

Genetic Association of rs10757278 on Chromosome 9p21 and Coronary Artery Disease in a Saudi Population

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Purpose: Coronary artery disease (CAD) is one of the most important leading causes of morbidity and mortality worldwide. Few studies have been carried out in the Saudi population regarding the association of rs10757278 polymorphism with CAD. This study aimed to investigate the association of the rs10757278 polymorphism with CAD in Saudi population.

Materials and Methods: In this case-control study, we recruited 437 patients with CAD and 251 cross-matched healthy controls and performed polymorphism genotyping for rs10757278 using a polymerase chain reaction followed by a restriction fragment length polymorphism analysis.

Results: The G allele (OR-1.44; 95% CI: 1.15–1.80; $p=0.001$), as GG (OR-2.13; 95% CI: 1.35–3.36; $p=0.0009$), in the dominant (OR-1.47; 95% CI: 1.03–2.10; $p=0.03$) and recessive mode (OR-1.84; 95% CI: 1.26–2.70; $p=0.001$) of inheritance showed a high-risk association. A disease stratified risk analysis was conducted and comparisons were made using an ANOVA analysis. Diabetes showed a risk association ($p=0.001$). However, a regression analysis confirmed that for the CAD cases, there was an association between the GG genotype and diabetes ($p=0.005$).

Conclusion: The results of this study suggest that the polymorphism rs10757278 is related to a high risk of CAD in a Saudi population.

Keywords: coronary artery disease, 9p21, rs10757278, gene polymorphism and Saudi population

Introduction

Coronary artery disease (CAD) is a chronic, complex, multifactorial, continuous inflammatory condition that results from the accumulation of atheromatous plaques in intima layer of the coronary arteries. CAD is responsible for numerous acute coronary syndromes and is the main cause of death in the Western hemisphere.¹ CAD is a multifactorial illness that is affected by both acquired and inherited factors. Certain risk factors for CAD are modifiable, such as lifestyle changes and medication management.³ However, there are numerous genetic and environmental risk factors contributing to CAD with high mortality and morbidity rate in most developed and developing countries.⁴ Myocardial infarction and angina are considered coronary vascular diseases (CVD), reported in 31% of the population worldwide.⁵ Specifically, the World Health Organization (WHO) reported that around 17.9 million people succumbed to CVD in 2016.⁶ Thus, a better understanding of the various CVDs is of great interest.

CAD is associated with conventional risk factors, including smoking, hypertension (HTN), diabetes mellitus (DM), poor diet and advanced age.⁷ However, the etiology of CAD remains obscure so far.⁸ The lesion incidence and long-term prognosis of CAD is variable. For example, patients with similar lesions may become stable or progressive and receive similar treatments. In addition, a small proportion of CAD patients show none of the classical risk factors. Thus, these observations support the unclear etiology. However, researchers suggest that genetic factors also contribute not only to the development of atherosclerosis but also to the development of the classical risk factors.⁹ In fact, CAD family aggregation has been recognized for quite some time, and the genetic predisposition to CAD is confirmed by conclusive evidence. For example, twin studies reveal that the estimates of heritability for CAD range from 41 to 77%, and the significance of genetic factors in CAD led to the extensive discovery of significant candidate genes and multiple small nucleotide polymorphisms (SNPs) being linked to this condition.^{10,11} The exact mechanism that underlies the impact of polymorphisms on the pathogenesis of CAD is not fully understood. However, polymorphisms in genes related to inflammation, lipid and glucose metabolism, blood, coagulation and homocysteine can affect the susceptibility to CAD.¹³ Thus, genotyping specific SNPs within a potential CAD-related gene is an essential and effective method to detect genetic risk markers, and numerous significant CAD-related SNPs have been reported.¹⁴ In particular, one SNP at loci 9p21.3 has been identified as a global population hotspot and is linked to CAD.¹⁵ Helgadottir et al¹⁶ identified the SNP variant of rs10757278 on chromosome 9p21.3 using genome-wide association studies (GWAS) that included both myocardial infarction and CAD patients. In addition, a meta-analysis by Hu et al¹⁷ reported that the rs10757278 polymorphism was significantly linked to CAD. Nevertheless, the rs10757278 polymorphism studies related to CAD are very few, if present, in Saudi population and this area of research is not fully elucidated. Moreover, the vast area of Saudi Arabia and far distances among its governorates necessitates wider research on CAD in different localities inside Saudi Arabia. Therefore, the current study aimed to investigate the genetic association of the rs10757278 polymorphism in Saudi patients with confirmed CAD.

