

# Association of rs2954029 and rs6982502 Variants with Coronary Artery Disease by HRM Technique: A GWAS Replication Study in an Iranian Population

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## Abstract

**Background:** Genome-wide association studies (GWAS) have been the primary tool for an unbiased study of the genetic background of coronary artery disease (CAD). They have identified a list of single-nucleotide polymorphisms (SNPs) associated with coronary artery disease (CAD). In this study, we aimed to replicate the association of rs2954029 and rs6982502, a GWAS identified SNP, to CAD in an Iranian population.

**Methods:** A sample of 285 subjects undergoing coronary angiography, including 134 CAD patients and 151 healthy. The genotype determination of rs2954029 and rs6982502 SNPs performed using the high-resolution melting analysis (HRM) technique.

**Results:** Our results revealed that the TT genotype of rs2954029 ( $p= 0.009$ ) and rs6982502 ( $p< 0.001$ ) were significantly higher in CAD patients compared with controls. Binary logistic regression showed that rs6982502 and rs2954029 increase the risk of CAD incidence (2.470 times,  $p= 0.011$ , 95% CI= [1.219-4.751], and 2.174 times,  $p= 0.033$ , 95% CI= [1.066-4.433] respectively). After adjusting for confounders, we found that rs6982502 and rs2954029 are significantly associated with CAD risk.

**Conclusions:** These data showed that the TT genotype of rs2954029 and rs6982502 is associated with the risk of CAD in a hospital-based sample of the Iranian population, which has replicated the result of recent GWAS studies.

**Keywords:** Coronary Artery Disease (CAD), Genome-Wide Association Studies (GWAS), High-Resolution Melting (HRM), Single-Nucleotide Polymorphisms (SNP).

## Introduction

Coronary Artery Disease (CAD) is considered as a prominent cause of premature death in the world (1, 2). CAD is a chronic inflammatory disease in which immune function cells produce pro-inflammatory cytokines. The disease's main feature is the accumulation of lipids in the arterial wall, which leads to arterial blockage due to the formation of foam cells (3).

Risk factors for atherosclerosis include age, obesity, diabetes, high blood pressure, smoking, lifestyle, high cholesterol, and Low-Density Lipoprotein Cholesterol (LDL-C) content, and low blood High-Density Lipoprotein Cholesterol (HDL-C) content (4, 5).

Moreover, oxidative stress plays a crucial role in the progression of atherosclerosis (6).

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Received: 1 Mar, 2021; Accepted: 8 May, 2021

Collected evidence suggests that the complex interactions between environmental risk factors and genes susceptible to CAD have a substantial role in CAD's pathogenesis. Most importantly, we cannot exclude the potential role of Single Nucleotide Polymorphism (SNP) in CAD's pathogenesis as the most common genetic variation among populations (7-9).

Genome-wide association studies (GWAS) have identified innumerable candidate genetic loci associated with the risk of CAD (10-13). In 2018, a study conducted a 1000 genome-based GWAS in which they identified 64 novel genetic risk loci for CAD (14). According to GWAS, TRIB1 is a CAD-associated locus (11, 15, 16). TRIB1, which is located on chromosome 8q24, encodes the protein tribbles homolog one that is recognized as an intriguing protein that is involved in the etiology of multiple human diseases; including myeloid leukemia, Crohn's disease, non-alcoholic fatty liver disease (NAFLD), dyslipidemia, and coronary artery disease (CAD) (17).

Based on GWAS, rs2954029 and rs6982502 are two intron variants near TRIB1 locus and are associated with cardiometabolic parameters, CAD, and other metabolic diseases (11). There are several studies on the relationship between CAD and polymorphisms in different populations. Still, to the best of our knowledge, few GWAS studies evaluated the association of these two SNPs with CAD in the Iranian population. Therefore, in this study, we investigate the correlation between TRIB1 genomic region SNPs, rs2954029 and rs6982502, with CAD susceptibility in the Iranian population as a replication study.

