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ORIGINAL ARTICLE

Association of AHSG gene polymorphisms with serum Fetuin-A levels in individuals with cardiovascular calcification in west of Iran

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Abstract

Fetuin-A (AHSG) is a multifunctional secretory protein and acts as an ectopic valve and artery calcification inhibitor. We assessed the correlation between serum levels of Fetuin-A and both exon 6 (248 C/T) and exon 7 (256 C/G) mutations in patients with coronary artery calcification (CAC), mitral annular calcification (MAC), and aortic valve calcification (AVC). 184 patients and 184 healthy individuals as control group were included. The genetic variants of rs4917 and rs4918 for the AHSG gene were determined by PCR-RFLP and T-ARMS PCR techniques. Fetuin-A levels, fasting blood sugar (FBS), urea, creatinine, calcium phosphorus, and lipid profile were measured. Fetuin-A levels were remarkedly lower in individuals with AVC, MAC, and CAC comparing to the control group ($p < 0.001$). The CT + TT genotypes and the T allele (AHSG Thr248Met) were associated with the risk of calcification of heart valves and coronary artery by 1.31 and 1.27 times in the patient group, respectively. The frequency of CT genotype and T allele was considerably higher in the patient group comparing to the control group. Patients with T allele (CT + TT) had higher levels of FBS, urea, low-density lipoproteins (LDL)-C, phosphorus, systolic blood pressure (SBP), diastolic blood pressure (DBP) while decreased levels of triglyceride, high-density lipoproteins (HDL)-C, calcium and fetuin-A in comparison to control group. Additionally, there was a positive correlation between serum FBS, urea, creatinine, HDL-C, calcium with fetuin-A, and a negative correlation between phosphorous level, SBP, and DBP with fetuin-A. T allele in rs4917 Single nucleotide polymorphism (SNP) is the risk allele of calcification of heart valves and coronary arteries and fetuin-A levels correlates negatively with the occurrence of the disease.

Keywords Aortic valve calcification · α2-HS glycoprotein · Coronary artery calcification · Fetuin-A · Mitral annular calcification · Polymorphism

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Abbreviations

AHSG	α2-Heremance-Schmid –glycoprotein
AVC	Aortic valve calcification
BMI	Body mass index
CAC	Coronary artery calcification
CPPs	Calciprotein particles
DBP	Diastolic blood pressure
FBS	Fasting blood sugar
HDL	High-density lipoprotein
LDL	Low-density lipoprotein
MAC	Mitral annular calcification
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
SBP	Systolic blood pressure
SNP	Single nucleotide polymorphism
TG	Triglyceride
WT	Wild type

Introduction

Coronary artery calcification (CAC) is a concomitant finding in developing advanced atherosclerosis. CAC is a risk factor in patients who suffer from coronary artery disease [1–3] .The disease is related to age and gender and has a distribution of above 90% in men and 67% in women over 70 years of age [4]. Calcification of heart valves is an active cellular process almost identical to atherosclerosis, characterized by the accumulation of lipoproteins, chronic inflammation and calcium deposition in valve cusps [5, 6]. Aortic valve calcification (AVC) is the most common heart valve disease in older adults with a 2–7% prevalence in people over 65 who may undergo valve replacement surgery [7–9]. The incidence of mitral annulus calcification (MAC) is age-related and between 8 and 15%. It has a high incidence in patients with multiple cardiovascular risk factors or chronic kidney disease (CKD) [5]. Hypertension, hyperlipidemia, diabetes, and obesity are common risk factors for calcification of heart valves and coronary arteries [10]. The serum concentration of calcium and phosphorus in the normal range is roughly high enough to precipitate itself; therefore, the presence of serum inhibitors is necessary to prevent this occurrence [11, 12]. Fetuin-A (also called α2-Heremans Schmid glycoprotein or AHSG) has recently been described as a serum calcification inhibitor. This protein is synthesized in the liver and is found in high concentrations in the human serum [13–15]. Fetuin-A is a negative acute-phase protein, which forms colloidal complexes called calciprotein particles (CPPs) in plasma along with calcium and phosphorus to prevent the formation of calcium-phosphate crystals in the blood and the sedimentation on soft tissues [11, 14, 16–18]. The AHSG gene is located on chromosome 3 (3q27). It contains 7 exons and has a range of 8.2 kb of genomic DNA [19]. There are controversial studies about the correlation between serum Fetuin-A and cardiovascular disease [20, 21]. Of 30 SNPs detected in the Fetuin-A gene, Thr256Ser and Thr248Met polymorphisms have the highest correlation with serum Fetuin-A levels [22]. Fisher et al. showed that AHSG rs4917 C > T correlates strongly with the plasma level of AHSG [23]. The present study was conducted to determine the two Fetuin-A gene polymorphisms and to investigate the hypothesis that the mutation in the AHSG gene correlates with the risk of calcification of the heart valves and coronary artery due to changes in the Fetuin-A level.

Material and methods

Demographic features of patients

In the present case–control study, 92 patients were diagnosed with AVC or MAC and 92 individuals out of 184 patients were diagnosed with CAC. The mean age was 64.6 ± 9.8 years in the patient group (40–88 years). Additionally, 184 healthy subjects with a mean age of 63.7 ± 7.2 years (47–81 years) who were matched for the age and sex with the patient group, were selected as the control group. The patients with AVC and MAC were those referred to Imam Ali hospital of Kermanshah and diagnosed by a cardiologist. All included individuals in the study were from Kermanshah province located in western Iran with Kurd ethnicity. The pharmacological history of patients with cardiac calcification is provided in Table 1.