Materials and Methods

Recruitment of the Participants

This case-control study included 688 patients, consisting of 437 CAD subjects and 251 healthy subjects from King Abdullah Medical City (KAMC) and Al-Noor specialized hospitals in the Jeddah and Mecca regions of Saudi Arabia. Sample size was calculated by statistical expert using open Epi version 6 program, at 80% power of study, 95% confidence level,¹⁸ as the total number of patients with ischemic heart diseases mounts to 167,499 people according to the Ministry of Health (MOH) Statistical Yearbook and the mutant genotype frequency in Eastern area of Saudi Arabia was found to be around 50%.¹⁹ We add 10% sample to compensate for drop-out and missed cases. The research protocol was approved by the Umm Al-Qura (43430838-05/05/1434H) and KAMC (13/043) institutes. Before enrolling in the study, all the participants signed an informed consent form. The study was conducted as per the Helsinki Declaration. Our recent publication²⁰ described the selection of the cases and controls in detail. The inclusion criteria for the CAD cases were selected based on full assessments in the hospital premises. In the age range of 30–85 years of age, the reported CAD cases were 50% higher than coronary artery stenosis. The CAD exclusion criteria were non-Saudi nativity, malignant diseases and/or concomitant inflammatory diseases. The type 2 diabetes mellitus (T2DM) cases were defined based on the American Diabetes Association criteria.^{22–24} The 251 apparently healthy controls were chosen among blood donors visiting same hospitals with no family history of CAD, heart pain, stroke or peripheral vascular diseases. Cross-matching was done according to age, sex and residence. The control subjects' exclusion criteria were malignancy and/or with autoimmune diseases.

Blood and Serum Analysis

Total cholesterol (TC), triglycerides (TG), high-density lipoprotein-cholesterol (HDL - c) and low-density lipoprotein-cholesterol (LDL - c) were obtained from the enrolled (n=688) subjects, and 5 mL of coagulant blood was transferred for the biochemical analysis of the fasting blood glucose (FBG) and the lipid profile analysis (LDL - c).²⁵ The samples for FBG were collected after an overnight fasting.

Molecular Analysis

A total of 5 mL of EDTA anticoagulant blood was used to isolate the genomic DNA using the Promega Wizard Genomic DNA purification kit as recommended by the manufacturers, and it was stored at -20°C until further use. In order to measure the quality and quantity of the genomic DNA, it was analyzed using a NanoDrop Spectrophotometer. The genotyping for rs10757278 was performed using an amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) with the following primer sequences:

Forward Outside: 5'GCTGTCCAGCAGCAGCAGCA GCTCTCT-3';

Reverse Outside: 5'AAGGGCATTAAAGGGTGGTGGT AGACAA-3';

A allele Primer: 5'ACTACTCTGTCTGCTGCTGCTT-3'; and

G Allele Primer: 5'AGTCAGGTGTGTGCATTCGG GAA-3'.

The PCR amplification was carried out on a Veriti Life Technology Thermal Cycler. The GoTaq Green Master Mix (Promega Co. Ltd) was used for the reaction, and the

conditions were an initial denaturation at 95°C for 5 min, a subsequent denaturation at 95°C for 30 s, annealing at 60°C for 1 min, extension at 72°C for 45 s and a final extension at 72°C for 7 min for 35 cycles and a final 4°C hold. The undigested PCR products were run on a 3% ethidium bromide agarose gel. A PCR fragment of 445 bp was observed along with an A allele fragment of 263 bp and a G allele fragment of 238 bp. The AA genotype produced bands of 445/263 bp, AG showed bands at 445/263/238 bp and GG had bands of 445/238 bp (Figure 1). Ten percent of the study samples were validated by DNA/sanger sequencing for quality control. The findings from the study of ARMS-PCR and sequencing were 100% concordant, and the representative chromatograms of the wild-type (AA), heterozygous (AG) and homozygous (GG) genotypes (Figure 2).^{26,27}

