kidney stone disease.

5. Discussion

Fetuin-A, a protein primarily synthesized in the liver, serves as a valuable biomarker in the context of kidney stone disease. It plays a significant role in forming calciprotein particles in collaboration with phosphate and calcium, a process that increases the solubility of these particles and disrupts hydroxyapatite formation, ultimately inhibiting calcification. However, high calcium deposition can gradually deplete fetuin-A levels in the serum, which may lead to mineral precipitation and, consequently, the formation of kidney stones in the renal system (Al-Shuhaib et al.,2019; Roy et al., 2010).

To explore the potential genetic factors involved in kidney stone disease, the current study employed a selection of Single Nucleotide Polymorphisms (SNPs) using bioinformatics tools like SIFT Analysis, Polyphen2, Provean, and I mutant3.0. These tools are instrumental in evaluating the consequences of amino acid substitutions on protein structure, function, and stability. The selected SNPs were assessed based on specific scoring criteria to

determine their impact on protein function and stability (Choi et al., 2012; Kono et al., 2018).

Several prior studies have highlighted the association between the fetuin-A c.766C>G polymorphism and serum fetuin-A levels. In certain populations, G carriers were found to have lower serum fetuin-A concentrations than non-G carriers, and this was observed in diabetic patients and those with coronary artery calcification (Temesszentandrási et al.,2016, Bellia et al.,2012). Additionally, the mutant GG genotype of fetuin-A c.766C>G was linked to lower circulatory fetuin-A levels in patients with type 2 diabetes, particularly in the context of diabetic nephropathy(Umapathy et al.,2022). Furthermore, alleles T and G in the fetuin-A gene were associated with lower serum fetuin-A levels, higher occurrence of coronary artery calcification, and increased mortality rates in individuals with renal transplantation and chronic kidney disease (Jovičić-Pavlović et al.,2022). Patients carrying the FETUIN A 256Ser allele exhibited lower serum fetuin-A levels and higher all-cause and cardiovascular mortality rates if they were inflamed (Stenvinkel et al.,2005).

A significant finding in this study was the dominance of the GG polymorphism of fetuin-A c.766C>G in patients with kidney stone disease, signifying a potential genetic predisposition. It is important to note that the role of fetuin-A gene polymorphisms in kidney stone disease has been a subject of limited exploration, and this study contributes valuable insights(Schafer et al.,2003).

The potential interplay between fetuin-A and kidney stone disease involves complex mechanisms. Fetuin-A appears to regulate calcium burden and deposition, with higher calcium burden potentially leading to reduced fetuin-A protein levels. Conversely, low fetuin-A levels may promote crystal deposition, induce cell injury, and trigger oxidative stress, which can, in turn, lead to crystal adherence, aggregation, and deposition in the kidneys. Fetuin-A is also involved in inhibiting inflammation and oxidative stress, and its levels are negatively correlated with inflammatory markers in various inflammatory diseases (Noori et al.,2020).

To investigate the genetic factors further, gene-based association studies were conducted using the "SHEsis" tool, which allowed for haplotype inference, linkage disequilibrium analysis, and single locus association tests. The strong linkage observed between the two SNPs (rs4917, rs4918) in the FETUIN A gene provides a basis for future research into their co-inheritance and potential implications in kidney stone disease(Aksoy et al.,2010).

In addition to genetic analysis, structural impact assessments of the most damaging nsSNPs were performed using protein modeling tools such as HOPE and I-TASSER. These tools offer insights into how amino acid substitutions can affect protein structure. The structural analysis revealed the specific changes in amino acids and their potential structural consequences, providing valuable information about the genetic variants' effects on fetuin-A(Aksoy et al.,2010).

In summary, this study sheds light on the genetic and structural aspects of fetuin-A in the context of kidney stone disease, offering insights into potential genetic predispositions and the structural consequences of specific genetic variants. The findings emphasize the complex interplay between fetuin-A, genetics, and kidney stone formation, providing a foundation for further research in this area(Aksoy et al.,2010).

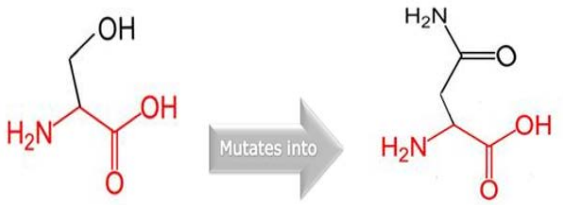

Figure 4. HOPE modelling of c.766C>T

Amino acids exhibit distinctive characteristics, including size, charge, and hydrophobicity. Mutations often involve replacing the original wild-type amino acid with a mutant amino acid that differs in size and hydrophobicity. Typically, the mutant amino acid is larger and less hydrophobic than the wild-type amino acid. These variations in size and hydrophobicity can lead to the disruption of hydrogen bonds within the protein's core, thereby affecting its proper folding process.

For example, in the case of a mutation where Methionine is replaced by Threonine at position 248 (as shown in Figure 5), the HOPE analysis identified a change in charge between the wild-type and mutant amino acids, which stemmed from the hydrophobicity differences between them. This alteration in hydrophobic interactions, whether occurring within the protein's core or on its surface, can hinder the protein's folding. Furthermore, the mutant amino acid's smaller size may pose challenges, particularly if the wild-type amino acid was originally situated deep within the protein's core, as the mutant's larger size might not fit appropriately. This discrepancy in size, charge, and hydrophobicity underscores the potential structural consequences of amino acid mutations in proteins.

Figure 5. HOPE modelling of c.742C>T

The mutated residue is in close proximity to a residue involved in forming a cysteine bond. Although the cysteine bond itself remains unchanged, the nearby mutation could potentially impact its function. This mutation takes place within a domain known as Cystatin fetuin-A-type 2, and the introduction of an amino acid with differing properties may disrupt the function of this specific domain.

It is worth noting that the wild-type residue at this position is not conserved, meaning that it is not consistently preserved across related protein sequences. Conversely, the mutant residue is more frequently observed in other homologous sequences, suggesting that it is a common variant in proteins similar to the one under study. This indicates that the mutation may not have a detrimental effect on the protein's function. Additionally, the mutant residue is positioned near a highly conserved site, indicating potential stability within the protein structure.

In the I-TASSER analysis, the confidence score (C-score) is employed to predict each protein structure model. A C-score greater than -1.5 indicates a correctly folded structure. Moreover, the Tm score serves as a measure of structural similarity, and a Tm score above 0.5 indicates similarity to other proteins within the same structural classification in the protein database family.

6. Conclusion

In summary, based on the analysis, there is no evidence supporting an association between FETUIN A2 gene polymorphisms and reduced levels of Fetuin-A. The structural changes in the amino acid sequence of fetuin A do not appear to have a significant impact on its function.

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Conflicts of interest:

None

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