Before participating in this research, all included individuals completed and signed a consent form. The study was done according to the guidelines of the 1975 Declaration of Helsinki and the ethical committee of Kermanshah University of Medical Sciences.

Table 1 Pharmacological history in cardiac calcification patients

Drug	Patients n (%)
Losartan	24 (13%)
Amlodipin	5 (2.7%)
Metoral	15 (8.2%)
Digoxin	2 (1.1%)
Carvedilol	3 (1.6%)
Captopril	3 (1.6%)
Nitroglycerin	5 (2.7%)
Atorvastatin	42 (22.8%)
Aspirin	33 (17.9%)
Glibotex	10 (5.4%)
Metformin	5 (2.7%)
Glibotex and Metformin	9 (4.9%)

Sample collection and DNA extraction

7 mL of fasting blood specimen was collected from individuals of both groups, 3 cc of which was poured into tubes containing EDTA anticoagulant for extracting DNA and 4 cc in anticoagulant-free tubes to isolate serum samples for measuring serum levels of fetuin-A.

Measurement of serum fetuin-A levels

The serum levels of Fetuin-A were measured in both groups using ELISA commercial sandwich kit (R & D System, USA). The results were detected using the Awareness Elisa Reader Version Stat fax 2100 and calculated in grams/ Liter.

Measurement of other biochemical parameters

The serum levels of FBS, urea, creatinine, total cholesterol, HDL-C, LDL-C, TG, calcium, and phosphorus were measured by standard enzymatic methods using automated RA-1000 (AutoTechnicon, USA).

Echocardiography

In this study, the patients with AVC and MAC were diagnosed using a Doppler echocardiography technique by an experienced cardiologist. The criteria for detecting the amount of aortic valve stenosis are aortic jet velocity 2.5–2.9 m/s, mean gradient < 25 mmHg and aortic valve area of 1.5–2 cm^2 in mild type; velocity of aortic jet 3–4 m/s, mean gradient of 25–49 mmHg and aortic valve area of 1–1.5 cm^2 in medium type, and aortic jet velocity > 4 m/s, mean gradient > 40 mmHg, and aortic valve area < 1 cm^2 in severe type [24]. The criteria for detecting the amount of mitral valve stenosis include pulmonary artery pressure < 30 mmHg, mean gradient < 5 mmHg and mitral valve area > 1.5 cm^2 in mild type; pulmonary artery pressure of 30–50 mmHg, mean gradient of 5–10 mmHg and mitral valve area of 1–1.5 cm^2 in the middle type and pulmonary artery pressure > 50 mmHg, Mean gradient > 10 mmHg and mitral valve area < 1 cm^2 in severe type [25].

Coronary artery angiography

The CAC patients were diagnosed using a coronary angiography technique by a cardiologist. The severity of the CAC disease was considered based on the number of calcified arteries [26].

DNA extraction and genotyping

DNA was extracted from the entire population of peripheral blood leukocytes using a standard phenol–chloroform technique [27] and the PCR–RFLP technique was used to determine the genotypes of single-nucleotide polymorphism of the AHSG Thr256Ser C/G gene (SNPrs4918). A pair of primers was designed based on gene sequences in the GeneBank and using the Primer3 online website (https://primer3.ut.ee/) to amplify the 361 bp fragment of the AHSG gene. The sequences of the primers were as the following: P1: 5′-ATAGGCCAGTCACCCCTCCTTG-3′ (Forward primer) and P2:5′- TGCAACTGGTGTCCTGGAGGAG-3′ (Reverse primer). PCR reaction was performed with 25 µL volume (1 µL of DNA at the concentrations of 200–600 ng, 0.6 µL of each primer at a concentration of 10 pmol, 0.75 µL of $MgCl_2$ at a concentration of 50 mM, 0.5 µL of dNTPs at a concentration of 200 µM, 2.5 µL of 10X PCR Buffer, 19.05 µL of ddH_2O and 0.2 µL of Taq DNA Polymerase at a concentration of 5 U/µL). The PCR process was performed in 40 cycles under initial denaturation at 95 °C for five minutes, denaturation at 95 °C for thirty seconds, annealing at 63.7 °C for 30 s, extension at 72 °C for 40 s, and a final extension at 72 °C for 10 min. About 15 µL of PCR product was digested with SacI enzyme. The RFLP product has been electrophoresed in 2.5% agarose gel. Since there was no mutation in the form of wild allele (C/C), the PCR product in this site was not cleaved by the SacI enzyme and a fragment with a length of 361 bp was created. Three fragments of 361 bp, 201 bp, 160 bp were developed in the heterozygote form (C/G), and two fragments of 201 bp and 160 bp in the mutant homozygote form (G/G). The T-ARMS PCR (Tetra Amplification Refractory Mutation System) technique was used to determine the genotypes of the single nucleotide polymorphism of AHSG Thr248Met C/T gene (SNPrs4917). In this technique, 4 primers were used in a PCR reaction (Table 2).