Statistical Analysis

The statistical data analyses were performed using version 21 of SPSS (Chicago, IL, United States). The continuous variables were tested for normality using a Kolmogorov-Smirnov test. The normally distributed variables are

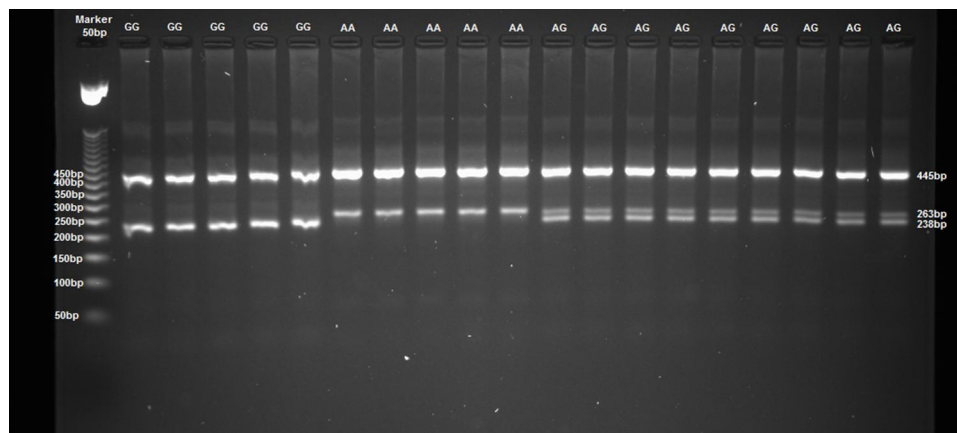


Figure 1 The rs10757278 genotyping for the G/A alleles using ARMS-PCR. Lane 1: 50 bp ladder. Lane 2–6: GG genotypes. Lane 7–11: AA genotypes. Lane 12–20: AG genotypes.

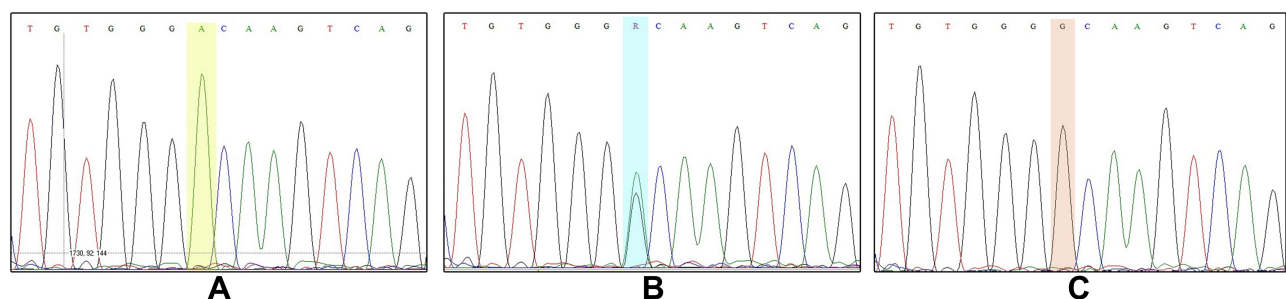


Figure 2 Representation of the validation by DNA sequencing for rs10757278.

expressed as the mean \pm standard deviation (SD) and were assessed using a Student's *t*-test. The non-normally distributed variables are expressed as the median \pm standard error of mean (SEM) and were assessed using Mann–Whitney *U*-tests. The nominal variables are presented as the number and percentage frequency and were assessed using a Pearson's Chi-square (χ^2) test. For the CAD instances, the genotypes were evaluated for Hardy Weinberg-equilibrium (HWE), and a chi-square (χ^2) analysis was used for the controls. The genotype and allele frequencies were calculated using Openepi software, and the cases and controls were evaluated using chi-square, odds ratio (OR) and 95% confidence intervals (CI's). In an independent group, the data for 3 genotypes were analyzed using the means of a one-way variance analysis (ANOVA) or Kruskal–Wallis with a post-hoc test for significance. In addition, a multiple regression analysis was performed using the GG genotypes and the associated risk factors. A *P*-value <0.05 was considered statistically significant.^{28,29}