## Materials and Methods

### *Study population*

This case-control study was performed on 134 Iranian patients with CAD and 151 healthy individuals whom all underwent angiography in Tehran Heart Center (Tehran University of Medical Sciences), Tehran, Iran. The ethics committee of Shahid Beheshti University of Medical Sciences approved the study protocols and methods

(IR.SBMU.RETECH.REC.1397.1177). The samples were collected between 2019 and 2020. Also, the study was following the declaration of Helsinki, and all patients signed written consent. The definitive diagnosis of CAD was made by a cardiologist using angiography. These patients were diagnosed with three-vessel CAD and candidates for coronary artery bypass graft (CABG) based on angiographic results. The severity of CAD was defined by  $\geq 50\%$  stenosis in at least one of the major coronary arteries. All participants were matched for age ( $50 \pm 10$ ). We selected non-CAD subjects from individuals with no coronary artery obstruction (zero) or a maximum of 5-10% stenosis in their coronary arteries referred to the mentioned clinic. We recruited all control subjects after careful examination by an angiography. All subjects were asked questions about their smoking habits, history of hypertension and diabetes, and complete medical history was obtained from their clinical records. We did not include the individuals with diabetes Mellitus and people with chronic diseases such as liver, kidney, stroke, myocardial infarction, open-heart surgery, and unstable angina. People who received vitamin D and antioxidant supplements in the previous 12 months and smokers were also not included.

### *Measurements of biochemical parameters*

We measured systolic and diastolic blood pressure twice from each person's right arm. Subjects were instructed to rest for at least 10 minutes in a comfortable place; then, measurements were made (Omron, M6 Comfort HEM7321-E, Japan). Fasting blood sugar (FBS) was taken from CAD patients before coronary artery bypass graft (CABG). The assessment of fasting blood samples in the healthy control group was also carried out following the samples' collection after angiography. Total Cholesterol (TC), LDL-C, HDL-C, Triglyceride (TG), and FBS were quantified by routine laboratory kits (Pars Azmon Inc., Iran) in an auto-analyzer (Hitachi 917 Ltd, Tokyo, Japan).

### DNA extraction

DNA was separated by the salting-out method from whole blood (18, 19). After collecting DNA, the DNA concentration was specified by Nano Drop<sup>TM</sup> then the samples were stored at -20 °C until utilization.

### Real-time PCR-HRM

Real-time Polymerase Chain Reaction High-Resolution Melting (real-time PCR-HRM) analysis was utilized to detect the TRIB1 - LOC105375746 - rs2954029 polymorphism using forward: 5'- GCC ATT TAC AAA GCT GCT GAT GGT -3' and reverse: 5'-TTG TGT CAT GAG GGG AGA GAT ACA A -3' primers and to detect the TRIB1 - LOC105375746 - rs6982502 polymorphism using forward: 5'- GAC GAC AGT TCC CTC CTT GG -3' and reverse: 5'-TGT CCT AGA TGT CCT TCA GCT C -3' primers. Primers for HRM were designed by Primer3web software (version 4.1.0.), and their specificity for PCR was checked by nucleotide BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

To this end, the thermal cycling conditions were as follows: holding time, 95 °C for 12 min; followed by 35 denaturation cycles at 95 °C for 20 s and annealing for rs2954029 at 58 °C and rs6982502 at 61 °C for 20 s; extension at 72 °C for 20 s; and HRM, 75–90 °C. Then the HRM curves were normalized, and three different classes of melting curves were observed. Three samples with defined genotypes were subjected to Sanger sequencing to evaluate the reliability of the HRM method.

### Statistical Analysis

We used the statistical software SPSS 26 (SPSS, Chicago, IL) for data analysis. Quantitative variables were tested for normality by Kolmogorov–Smirnov test. Quantitative variables were presented as median (min–max) deviation and tested by Mann–Whitney U test. Also, the  $\chi^2$  test was employed to evaluate the deviation of genotype distribution from the Hardy–Weinberg equilibrium in both groups. We used binary logistic regression to assess the

association of each SNP with the incidence of CAD. The association between CAD and the SNP genotypes and allelic frequencies was measured by the odds ratio (OR) with 95% confidence intervals (CI). The limit for statistical significance was maintained at P values  $\leq 0.05$ .

## Results

### Demographics and clinical characteristics

The clinical characteristics of CAD patients and the control group are shown in Table 1. The study groups were matched for age (p= 0.082). The subjects included 134 cases and 151 controls: 14.0% of the subjects were female, and 86.0% were male. Unfortunately, for the rs2954029, nine genotypes in the patient group and two genotypes in the control group could not be detected. The genotypes of these individuals were not mentioned in this article. Laboratory analysis showed that the serum level of LDL-C, WC, TC, and TG significantly increased in the patient group compared to the healthy subjects (p< 0.001, p= 0.001, p< 0.001, and p= 0.001 respectively). Also, systolic, and diastolic blood pressures were significantly higher in CAD patients than in the control group (p= 0.035, p= 0.003, respectively). Also, the healthy group showed significantly higher HDL-C (p< 0.001 and p= 0.001, respectively) compared to CAD patients.