The 320 bp fragment from the external forward and reverse primers was used as a control for checking the accuracy of the PCR reaction. The internal forward primer was considered to be specific to the mutant allele (T allele), and the internal reverse primer as specific to the wild allele (normal). These primers were designed based on gene sequences in GeneBank and using the Primer1 online site (https://primer1.soton.ac.uk/primer1.html). The T-ARMS PCR in a total volume of 25 µL contained 1 µL of DNA at the concentrations of 200–600 ng, 1 µL of each primer at a concentration of 10 pmol, 0.75 µL of $MgCl_2$ at a concentration of 50 mM,

Table 2 T-ARMS-PCR primers for SNPrs4917 of the AHSG gene

Primers sequence
Fo: 5′-TTTAAGGTTGGCGCGTCAATGAAATTGG-3′
Fi: 5′-GGGGCAGAGGTTGCAGTGACCTGAAC-3′
Ro: 5′-TTTGTTGATGATTCCGCATACCCCAGTG-3′
Ri: 5′-ACGGAGCTGTTACCTGTGTTTGGAACAACA-3′

0.5 µL of dNTPs at a concentration of 200 µM, 2.5 µL of 10X PCR Buffer, 16 µL of ddH_2O and 0.2 µL of Taq DNA Polymerase at a concentration of 5 U/µL. The PCR process was performed for 35 cycles under initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 45 s, annealing at 61 °C for 45 s, extension at 72 °C for 45 s, and a final extension at 72 °C for 10 min. 3% agarose gel electrophoresis was then used to detect the PCR product. Individuals with a genotype of CC (WT) showed bands of 320 bp and 198 bp, those with a genotype of CT had three bands of 320 bp, 198 bp, and 178 bp, as well as the subjects with a genotype of TT (mutated homozygous), indicated two bands of 320 bp and 178 bp. To confirm the mutations seen in both polymorphisms, DNA sequencing on the resulting PCR products was performed by Nedayefan Co (Figs. 1, 2).

Statistical analysis

The distribution of different qualitative data was compared between the studied groups using Chi-square test (χ^2). The normal distribution of quantitative data was investigated using the One-Sample Kolmogorov -Smirnov test. Comparison of mean age, BMI and serum levels of fetuin-A, creatinine, TG, HDL-C, LDL-C, FBS, total cholesterol, calcium, phosphorus, systolic and diastolic blood pressure were performed between groups using independent *t* test. Correlation between genotypes and biochemical parameters was explored using independent *t* test. Correlating between the levels of biochemical parameters and severity of disease was tested by independent *t* test. Odds ratio (OR) was estimated for the disease with 95% confidence interval (CI) by using logistic regression analysis. Correlation between fetuin-A level and age, BMI and serum levels of biochemical parameters were measured using Pearson correlation. Haplotype analysis was done by SNPSTATS (https://bioinfo.iconcologia.net/snpstats/start.htm). SPSS16 was used for analyzing data at a significance level of $p < 0.05$.

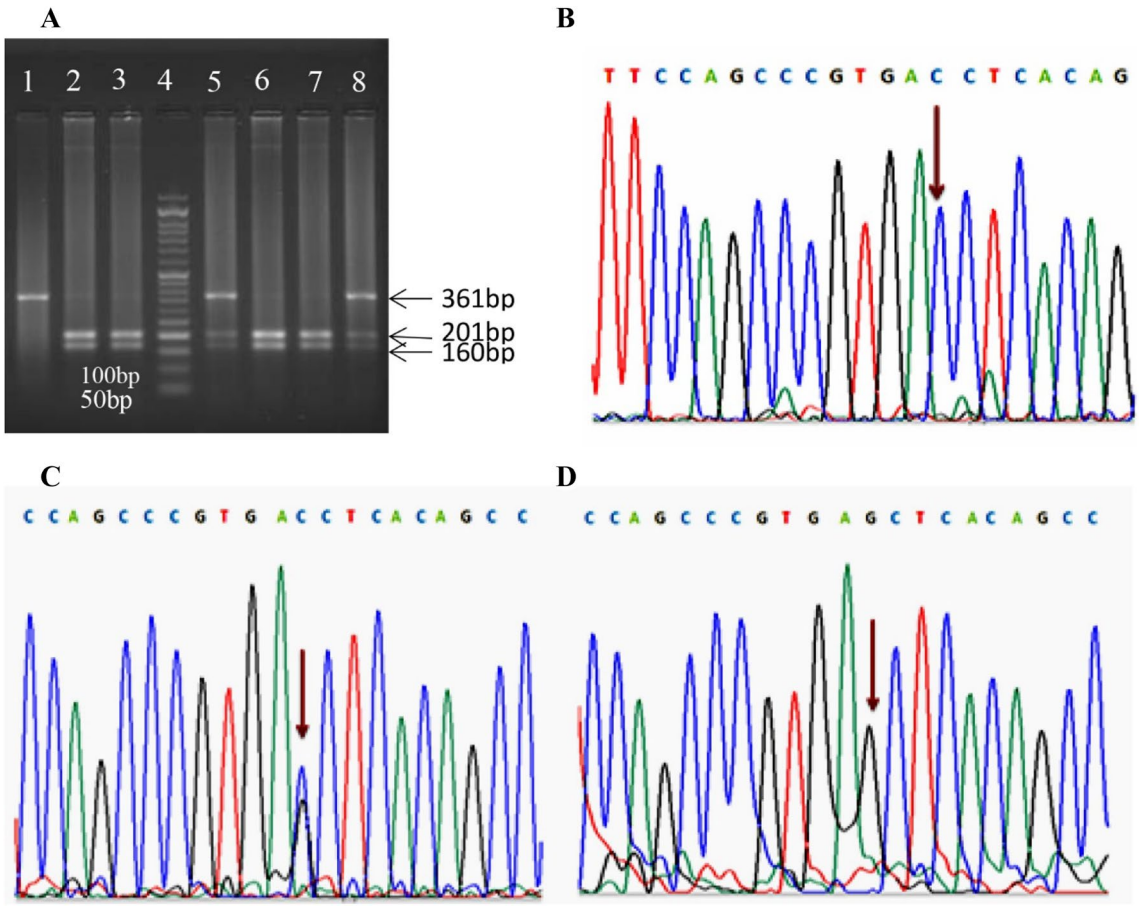

Fig. 1 PCR–RFLP results from Fetuin-A Thr 256 Ser (rs4918) polymorphism: Lane1 CC; Lane2 and 3 GG; Lane4 molecular marker; Lane5 CG; Lane6 and 7 GG; Lane8 CG. **b**, **c**, and **d** respectively confirm the genotypes CC, CG, and GG of SNPrs4918 in DNA sequencing

