Original Article

Genotyping And Haplotype Analysis Of Fetuin A In Kidney Stone Disease

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Abstract

Background: The polymorphism of the plasma glycoprotein fetuin-A, a powerful inhibitor of calcification, is least studied in kidney stone disease. The purpose of the study is to compare patients with and without urinary oxalate stones in terms of the distribution of fetuin A gene polymorphisms and associated linkage disequilibrium (LD).

Methods: 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 analyse the fetuin-A c.742C>T and c.766C>G Single Nucleotide Polymorphisms (SNPs) (PCR–RFLP). SHE sisplus and SNP stat online were used to analyse the linkage disequilibrium between the SNPs.

Results: 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 fetuin A 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.

Conclusion: The study's findings suggest that there is no conclusive evidence linking renal stone disease with fetuin A 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 significantly significant connection with the CT and GC haplotypes of c.742C>T and c.766C>G.

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

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].

Over 80% of all urinary calculi, or kidney stones, are calcium stones, making them the most prevalent type of kidney stone. The majority of calcium stones may be composed of calcium oxalate (50%) calcium phosphate (5%), or a combination of both (45%) [5]. The most prevalent component of calcium stones is calcium hydrogen phosphate, which is frequently referred to as hydroxyapatite [6,7]. More than 60% of all kidney stones contain calcium oxalate, either in the form of calcium oxalate monohydrate (COM), calcium oxalate dihydrate (COD), or a combination of both [8]. Stone of the COM type is the most thermodynamically stable type. COM is more frequently seen than COD in clinical stones [9]. There are a number of variables that can affect the development of calcium oxalate stones, including hypercalciuria, hypereroxaluria, hypocitraturia, and hypomagnesuria[10].

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[11,12]. These stages are controlled by an imbalance between the chemicals that promote or prevent urine crystallization[13].

Finding the chemicals and metabolic modifications that affect stone formation may enable intervention. One of these compounds that has gotten the least attention is fetuin A. It may be possible to use fetuin A as a biomarker to foretell the development of calcium oxalate stones.

A 45-kDa plasma glycoprotein called fetuin-A is mostly produced by the liver[14]. Two polypeptide chains from posttranscriptional cleavage make up its structure. With calcium and phosphorus, it creates reversible compounds that increase the solubility of those elements in blood[15,16].

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[17]. 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% [18].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[19]. There is little information on gene polymorphisms and their linkage disequilibrium in nephrolithiasis.

OBJECTIVES

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

METHODS

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.
- Patients who met even one of the following requirements were disqualified;
- Patients with cysteine/uric acid stones identified with qualitative testing
- 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
  Subjects with the history of urinary stone or family history of urinary stone and Gout were excluded.

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[20].
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 c.742C>T polymorphism was amplified using Primer 3Plus utilising the forward and reverse oligonucleotide primers 5'-CCTCCCACAAGCAGAAAC-3' and 5'-TGATGATTC-CGCATACCC-3', respectively.
Mini Amp + 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 Doc TM EZ imager from Biorad 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 c.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 FETUIN A 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 semi automated chemistry analyser. Estimated Glomerular Filtration Rate (eGFR) was calculated using Modification of Diet in Renal Disease (MDRD) formula.

STATISTICAL ANALYSIS

The statistical analysis was carried out utilising 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 ShesisPlus (http://shesisplus.bio-x.cn/SHEsis.html) software was used for the LD analysis, and SNPstat (https://snpstats.net/start.htm) was used for the online haplotype analysis.

RESULTS

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 fetuin A 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.

Table 1. Distribution of genotypes and allele frequencies of Fetuin-A c.742C>T

Fetuin A (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%) x^2=0.891, df=2 ,p=0.640	30(60%) 18(36%) 2(4%)	
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 c.766C>G

Fetuin A (c.766 C>G) Genotype	Control (%)	Case (%)	P value
CC CG GG	32(64%) 17(32%) 1(4%) x^2=0.426,df=2,p=0.808	30(60%) 18(36%) 2(4%)	
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.