Results

Clinical and Biochemical Data

A case-control study was conducted that consisted of 437 CAD cases and 251 controls in a Saudi population. The patients with CAD had a mean age (SD) of 59.04 (5.19) and 66% were males, 57.2% were diabetic and 68% hypertensive. The subjects in the control group were age-matched and 45.4% were males. The lifestyle and clinical characteristics of the participants involved in the study are shown in (Table 1). There was a highly significant difference between the cases and controls with regard to weight, BMI, smoking, diabetes, hypertension, stroke, heart failure, blood glucose level and all of the lipid parameters evaluated.

Genotype and Allele Frequencies

For the CAD cases and controls, the genotype distribution showed no evidence of divergence from HWE ($p>0.05$). The distribution of both alleles and the genotype frequencies of the polymorphism rs10757278 at the 9p21.3 locus for the CAD cases and controls is shown in Table 2 and Figure 3. Among the CAD cases, the frequencies of the AA, AG and GG genotypes were 21.7%, 49% and 29.3% and were 29.1%, 52.6% and 18.3% for the control subjects, respectively. The mutant GG genotype significantly doubled the risk of CAD among the cases when compared to the controls (OR-2.13; 95% CI (1.35–3.36); $p=0.0009$).

Table 1 Characteristics of the Study Population Cases and Controls

Variables	Cases (n=437)	Controls (n=251)	P
Age (Years)	59.04 (5.19)	57.94 (5)	0.08
Weight (Kg)	88.85 (9.92)	76.96 (11.25)	0.000
BMI (Kg/m ²)	29.63 (3.08)	18.91 (3.09)	0.000
Systolic BP (mmHg)	142.85 (9.92)	118.97 (9.21)	0.000
Diastolic BP (mmHg)	84.94 (3.97)	78.98 (6.14)	0.000
Blood glucose (mg/dl)	163.84 (10.91)	111.97 (10.23)	0.000
TC (mg/dl)	242.03 (7.35)	171.87 (5.34)	0.000
HDL-c (mg/dl)	39.1 (3.14)	58.27 (4.39)	0.000
LDL-c (mg/dl)	173.04 (4.87)	120.89 (3.86)	0.000
TG (mg/dl) *	223.18 (7.04)	142.42 (5.34)	0.000*
Gender (male%)	289 (66.1)	114 (45.4)	0.055
BMI group: Normal	114 (26.1)	105 (41.9)	0.000
Overweight	143 (32.7)	69 (27.5)	
Obese	180 (41.2)	77 (30.6)	
Smoking	203 (46.5)	69 (27.5)	0.000
Exercise	107 (24.5)	72 (28.5)	0.132
Diabetes	252 (57.7)	42 (16.7)	0.000
Hypertension	297 (68)	54 (21.5)	0.000
Event: Stroke	109 (24.9)	9 (3.6)	0.000
Heart failure	30 (6.9)	6 (2.4)	0.007
CABG	36 (8.2)	–	–

Notes: Continuous variables are presented as [mean (SD)] \rightarrow compared by a Student's *t*-test. Nominal variables are presented as frequency, No. (%) \rightarrow compared by a Pearson's chi square test. *Median (SEM) \rightarrow Mann–Whitney *U*-test. **Abbreviations:** BMI, body mass index; TC, total cholesterol; TG, triglycerides. HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; CABG, coronary artery bypass graft.

Also, the subjects that carried the G allele was more 1.44 times likely to develop CAD (OR-1.44; 95% CI (1.15–1.80); $p=0.001$). The AA genotype was protective against CAD when compared to the GG and AG genotypes (OR-0.6773; 95% CI (0.475–0.965); $p=0.03$). While for the recessive model, the GG genotype significantly increases the risk of CAD by 1.84 times compared to the AA and AG genotypes (OR-1.84; 95% CI (1.26–2.70); $p=0.001$). There was no association between CAD and the heterozygous genotype when it was compared to either the wild-type genotype alone or inside the co-dominant genetic model ($p=0.250$ and 0.360 , respectively).