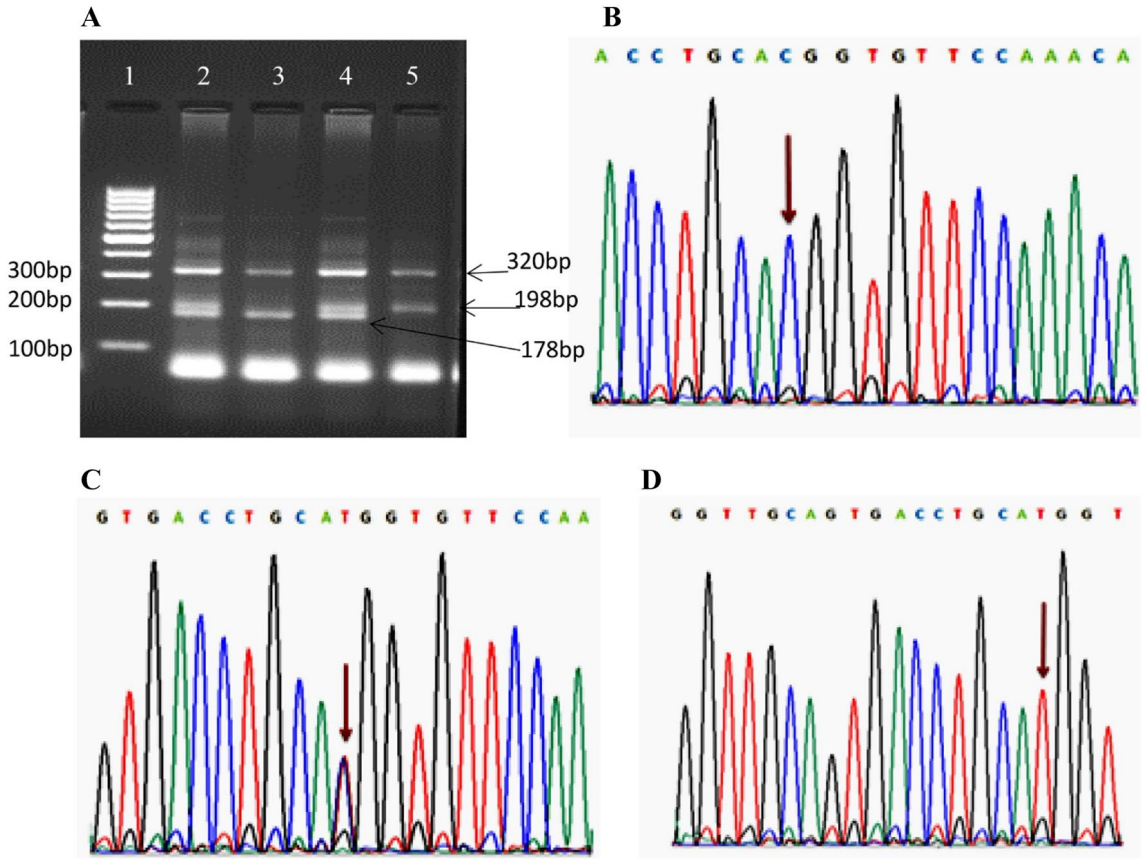

Fig. 2 T-ARMS-PCR results from Fetuin-A Thr 248 Met (rs4917) polymorphism: Lane1 molecular marker; Lane2 CT; Lane3 TT; Lane4 CT; Lane5 CC. **b**, **c**, and **d** respectively confirm the genotypes CC, CT, and TT of SNPrs4917 in DNA sequencing

Results

Table 3 presents the demographic characteristics and clinical information of participants. The mean of FBS (102.29 ± 22.55 vs 92.27 ± 7.66, $p < 0.001$), urea (33.79 ± 7.42 vs 28.27 ± 5.60, $p < 0.001$), creatinine (0.93 ± 0.16 vs 0.89 ± 0.15, $p = 0.016$),phosphorus (4.55 ± 0.63 vs 4.32 ± 0.44, $p < 0.001$), LDL-C(94.13 ± 41.08 vs 84.10 ± 26.10,$p = 0.005$), systolic blood pressure (125.90 ± 18.20 vs 108.53 ± 8.20, $p < 0.001$) and DBP (80.30 ± 10.14 vs 71.41 ± 5.60, $p < 0.001$) is considerably greater in the patients group in comparison with the control group, respectively. The serum levels of HDL-C (43.45 ± 7.60 vs 54.69 ± 11.98, $p < 0.001$), calcium (8.55 ± 0.94 vs 9.22 ± 1.09, $p < 0.001$) and Fetuin-A (0.05 ± 0.03 vs 0.08 ± 0.04, $p < 0.001$), are significantly lower in the patient group in comparison with the control group, respectively. A significant difference was not observed in the mean of age, BMI, total serum cholesterol and triglyceride levels between patients and control groups (Table 3).

The lipid profile of patients treated with Atorvastatin comparing with the control group is shown in Table 4. The levels of total cholesterol and LDL-C were higher in patients taking Atorvastatin comparing to the control group that could be the result of the cholesterol-lowering effect of the drug.

Table 5 reports the frequency of genotype and alleles for the AHSG gene polymorphisms (rs4917 and rs4918) in the patient and control groups. Alleles and genotype distribution of AHSG Thr256Ser polymorphism was in Hardy–Weinberg equilibrium (HWE) in the patient ($p = 0.63$) and control groups ($p = 0.56$).