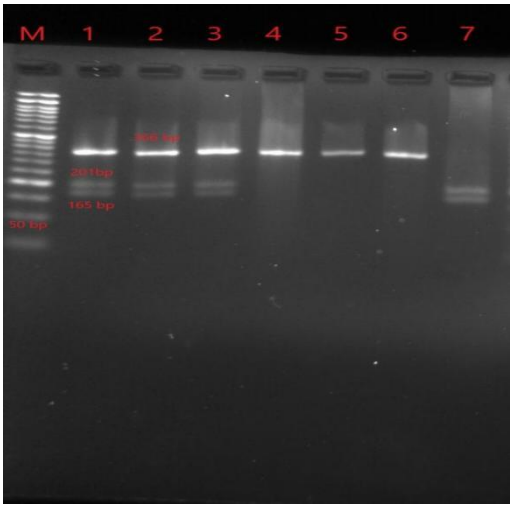

Fig 1: PCR RFLP analysis c.742C>T polymorphism in the of Fetuin A. Lane M: 50 bp marker: Lanes 1,2,3: CT alleles ;Lanes 4,5,6: CC alleles ; Lanes 7:TT alleles

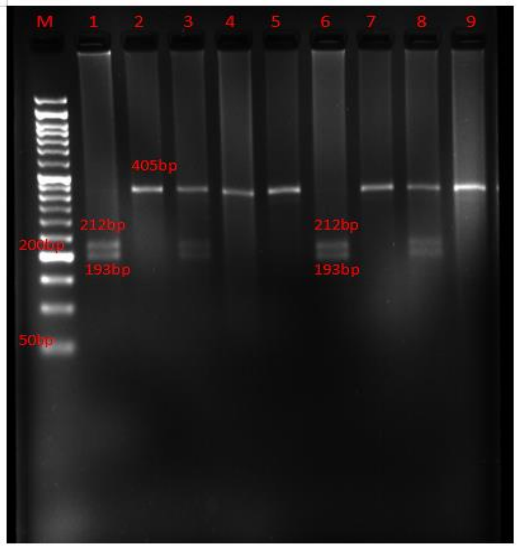

Fig 2 : PCR RFLP analysis c.766C>G polymorphism in the of Fetuin A. Lane M: 50 bp marker: Lanes 1,6: GG alleles ;Lanes 2,4,5,7,9: CC alleles ; Lanes 3,8 :CG alleles

D' and r^2 were calculated to determine the degree of LD in paired combinations of SNPs. LD between the c.742C>T and c.766C>G fetuin A haplotypes was discovered utilising the SHE sisplus 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 fetuin A were found to have higher D' values, indicating their co-inheritance.

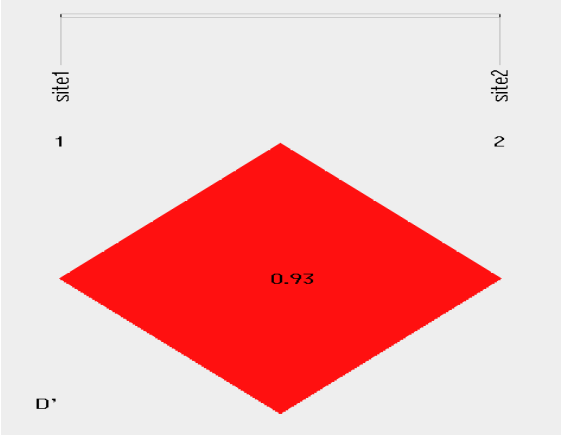

Fig3A: Haplotype block (D') of two sites, fetuin A **c.742C>T(** rs4917) & fetuin A **c.766C>G (**rs4918) SNPs

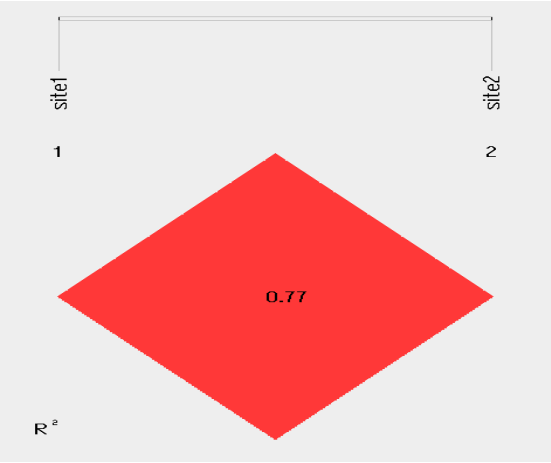

Fig3B: Haplotype block (R^2) of two sites, fetuin A **c.742C>T(** rs4917) & fetuin A **c.766C>G (**rs4918) SNPs

A ‘highly significant association was observed between kidney stone disease and Haplotypes C,T,G,C SNPs (p<0.0001) (table 3).

Table 3: Association of single haplotypes of 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 4).