Table 2 Comparison of the Different Mode of Inheritance of the Genotypes and Allele Frequencies with the rs10757278 Polymorphism in CAD Cases and Control Subjects

Genotype	Case (437)		Control (251)		OR	95% CI	p
	No.	%	No.	%			
AA	95	21.7	73	29.1	1		Reference
AG	214	49	132	52.6	1.246	0.856–1.811	0.250
GG	128	29.3	46	18.3	2.138	1.357–3.369	0.0009
Dominant (AA vs AG + GG)							
	95	21.7	73	29.1	0.6773	0.475–0.965	0.030
Co-dominant (AG vs GG+AA)							
	214	49	132	52.6	0.8651	0.6341–1.18	0.360
Recessive (GG vs AG+AA)							
	128	29.3	46	18.3	1.846	1.262–2.7	0.001
Alleles							
A	404	46.2	278	55.4	1		Reference
G	470	53.8	224	44.6	1.444	1.158–1.8	0.001

Note: OR, odds ratio by Pearson's chi square test.

Genotype Distribution Among the Various Risk Factors

To study the distribution of the genotype among various risk factors, an ANOVA and Kruskal–Wallis analyses were performed, and the results are displayed in Table 3. In comparison, both physical activity and diabetes, which are modifiable risk factors for CAD, were statistically significant among the three genotypes ($p < 0.05$). In addition, the post-hoc test revealed that the difference was attributed to the mutant GG genotype. Gender is one of the non-modifiable risk factors which, along with the other modifiable risk factors, such as HTN and DM, was not significant among the various genotypes ($p > 0.05$).

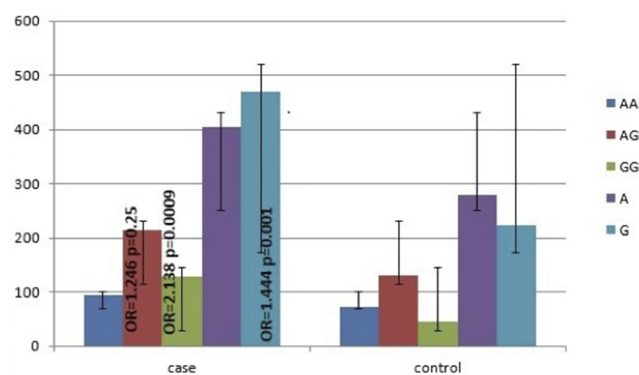


Figure 3 Genotype and allele frequencies of the CAD cases and control subjects.

Characteristics of the Lipid Risk Factors Among the Genotypes

The mean differences among different genotypes were not statistically significant regarding lipid parameters, triglycerides, total cholesterol, LDL-c and HDL-c (Table 3).

Independent Associations with CAD

To minimize the effect of the confounding risk factors, a multiple regression analysis was conducted with the GG genotype and the variable risk factors, including DM, HTN, physical activity and the lipid parameters, as listed in Table 4. The rs10757278 polymorphism was an independent risk factor for CAD. The multiple regression analysis, along with the associated risk factors, indicated that GG genotype, DM, HTN, TC, LDL-c and TG showed significant associations ($p < 0.05$).

Discussion

In this case-control study, the rs10757278 polymorphism on the 9p21 locus was confirmed to be associated with CAD in Saudi patients. The GG genotype, G allele and the dominant and recessive models of rs10757278 polymorphism were strongly associated with CAD in Saudi population. The main findings of the present study were as follows: (1) this was the first study conducted in a Saudi