The genotype and alleles frequency observed for AHSG Thr248Met were consistent with the HWE equilibrium in the control group ($p = 1$), but out of equilibrium in the patient group ($p = 0.03$). Distribution of rs4917 AHSG genotypes and their alleles showed a significant difference in the patient group in comparison with the control group. T allele frequency for the patient and control groups was 16.3% and 11%, respectively. Frequency of CT + TT genotype was higher in patients compared to control subjects (37.5% vs 24.5%). On the contrary, a significant difference was not found in the distribution of genotypes and alleles of AHSG rs4918 ($p > 0.05$). The logistic regression analysis revealed the importance of AHSG rs4917 in patients

Table 3 Clinical and demographic data in patient and control groups

Parameters	Control group	Patient group	p value
Sex (male/female)	92(50%)/92(50%)	92(50%)/92(50%)	1
Smoking (yes/no)	28(15.2%)/156(84.8)	37(20.1)/147(79.9)	0.219
DM (yes/no)	–/184(100%)	24(13%)/160(87%)	< 0.001
MAC (yes/no)	–/184(100)	32(17.4%)/152(82.6%)	< 0.001
AVC (yes/no)	–/184(100%)	48(26.2%)/136(73.9%)	< 0.001
CAC (yes/no)	–/184(100)	92(50%)/92(50%)	< 0.001
MAC and AVC (yes/no)	–/184(100%)	12(6.5%)/172(93.5%)	< 0.001
History of hyperlipidemia (yes/no)	–/184(100%)	41(22.3%)/143(77.7%)	< 0.001
Age	63.72 ± 7.29	64.63 ± 9.82	0.315
BMI (kg/m^2)	24.57 ± 2.46	24.22 ± 3.10	0.233
FBS (mg/dl)	92.27 ± 7.66	102.29 ± 22.55	< 0.001
Urea (mg/dl)	28.27 ± 5.60	33.79 ± 7.42	< 0.001
Creatinine (mg/dl)	0.89 ± 0.15	0.93 ± 0.16	0.016
Total cholesterol (mg/dl)	160.92 ± 27.30	162.28 ± 43.34	0.718
Triglyceride (mg/dl)	119.11 ± 22.36	126.42 ± 52.16	0.082
HDL-C (mg/dl)	54.69 ± 11.98	43.45 ± 7.60	< 0.001
LDL-C (mg/dl)	84.10 ± 26.10	94.13 ± 41.08	0.005
Calcium (mg/dl)	9.22 ± 1.09	8.55 ± 0.94	< 0.001
Phosphorus (mg/dl)	4.32 ± 0.44	4.55 ± 0.63	< 0.001
SBP (mmHg)	108.53 ± 8.20	125.90 ± 18.20	< 0.001
DBP (mmHg)	71.41 ± 5.60	80.30 ± 10.14	< 0.001
Fetuin-A (g/l)	0.08 ± 0.04	0.05 ± 0.03	< 0.001

Values are written as mean ± SD or n(%)

DM diabetes mellitus, *MAC* mitral annular calcification, *AVC* aortic valve calcification, *CAC* coronary artery calcification, *BMI* body mass index, *FBS* fasting blood sugar, *HDL* high-density lipoprotein cholesterol, *LDL* low-density lipoprotein cholesterol, *SBP* systolic blood pressure, *DBP* diastolic blood pressure; Statistically significant at $p < 0.05$ was considered significant

Table 4 The lipid profile of patients treated with Atorvastatin comparing with the control group

Individuals	CHO	LDL	HDL
Diabetic	201.83 ± 47.98*	132.67 ± 49.67*	41.50 ± 7.14**
Nondiabetic	231.50 ± 30.25*	152 ± 34.48*	44.37 ± 8.80*
Diabetic + non-diabetic	225.57 ± 35.61*	148.13 ± 37.82*	43.80 ± 8.46*
Control	160.92 ± 27.30*	84.10 ± 26.10*	54.69 ± 11.98*

*$p < 0.001$ compared to the control group, **$p = 0.008$ compared to the control group

with calcification of heart valves and coronary arteries (Table 6).

The OR for T allele and CT + TT genotype from Thr248Met C > T was found to be 1.27 (1.046–1.561, $p = 0.017$) and 1.31(1.087–1.704, $p = 0.007$) in the patient group. The correlation of rs4917 and rs4918 genotypes with the levels of biochemical parameters between the patient and control groups is shown in Tables 6 and 7.

The patients with CT + TT genotype for rs4917 and CG + GG genotype for rs4918 possessed significantly greater FBS, urea, low-density lipoprotein, phosphorus, SBP, and DBP, and lower serum levels of HDL, triglyceride (significant decrease only in the CT + TT genotype), calcium and Fetuin-A when compared to control group with the same genotype. The correlation of the levels of biochemical parameters with the severity of disease in the patients with calcification of heart valves and coronary artery showed that there was a significant decrease in serum phosphorus levels in patients with 1 calcified vein versus 2 and 3 calcified veins (4.75 0.56 vs 5.27 ± 1.56, $p = 0.01$). There was a positive correlation between the serum levels of FBS ($r = 0.547$, $p < 0.001$), urea ($r = 0.177$, $p = 0.016$) and creatinine ($r = 0.257$, $p = 0.016$) with fetuin-A level in the patient group. In the total group, a positive correlation has been found between serum FBS($r = 0.231$, $p < 0.001$), HDL-C ($r = 0.128$, $p = 0.014$) and calcium($r = 0.202$, $p < 0.001$) as well as a negative correlation between serum phosphorus ($r = -0.0022$, $p = 0.767$), systolic ($r = -0.136$, $p = 0.009$) and DBP ($r = -0.146$, $p = 0.005$) with fetuin-A level (Table 8).The haplotyping of the two SNPs is shown in Table 9. The presence of haplotypes rs4918 G, rs4917 C (GC) and haplotypes rs4918 C, rs4917 T (CT) in comparison with CC increases the risk of calcification of the heart valves and coronary artery by 1.78 and 2.38- fold, respectively.