Table 4: Association of haplotypes of 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 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 r^2 values. r^2 values between 0 and 1 suggest bright coloured squares, whereas r^2= 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 Shesisplus and SNP Stat online analysis allowed us to determine that the fetuin-A alleles were in equilibrium. High D' and r^2 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 utilised 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 a study by Hulya et al., it was discovered that kidney stone disease significantly affected the genotypic frequencies of fetuin-A c.766C>G between the control and patient groups (p=0.003) [15]. In both cases and controls, the fetuin-A c.742C>T polymorphism did not have a statistically significant distribution (p=0.77). The presence of kidney stones was not substantially correlated with any of the fetuin-A SNPs (p>0.05). The kidney stone disease (KSD) odds ratios for the dominant, recessive, and co-dominant alleles of the c.766C>G mutation were 0.94, 0.47, and 0.47 times, respectively, with 95% confidence intervals (CI) of (0.42-2.1), (0.04-5.36), and (0.04-5.44).

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).

DISCUSSION

Fetuin-A is one of the numerous non-collagenous proteins involved in osteogenesis and is known to have a strong affinity for calcium ions [21,22]. It has been demonstrated that fetuin-A prevents calcium phosphate crystals from growing and forming [23–25]. By generating a fetuin-mineral complex, hydroxyapatite crystals appear to be absorbed by fetuin-A, preventing its deposition and inhibiting non-bone calcification [26,27,28]. Fetuin-A has been proposed as a predictor of poor prognosis in persons with acute atherosclerosis and patients receiving hemodialysis [16]. Rats' heart, lungs, kidneys, and skin have all shown extensive calcification in response to low serum fetuin-A levels [29]. Additionally, it stops the calcification of vascular smooth muscle cells [28].

According to Cai et al., this protein may be important in the malfunction of mineral deposition or other mechanisms that lead to defective mineralization [30]. 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 [31,32].
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.
Fetuin A 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[33].
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 single haplotype G C and T C increases the risk of calcification of the heart valves and coronary artery by 1.78 and 2.38-fold, respectively[34].

CONCLUSION

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 fetuin A 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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REFERENCES:

1. Alelign T, Petros B. Kidney stone disease: an update on current concepts. Advances in urology. 2018;2018.
2. Liu Y, Chen Y, Liao B, Luo D, Wang K, Li H, Zeng G. Epidemiology of urolithiasis in Asia. Asian journal of urology. 2018;**5**(4):205-14.
3. Faridi MS, Singh KS. Preliminary study of prevalence of urolithiasis in North-Eastern city of India. Journal of Family Medicine and Primary Care. 2020;9(12):5939.
4. Khan SR, Pearle MS, Robertson WG, Gambaro G, Canales BK, Doizi S, Traxer O, Tiselius HG. Kidney stones. Nature reviews Disease primers. 2016;2(1):1-23.
5. Chaudhary A,Singla SK, Tandon C. In vitro evaluation of Terminalia arjuna on calcium phosphate and calcium oxalate crystallization. Indian journal of pharmaceutical sciences. 2010;**72**(3):340.
6. Coe FL, Parks JH, Asplin JR. The pathogenesis and treatment of kidney stones. New England Journal of Medicine.1992;**327**(16):1141-52.
7. Skolarikos A, Straub M, Knoll T, Sarica K, Seitz C, Petřík A, et al. Metabolic evaluation and recurrence prevention for urinary stone patients: EAU guidelines. European urology. 2015;**67**(4):750-63.
8. Bensatal A, Ouahrani MR. Inhibition of crystallization of calcium oxalate by the extraction of Tamarix gallica L. Urological Research. 2008;**36**(6):283-7.
9. Basavaraj DR, Biyani CS, Browning AJ, Cartledge JJ. The role of urinary kidney stone inhibitors and promoters in the pathogenesis of calcium containing renal stones. EAU-EBU update series. 2007;**5**(3):126-36.
10. Dal Moro F, Mancini M, Tavolini IM, De Marco V, Bassi P. Cellular and molecular gateways to urolithiasis: a new insight. Urologia internationalis. 2005;**74**(3):193-7.
11. Kishore DV, Moosavi F, Varma RK. Effect of ethanolic extract of Portulaca oleracea linn. on ethylene glycol and ammonium chloride induced urolithiasis. International Journal of Pharmacy and Pharmaceutical Sciences. 2013;**5**(2):134-40.
12. Kumar SB, Kumar KG, Srinivasa V, Bilal S. A review on urolithiasis. International Journal of Universal Pharmacy and Life Sciences. 2012;**2**(2):269-80.
13. Chung HJ. The role of Randall plaques on kidney stone formation. Translational Andrology and Urology. 2014;**3**(3):251.
14. Nawratil P, Lenzen S, Kellermann J, Haupt H, Schinke T, Müller-Esterl W, et al. Limited proteolysis of human alpha 2-HS glycoprotein/fetuin. Evidence that a chymotryptic activity can release the connecting peptide. J Biol Chem.1996;**271**:31735-31741.
15. Schäfer C, Heiss A, Schwarz A, Westenfeld R, Ketteler M, Floege J, et al. The serum Protein α2–Heremans-Schmid glycoprotein/fetuin-A is a systemically acting inhibitor of ectopic calcification. The Journal of clinical investigation 2003;**112**(3):357-66
16. Ketteler M, Bongartz P, Westenfeld R, Wildberger JE, Mahnken AH, Böhm R, et al, Association of low fetuin-A (AHSG) concentrations in serum with cardiovascular mortality in patients on dialysis: a cross-sectional study. The Lancet 2003;**361**(9360):827-33.
17. Price PA, Lim JE. The inhibition of calcium phosphate precipitation by fetuin is accompanied by the formation of a fetuin-mineral complex. Journal of Biological Chemistry 2003;**278**(24):22144-52.
18. Stejskal D, Karpisek M, Vrtal R, Student V, Solichova P, Fiala R, et al. Urine fetuin-A values in relation to the presence of urolithiasis. BJU international. 2008;**101**(9):1151-4.
19. Aksoy H, Aksoy Y, Ozturk N, Aydin HR, Yildirim AK, Akçay F. Fetuin-A gene polymorphism in patients with calcium oxalate stone disease. Urology. 2010;**75**(4):928-32.
20. MWer S, Dykes D, Polesky H. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic acids res. 1988;**16**(3):1215.
21. Triffitt J, Gebauer U, Ashton B, Owen M, Reynolds J. Origin of plasma α2HSglycoprotein and its accumulation in bone. Nature. 1976;**262**: 226-227.
22. Dickson IR, Bagga M, Paterson CR. Variations in the serum concentration and urine excretion ofα 2HS-glycoprotein,a bone-related protein, in normal individuals and in patients with osteogenesis imperfecta. Calcif Tissue Int. 1983; **35**: 16-20.
23. Heiss A, Du Chesne A, Denecke B, Grotzinger J, Yamamoto K, Renné T, et al, Structural basis of calcification inhibition by α2-HS glycoprotein/fetuin-A formation of colloidal calciprotein particles. J Biol Chem. 2003; **278**: 13333-13341.
24. Vitorino R, Lobo MJC, Duarte J, Ferrer-Correia AJ, Tomer KB, Dubin JR, et al., In vitro hydroxyapatite adsorbed salivary proteins. Biochem Biophys Res Commun. 2004; **320**: 342-346.
25. Armstrong WG. Characterisation studies on the specific human salivary proteins adsorbed in vitro by hydroxyapatite. Caries Res. 1971; **5**:215-227.