### Genotype and Allele Frequencies of rs2954029 and rs6982502

Tables 2 and 3 lists the frequency of alleles and Hardy Weinberg equilibrium for variants rs2954029 and rs6982502, respectively. According to these tables, the genotype frequency of rs2954029 (p= 0.168) and rs6982502 (p= 0.126) agreed with the Hardy–Weinberg equilibrium. In addition, the T allele of rs2954029 and rs6982502 was higher in CAD compared to control group (OR 1.537; 95% CI= [1.088-2.170], p= 0.015 and OR 1.904; 95% CI= [1.360-2.667], p= 0.001 respectively). Based on our analysis, the TT genotype frequency was significantly higher in CAD patients compared with controls in both rs2954029 and rs6982502 variants (p= 0.009,

p<0.001). Table 4 analyzed the association of rs2954029 and rs6982502 genotype with CAD and presented three genetic models (Dominant, Recessive, and Over-dominant) for these SNPs. Moreover, our results showed that the TT+AT genotypes in rs2954029 and the TT+CT in rs6982502 were more frequent in CAD patients compared to controls under

the dominant model (OR 0.60; 95% CI= [0.361- 0.996], p= 0.047 and OR 2.286; 95% CI= [1.358-3.848], p= 0.002) (Table 4). In table 5, binary logistic regression results showed that rs6982502, 2.470 times (p= 0.011, 95% CI= [1.219-4.751]) and rs2954029, 2.174 times (p= 0.033, 95% CI= [1.066-4.433]) increases the risk of CAD incidence.

**Table 1.** Demographic and clinical characteristics of the study population.

Parameter	CAD patients (n= 134) Median (min-max)	Controls (n= 151) Median (min-max)	p-value Mann-Whitney test
Sex (male/female)	116/18	129/22	0.783
Age (year)	56(45-60)	55(45-60)	0.082
FBS (mg/dL)	92(68-173)	93(63-194)	0.926
BMI (kg/m <sup>2</sup> )	26.29(19.14-38.80)	26.15(19-37.11)	0.995
WC (cm)	98.5(84-117)	91.5(72-114)	<b>0.001</b>
LDL-C (mg/dL)	102.67(63-233)	78.05(51-102)	<b>&lt;0.001</b>
HDL-C (mg/dL)	35(19-89)	40(17-480)	<b>&lt;0.001</b>
TC (mg/dL)	163.5(103-264)	137(76-282)	<b>&lt;0.001</b>
TG (mg/dL)	140(42-382)	111(23-487)	<b>0.001</b>
Ca (mg/dL)	9.3(7.8-10.4)	9.6(9.1-10.2)	0.051
Phos (mg/dL)	3.03(1.48-5.70)	3.26(2.03-5.50)	0.057
Vit D (ng/mL)	26.36(3.76-140.3)	36.39(7.15-120.68)	0.007
SBP (mmHg)	120(100-170)	120(110-130)	<b>0.035</b>
DBP (mmHg)	80(60-100)	75(60-80)	<b>0.003</b>

CAD: coronary artery disease, FBS: fast blood sugar, BMI: body mass index, WC: waist circumference, LDL-C: low-density lipoprotein cholesterol, HDL-C: high-density lipoprotein cholesterol, TC: total cholesterol, TG: triglycerides, Ca: calcium, Phos: phosphorous, Vit D: vitamin D, SBP: systolic blood pressure, DBP: diastolic blood pressure. Data are shown as median±IQR (interquartile range).

**Table 2.** Genotype distribution and relative allele frequencies of the rs2954029 A>T variants.

Genotypes	Control N= 149	CAD N= 125	p-value*	OR (95% CI)
	N (%)	N (%)		
AA	60 (40.30%)	36 (28.80%)	<b>0.035</b>	-
AT	75 (50.30%)	66 (52.80%)	0.153	0.682 (0.402-1.158)
TT	14 (9.40%)	23 (18.40%)	<b>0.009</b>	2.738 (1.252-5.987)
<b>Allele</b>				
A	195 (65.4%)	138 (55.2%)		
T	103 (34.6%)	112 (44.8%)	<b>0.015</b>	1.537 (1.088-2.170)
<b>HWE</b>				
			0.168	

\*p values and OR were computed by  $\chi^2$  tests. OR: odds ratio, CI: confidence interval.