Table 5 Genotype distribution and alleles frequency AHSG (rs4917 and rs4918) gene polymorphisms in the studied subjects

SNPrs4917 Genotypes	Control n (%)	Patient n (%)	OR (95%CI, p value)
CC	139 (75.5%)	115 (62.5%)	
CT	42 (22.8%)	67 (36.4%)	
TT	3 (1.6%)	2 (1.1%)	
	$\chi^2 = 8.20$, df = 2,p = 0.017		
CC	139 (75.5%)	115 (62.5%)	1.31(1.087–1.704,0.007)
CT + TT	45 (24.5%)	69 (37.5%)	
	$\chi^2 = 7.32$, df = 1,p = 0.007		
Alleles			
C	320 (87%)	297 (80.7%)	1.27 (1.046–1.561,0.017)
T	48 (13%)	71 (19.3%)	
	$\chi^2 = 5.30$, df = 1, p = 0.021		
SNPrs4918 Genotypes	Control n (%)	Patient n (%)	OR (95%CI, p value)
CC	134 (72.8%)	119 (64.7%)	
CT	45 (24.5%)	60 (32.6%)	
TT	5 (2.7%)	5 (2.7%)	
	$\chi^2 = 3.03$, df = 2, p = 0.22		
CC	134 (72.8%)	119 (64.7%)	1.20 (0.969–1.510,0.092)
CT + TT	50 (27.2%)	65 (35.3%)	
	$\chi^2 = 2.84$, df = 1, p = 0.09		
Alleles			
C	313 (85.1%)	298 (81%)	1.15 (0.953–1.403,0.142)
T	55 (14.9%)	70 (19%)	
	$\chi^2 = 2.16$, df = 1, p = 0.141		

Distributing alleles and genotype frequency of Fetuin-A in the patient group in comparison to the control group are done by χ^2 test analysis. Odd ratio (OR) as an indicator of the relative risks for the disease was calculated and 95% confidence interval was achieved via χ^2 regression binary logistic analysis. p value < 0.05 was considered significant

Discussion

In this study, we could demonstrate a significant relationship between low levels of fetuin-A and calcification of heart valves and coronary arteries. The results of some studies on the role of fetuin-A in CVD are contradictory. Several studies reported a significant reverse correlation between the serum levels of fetuin-A and AVC, MAC and CAC, which is consistent with our results [7, 10, 11, 13, 23]. Bellia et al. reported an association between fetuin-A levels and serum calcium when investigated AHSG T256S genotype in 74 patients in Italy but found no link between fetuin-A levels and CAC [28]. Mikami et al. reported that there is no significant correlation between serum levels of fetuin-A and CAC [29]. In contrast, Mehrotra et al. found a correlation between the increased plasma levels of fetuin-A and CAC in diabetic nephropathy [30]. In the study of Kocyigit et al., the serum fetuin-A had a significantly higher level in AVC patients with kidney transplantation compared to the ones with no valve calcification [31]. In vitro studies demonstrated that the fetuin-A could inhibit the calcification of vascular smooth muscle [32]. The deficiency of this protein in chronic kidney disease is associated with vascular complications [33]. In contrast, the level of fetuin-A was positively correlated with common carotid artery intima-media thickness [34]. Besides, in vivo animal studies and in vitro studies on human adipocytes revealed the direct role of AHSG in inducing inflammation and expression of cytokines [35, 36]. In the transgenic mouse model, the degradation of the AHSG gene led to the fatal and progressive calcification of soft tissues such as skin, kidneys, lungs, myocardium, and heart valves [37]. The results of previous studies indicate that fetuin-A acts as a dystrophic calcification inhibitor and a decrease in serum levels of this protein increases the risk of calcification of the heart valves [7]. In a study by Merx et al. on fetuin-A knockout mice, an increase was observed in cardiac fibrosis and calcification, diastolic dysfunction, ischemic tolerance, and resistance to catecholamines [38]. Considering the effect on signaling pathways, this protein inhibits the inductive effects of TGF-β and BMP2 that can stimulate calcification of the valves. Besides, the AHSG may regulate the release of TNF-α, which

Table 6 Relationship of Thr 248 Met (rs4917) genotypes with biochemical parameters in patient and control groups