Table 3 Risk Factor Distribution Across Genotypes

No.	AA (n=95)	AG (n=214)	GG (n=128)	p
Age	58.62 (5.36)	59.17 (4.89)	59.14 (5.58)	0.668
Weight (Kg)	87.09 (9.36)	88.97 (10.26)	89.95 (9.61)	0.101
BMI (Kg/m ²)	29.89 (3.04)	29.52 (3.22)	29.63 (2.89)	0.629
Systolic BP (mmHg)	144.58 (10.57)	142.65 (10.18)	141.89 (8.81)	0.124
Diastolic BP (mmHg)	85.63 (4.23)	84.86 (4.07)	84.56 (3.53)	0.124
Blood glucose (mg/dl)	161.48 (10.85)	163.81 (10.73)	167.40 (11.31)	0.07
TC (mg/dl)	242.28 (7.64)	242.12 (7.61)	242.71 (6.69)	0.827
LDL-c (mg/dl)	172.66 (4.96)	172.82 (4.94)	173.69 (4.67)	0.190
HDL-c (mg/dl)	39.11 (3.01)	39.25 (3.27)	39.86 (3.03)	0.827
TG (mg/dl) *	223.53 (6.6)	222.81 (7.16)	223.53 (7.2)	0.573*
Gender (male%)	67 (70.53)	138 (64.49)	84 (65.63)	0.579
BMI group:				
Normal	30 (31.58)	54 (25.23)	30 (23.44)	0.149
Overweight	29 (30.53)	79 (36.92)	35 (27.34)	–
Obese	36 (37.89)	81 (37.85)	63 (49.23)	–
Smoking	46 (48.42)	104 (48.60)	53 (41.42)	0.396
Exercise	35 (36.84)	42 (19.63)	30 (23.44)**	0.005
Hypertension	56 (58.95)	146 (68.22)	95 (74.23)	0.054
Diabetes	39 (41.05)	136 (63.55)	77 (60.16)**	0.001
History of:				
Stroke	31 (32.63)	53 (24.77)	25 (19.53)	0.082
Heart failure	5 (5.26)	16 (7.48)	9 (7.03)	0.774
CABG	9 (9.47)	19 (8.88)	8 (6.25)	0.613

Notes: Continuous variables are presented as [mean (SD)] → ANOVA test. Nominal variables are presented as frequency, No. (%) → Pearson's chi square test. *Median (SEM) → Kruskal–Wallis test. **Post Hoc test.

Abbreviations: BMI, body mass index; TC, total cholesterol; TG, triglycerides. HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; CABG, coronary artery bypass graft.

Table 4 Multiple Regression Analysis of the Associated Risk Factors

Variable	Unstandardized Coefficients		Standardized Coefficients	t	p
	B	SE	β		
GG genotype	0.619	0.09	0.33	4.81	0.005
TC	0.071	0.066	1.92	3.15	0.000
LDL-c	0.013	0.011	0.22	2.67	0.008
TG	0.025	0.006	1.89	5.12	0.000
BMI	0.618	0.12	0.18	3.06	0.03
DM	0.432	0.14	0.38	4.16	0.000
HTN	0.638	0.13	0.19	2.94	0.000
Physical activity	-0.271	0.11	0.09	1.82	0.323

Abbreviations: BMI, body mass index; TC, total cholesterol TG, triglycerides; LDL-c, low-density lipoprotein cholesterol; DM, diabetes mellitus; HTN, hypertension.

population with CAD assessing the rs10757278 polymorphism with separate inheritance and allele modes; (2) several population studies of European and Asian origin consider the GG genotype of the rs10757278 polymorphism to be a risk of CAD, which was confirmed in Saudi Arabia; (3) the trigger allele G for the SNP rs10757278 increased the risk of CAD with an OR of 1.44; (4) in Saudi CAD subjects, the rs10757278 polymorphism was strongly associated with the GG genotype, the G allele and a dominant and recessive mode of inheritance and (5) a regression analysis revealed that, together the GG genotype, DM, HTN and lipid parameters, as its associated risk factors, were significantly correlated in CAD patients.