26. Schmid K, Bürgi W. Preparation and properties of the human plasma Ba-α 2-glycoproteins. Biochim Biophys Acta 1961; **47**: 440-453.
27. Mosa O, Mohamad I, Salama M. Relationship between fetuin-A and systemic lupus erythematosus as a predictor marker for atherosclerosis. Am Med J. 2012; **3**: 249-254.
28. Reynolds JL, Skepper JN, McNair R, et al., Multifunctional roles for serum protein fetuin-a in inhibition of human vascular smooth muscle cell calcification. J Am Soc Nephrol. 2005; **16**: 2920-2930.
29. Ketteler M, Bongartz P, Westenfeld R, Kasama T, Gupta K, Weissberg PL, et al. Association of low fetuin-A (AHSG) concentrations in serum with cardiovascular mortality in patients on dialysis: a crosssectional study. Lancet. 2003; **361**: 827-833.
30. Cai MM, Smith ER, Holt SG. The role of fetuin-A in mineral trafficking and deposition. Bonekey Rep. 2015; **4**: 672.
31. Umekawa T, Iguchi M, Konya E, Yamate T, Amasaki N, Kurita T. Localization and inhibitory activity of α2HS-glycoprotein in the kidney. Urol Res. 1999; **27**: 315-318.
32. Nishio S, Hatanaka M, Takeda H, Aoki K, Iseda T, Iwata H,et al. Calcium phosphate crystal-associated proteins: α-2-HS-glycoprotein,prothrombin fragment 1 and osteopontin. Int J Urol. 2001; **8**: S58-S62.
33. Abrol N, Panda A, Kekre NS, Devasia A. Nanobacteria in the pathogenesis of urolithiasis: Myth or reality? Indian J Urol. 2015;31(1):3-7.
34. Mohammadi-Noori E, Salehi N, Mozafari H, Elieh Ali Komi D, Saidi M, Bahrehmand F, Vaisi-Raygani A, Elahirad S, Moini A, Kiani A. Association of AHSG gene polymorphisms with serum Fetuin-A levels in individuals with cardiovascular calcification in west of Iran. Molecular biology reports. 2020;47(3):1809-20.