Parameters	Control group CC	Patient group CC	p values	Control group CT + TT	Patient group CT + TT	p values
N (%)	139 (75.5%)	115 (62.5%)		42 (24.5%)	69 (37.5%)	
BMI (kg/m^2)	24.51 ± 2.41	24.23 ± 3.06	0.408	24.77 ± 2.64	24.22 ± 3.19	0.342
FBS (mg/dl)	92.24 ± 7.45	99.58 ± 19.20	< 0.001	92.37 ± 8.38	106.81 ± 26.78	0.001
Urea (mg/dl)	28.55 ± 5.61	33.92 ± 7.28	< 0.001	27.40 ± 5.52	33.59 ± 7.70	< 0.001
Creatinine (mg/dl)	0.89 ± 0.15	0.92 ± 0.17	0.121	0.90 ± 0.17	0.95 ± 0.16	0.085
Total cholesterol (mg/dl)	161.47 ± 27.47	166.59 ± 43.54	0.256	159.22 ± 26.99	155.11 ± 42.33	0.564
Triglyceride (mg/dl)	116.79 ± 22.85	127.36 ± 47.14	0.02	126.31 ± 19.25	124.85 ± 59.94	0.875
HDL-C (mg/dl)	54.89 ± 12.27	43.54 ± 7.69	< 0.001	54.06 ± 11.13	43.28 ± 7.50	< 0.001
LDL-C (mg/dl)	84.81 ± 25.87	99.83 ± 42.46	0.001	81.93 ± 27	84.64 ± 37.06	0.673
Calcium (mg/dl)	9.21 ± 1.16	8.49 ± 0.93	< 0.001	9.28 ± 0.82	8.65 ± 0.95	< 0.001
Phosphorus (mg/dl)	4.32 ± 0.45	4.55 ± 0.70	0.002	4.30 ± 0.43	4.55 ± 0.51	0.007
SBP (mmHg)	108.52 ± 8.11	126.53 ± 17.23	< 0.001	108.55 ± 8.58	124.85 ± 19.79	< 0.001
DBP (mmHg)	71.43 ± 5.52	81.40 ± 9.05	< 0.001	71.35 ± 5.92	78.47 ± 11.57	< 0.001
Fetuin-A (g/l)	0.08 ± 0.03	0.04 ± 0.03	< 0.001	0.10 ± 0.04	0.06 ± 0.03	< 0.001

A *t* test was applied for calculating the correlation values of serum biochemical parameters with Fetuin-A polymorphism (rs4917) between patients and controls

BMI body mass index, *FBS* fasting blood sugar, *HDL* high-density lipoprotein cholesterol, *LDL* low-density lipoprotein cholesterol, *SBP* systolic blood pressure, *DBP* diastolic blood pressure; p value < 0.05 has been significant

Table 7 Relationship of Thr 256 Ser (rs4918) genotypes with biochemical parameters in patient and control groups

Parameters	Control group CC	Patient group CC	p value	Control group CG + GG	Patient group CG + GG	p value
N (%)	139 (75.5%)	115 (62.5%)		42 (24.5%)	69 (37.5%)	
BMI (kg/m^2)	24.55 ± 2.52	24.29 ± 3.17	0.456	24.62 ± 2.32	24.11 ± 2.99	0.318
FBS (mg/dl)	92.03 ± 7.71	102.29 ± 19.20	< 0.001	92.92 ± 7.59	102.29 ± 22.71	0.006
Urea (mg/dl)	28.41 ± 56.61	33.76 ± 7.35	< 0.001	27.88 ± 5.23	33.86 ± 7.62	< 0.001
Creatinine (mg/dl)	0.88 ± 0.14	0.93 ± 0.16	0.009	0.92 ± 0.17	0.935 ± 0.17	0.708
Total cholesterol (mg/dl)	160.77 ± 27.01	158.77 ± 42.61	0.652	161.32 ± 28.35	168.72 ± 44.24	0.305
Triglyceride (mg/dl)	119.90 ± 23.05	125.03 ± 49.04	0.28	117.02 ± 20.44	128.96 ± 57.75	0.165
HDL-C (mg/dl)	55.08 ± 12.03	43.11 ± 7.34	< 0.001	53.64 ± 11.89	44.06 ± 8.07	< 0.001
LDL-C (mg/dl)	83.68 ± 26.03	91.98 ± 42.15	0.058	85.24 ± 26.52	98.08 ± 39.07	0.048
Calcium (mg/dl)	9.24 ± 1.08	8.54 ± 1	< 0.001	9.19 ± 1.13	8.57 ± 0.82	0.001
Phosphorus (mg/dl)	4.33 ± 0.45	4.55 ± 0.69	0.003	4.27 ± 0.41	4.55 ± 0.51	0.002
SBP (mmHg)	108.67 ± 8.43	126.42 ± 18.09	< 0.001	108.16 ± 7.61	124.93 ± 18.50	< 0.001
DBP (mmHg)	71.40 ± 5.60	79.46 ± 10.48	< 0.001	71.46 ± 5.68	81.84 ± 9.37	< 0.001
Fetuin-A (g/l)	0.08 ± 0.03	0.05 ± 0.03	< 0.001	0.08 ± 0.04	0.04 ± 0.03	< 0.001

A *t* test was applied for calculating the correlation values of serum biochemical parameters with Fetuin-A polymorphism (rs4918) between patients and controls

BMI body mass index, *FBS* fasting blood sugar, *HDL* high-density lipoprotein cholesterol, *LDL* low-density lipoprotein cholesterol, *SBP* systolic blood pressure; *DBP* diastolic blood pressure; p value < 0.05 has been significant

is a strong regulator for the calcification of heart valves and coronary arteries [7, 10]. Tuttolomondo et al. reported a pro-inflammatory role for both CD40L and Fetuin-A in patients diagnosed with acute ischemic stroke and that the levels of both markers were higher compared to healthy controls [39]. Our data showed significant differences between the genotypes and alleles frequency of rs4917 (C/T) polymorphism between the two groups of patients and control, but this difference was not significant for rs4918 (C/G) polymorphism. The T allele from AHSG Thr248Met could increase 1.27 times and the

Table 8 Correlation of fetuin-A level with demographic data and biochemical parameters in all groups