CAD is a complex, multifactorial cardiovascular disorder that is caused by an interaction between genetic and environmental risk factors. GWAS provides an excellent way for a complete genome assessment of the genomic variants associated with common, complex disorders, such as CAD.³⁰ Globally, CAD is reported as one of the most frequent causes of death, affecting 3.8 million men and 3.4 million women.³¹ GWAS demonstrates a significant relationship in different populations between the 9p21 nucleotide sequence and the incidence of CAD. This locus consists of ANRIL, a non-coding sequence of anti-sense RNA and no protein-coding genes.¹⁷ Advances in molecular and biochemical approaches have revised our knowledge of CVD and CAD-causing metabolic disorders. In addition, GWAS and meta-analysis studies also identify several disease-causing genes that are further correlated and linked to the molecular basis of CAD diseases.³² At the 9p21 locus, which lies outside of annotated genes, the genetic and molecular basis of CAD risk heterogeneity in the genetic locus is unclear. A study conducted by Broadbent et al³⁴ reported that a high-risk haplotype of 9p21 collocates within a large non-coding antisense RNA gene, which is expressed in tissues affected by atherosclerosis as well as cell types that act as an important regulatory element for growth.

The rs10757278 polymorphism shows both positive^{35,36} and negative associations.³¹ However, Hu et al¹⁷ and Xu et al³⁷ conducted meta-analysis studies and concluded that the rs10757278 polymorphism might serve as a genetic marker for CAD. Our research results are in line with the Niemiec et al³⁵ study, which showed that rs10757278, independent of traditional risk factors, affected CAD susceptibility.^{16,38–40} In 2011, the rs10757278 polymorphism was first evaluated in a PAGE analysis to assess its association in two populations according to linkage disequilibrium, and the correlation coefficient high. In addition, a large-scale meta-analysis study suggests that the rs10757278 polymorphism is again significantly associated with MI.⁴² In one of the Framingham Heart studies, a 13-Kb locus on chromosome 9 (9p21) was associated with MI, stroke and fatal heart disease outcome. This locus consists of several SNPs, including rs1333049 and rs10757278.⁴³ Importantly, the rs10727578 polymorphism has been connected to atherosclerosis, and this polymorphism is associated with progression in the severity and degree of atherosclerosis.⁴⁰ Scheffold et al indicated that rs10757278 had a greater impact on the risk of CAD in patients with a positive family history.

The strength of this study is that it included 437 Saudi patients diagnosed with CAD, and we included controls that were matched for age and sex. Another strength of this study is that we conducted confirmation analyses to support the correct genotyping results. One limitation of the current study was that it was carried out only with a single SNP using an ARMS-PCR analysis. The final limitation of this study is that this study was carried out in Saudi subjects confirmed with CAD in the western region of Saudi Arabia and to better interpret the results we will need to properly authenticate Saudi expatriate CAD patients.

In conclusion, the present study indicated that allele, genotypes and different genetic inheritance modes were associated with CAD cases compared to controls. Through the regression analysis, the GG genotype was strongly associated with CAD. The current study confirmed the association of the rs10757278 polymorphism with patients diagnosed with CAD in a Saudi population. Future studies conducting analysis of CAD severity association with rs10757278 polymorphism and other genetic polymorphisms in CAD patients with different clinical presentations are being worked on.

Acknowledgment

The authors would like to thank the Deanship of Scientific Research at Umm Al-Qura University for supporting this work by Grant Code 20UQU0068DSR. We are thankful to Dr. Ahmed Shawky and the staff of the Science and Technology Unit (STU) and Deanship of Scientific Research at Umm-Al-Qura University, Makkah, and Innovation (MAARIFAH)-King Abdulaziz City for Science and Technology-the Kingdom of Saudi Arabia for their support, as well as the Monzino Heart Center, the University of Milan, Milan, Italy, the King Fahd Armed Forces Hospital and King Abdullah Medical City Makkah for their continuous support. The authors also acknowledge the support of Mr. Abdulmoniem Gowda, Dr. Ahmed Fawzy, Dr. Dareen Ibrahim Rednah, Dr. Saad Alghamdi, Dr. Khalid Faruqi, Dr. Enas Alharthi and Dr. Elaf Almatrouk. We all are deeply appreciative of Mr. Soud Abdulraof A Khogeer, Mr. Abdulmonim Gowda, Mr. Sami Kalantan, Mr. Mustafa N Bogari, Miss Dema N Bogari and Mr. Ehab Melibary. Mr Abdulaziz Alhussini and Mr. Soud Abdulraof A. Khogeer from the Department of Biochemistry, Umm Al-Qura University, Makkah, KSA.

Disclosure

The authors report no conflicts of interest in this work.

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