Parameters	Control group		Patient group		Total	
	r*	p	r*	p	r*	p
Age	0.008	0.919	−0.065	0.384	−0.051	0.331
BMI (kg/m^2)	−0.016	0.827	−0.103	0.166	−0.032	0.536
FBS (mg/dl)	0.152	0.039	0.547	< 0.001	0.231	< 0.001
Urea (mg/dl)	0.09	0.224	0.177	0.016	−0.04	0.44
Creatinine (mg/dl)	0.003	0.968	0.257	< 0.001	0.071	0.174
Total cholesterol (mg/dl)	0.065	0.381	0.018	0.811	0.025	0.634
Triglyceride (mg/dl)	0.106	0.152	−0.032	0.663	−0.028	0.597
HDL-C (mg/dl)	−0.143	0.053	0.003	0.965	0.128	0.014
LDL-C (mg/dl)	0.111	0.132	−0.015	0.837	−0.027	0.607
Calcium (mg/dl)	0.109	0.062	0.062	0.404	0.202	< 0.001
Phosphorus (mg/dl)	−0.193	0.009	−0.022	0.767	−0.167	0.001
SBP (mmHg)	0.055	0.455	0.126	0.089	−0.136	0.009
DBP (mmHg)	−0.028	0.707	0.109	0.139	−0.146	0.005

BMI body mass index, *FBS* fasting blood sugar, *HDL* high-density lipoprotein cholesterol, *LDL* low-density lipoprotein cholesterol, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, p value < 0.05 has been significant

*Pearson correlation coefficient

Table 9 Haplotype analysis of two polymorphisms of Fetuin-A (rs4917 and rs4918) in studied individuals

SNPrs4918	SNPrs4917	Ferequency case	Ferequency control	OR (95% CI, p)
C	C	0.6453	0.7581	–
G	C	0.1618	0.1108	1.78 (1.11–2.94, 0.02)
C	T	0.1672	0.0917	2.38 (1.40–4, 0.001)
G	T	0.0257	0.0395	0.82 (0.29–2.32, 0.72)

Global haplotype association p value = 0.004

presence of CT + TT genotype could increase 1.31 times the risk of calcification of heart valves and coronary artery in comparison with CC genotype. Maharem et al. examined the correlation of fetuin-A gene genotypes with the serum levels of this protein in CKD patients and found no significant difference in the distribution of rs4918 genotypes between the patients and control groups [40]. In the study of Coker et al., the distribution of rs4917 C/T polymorphism genotypes had a significant correlation between heart attack patients and the control group, while the frequency of rs4918 C/G polymorphism had no significant difference between the two groups. In this study, only the C allele of rs4917 increased the risk of MI [36]. Laugsand et al. reported that rs4917 genotypes in Caucasians had a significant relationship with decreasing serum fetuin-A levels, and increased the risk of coronary artery disease by 1.02 times per T allele [41]. Contradictory reports about the correlation of AHSG polymorphisms (rs4917 and rs4918) with the risk of cardiovascular disease may be due to differences in race, lifestyle, environmental factors, and population size. Our study showed a significant correlation between AHSG gene polymorphisms and serum fetuin-A levels, in which the patient with CT + TT genotype from Thr248Met C/T polymorphism and patient with CG + GG genotype from Thr256Ser C/G polymorphism had lower fetuin-A level when compared to the control subjects having the same genotype. Verduijn et al. assessed the possible correlation of AHSG rs4918 polymorphism with serum fetuin-A levels in dialysis patients and reported that patients with Ser/Ser genotype had significantly lower levels of fetuin-A than Thr/Thr and Thr/Ser genotypes [42]. Axelsson et al. showed that the G allele of rs4918 was effective on the circulating fetuin-A levels [43]. Fisher et al., within the EPIC-Potsdam study, showed that the allele C of rs4917 (C/T) polymorphism correlates with the increased plasma fetuin-A levels in patients with MI [23], which is not consistent with our results. Inconsistent results may be due to differences in the size of the included population and various methods of measuring this protein. Our results showed that carriers of one or two copies of the T or G (CT + TT or CG + GG) allele possessed significantly greater levels of FBS, urea, low-density-lipoprotein, phosphorus, SBP, and DBP, and lower serum levels of HDL, triglyceride (only in CT + TT genotype) and calcium in comparison with the

control group with the same genotype. A prospective study reported that rs4917 polymorphism had no effect on lipid profile, SBP, and DBP, but had a significant relationship with serum fetuin-A levels [41]. Table 6 shows a positive correlation between serum AHSG level with FBS (in the patient and total groups), urea and creatinine in patients, HDL-C and calcium in the total group, as well as a significant negative relationship between SBP, DBP, phosphorus with the level of this protein. Afsar et al. observed a significant negative correlation between plasma glucose, urea, and creatinine in patients with the acute coronary syndrome with fetuin-A levels. They also reported a significant positive relationship between fetuin-A with HDL-C and calcium in all participants [10]. In contrast, our results showed a significant positive relationship between Fetuin-A and systolic blood pressure and diastolic blood pressure in both groups. The AHSG probably reduces calcium and phosphorus precipitation in soft tissues, which is associated with increased plasma concentrations of these elements. Besides, it may increase the HDL-C level with an unknown mechanism. In our study, a negative correlation was observed between fetuin-A level and the calcification of heart valves and coronary arteries. The genetic variants of AHSG (Thr248Met and Thr256Ser) were associated with a decrease in serum levels of Fetuin-A. The rs4917 polymorphism significantly increased the risk of calcification of the heart valves and coronary artery. In patients with moderate to severe calcification of heart valves, the serum calcium levels were lower compared to the patients with mild degrees. Our study lacks the results for the coronary artery calcium (CAC) score due to our applied techniques, however, the assessment of the CAC score helps to understand the pathology of a variety of diseases [44]. Finally, although the role of systemic Fetin-A in the pathogenesis of the calcification of the heart valves and coronary artery has been investigated, the role of local Fetuin-A needs to be investigated. In this regard, Mancio et al. studied the proteome of epicardial adipose tissue in patients with CAD and reported an upregulation of annexin-A2 and downregulation of fetuin-A [45].

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Compliance with ethical standards

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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