**Table 3.** Genotype distribution and relative allele frequencies of the rs6982502 C>T variants.

Genotypes	Control	CAD	p value *	OR (95% CI)
	N= 151	N= 134		
		N (%)		
CC	60 (39.70%)	30 (22.40%)	<b>0.000</b>	-
CT	77 (51.0%)	73 (54.50%)	<b>0.020</b>	1.896 (1.102-3.263)
TT	14 (9.30%)	31 (23.10%)	<b>0.000</b>	4.429 (2.054-9.549)
<b>Allele</b>				
C	197 (65.2%)	133 (49.6%)		
T	105 (34.8%)	135 (50.4%)	<b>0.000</b>	1.904 (1.360-2.667)
HWE			0.126	

\*p values and OR were computed by  $\chi^2$  tests. OR: odds ratio, CI: confidence interval.

**Table 4.** Dominant, recessive, and Over-dominant models in case-control studies.

Genotypes	Models	Control	CAD	OR (95%CI)	p-value*
		N (%)	N (%)		
Rs2954029	Dominant	89 (59.70%)	99 (71.20%)	0.60 (0.361-0.996)	<b>0.047</b>
		60 (40.30%)	36 (28.80%)		
	Recessive	14 (9.40%)	23 (18.40%)	2.174 (1.066-4.433)	<b>0.030</b>
		135 (90.60%)	102 (81.60%)		
Rs6982502	Over-dominant	75 (50.30%)	66 (52.80%)	0.906 (0.563-1.458)	0.684
		74 (49.70%)	59 (47.20%)		
	Dominant	91 (60.30%)	104 (77.60%)	2.286 (1.358-3.848)	<b>0.002</b>
		60 (39.70%)	30 (22.40%)		
	Recessive	14 (9.30%)	31 (23.10%)	2.945 (1.491-5.819)	<b>0.001</b>
		137 (90.70%)	103 (76.90%)		
	Over-dominant	77 (51.0%)	73 (54.50%)	0.869 (0.545-1.386)	0.557
		74 (49.00%)	61 (45.50%)		

\*p values and OR were computed by  $\chi^2$  tests. OR: odds ratio, CI: confidence interval.

**Table 5.** Logistic regression analysis of the rs2954029 and rs6982502 variants with the risk of CAD.

	p value	OR	95% CI
SNP rs6982502	0.011	2.470	1.219-4.751
SNP rs2954029	0.033	2.174	1.066-4.433

CI: confidence interval, OR: odds ratio.

## Discussion

Coronary artery disease (CAD) is, in fact, one of the most common causes of mortality worldwide. Besides lifestyle and environmental factors, which play an essential role in CAD development (20-22), a genetic basis has been established for CAD (23). GWAS studies have detected strong associations among different loci and CAD (24). Over the past decade, GWAS has shown that chromosomal locations play a vital role in increasing CAD sensitivity (24). In the present study, we surveyed the Iranian

population to evaluate CAD's association with TRIB1 rs2954029 and rs6982502 variants adjacent to the TRIB1 risk locus derived from GWAS based on 1000 genomes.

Most importantly, we observed that the genotypes of rs2954029 and rs6982502 were significantly associated with the risk of CAD. T allele and TT genotype of the two variants were significantly more frequent in CAD patients than healthy controls. Moreover, the TT+AT genotypes of rs2954029 and the TT+CT of rs6982502 were

more frequent in CAD patients than healthy controls under the dominant model.

These results were partly consistent with previous studies in the other populations. Ram et al. identified that rs6982502 is associated with hypertriglyceridemia in ethnic Arabs, a significant risk factor for CAD (25).

Regarding the observed higher frequency of rs6982502 T allele in our CAD patients, in a study conducted by Ishizuka et al. in the Japanese population, they concluded that the C minor allele rs6982502 increased the risk of non-alcoholic fatty liver disease (NAFLD), which is another metabolic disorder which has some shared pathophysiological mechanisms with CAD (26).

Although Varbo et al. and Zhang et al. showed in two different studies that TRIB1-rs2954029 was linked with the increased risk of ischemic heart disease in the general population and Chinese people, respectively (27, 28), in contrast to our results indicating a high frequency of TT genotype in CAD patients; Varbo et al. showed that TRIB1-rs2954029 TA genotype was associated with an increased level of TG and ischemic heart disease (27). Moreover, Zhang et al. showed that A allele of TRIB1-rs2954029 was associated with blood lipids and risk of ischemic heart disease (28). Contrary to our findings, Toshiyuki Ikeoka et al. showed that the AA genotype of TRIB1 rs2954029 was associated with TG serum level in Japanese women (29). Population differences in genotype frequencies of SNPs in TRIB1 might explain this discrepancy.

Like other complex disorders, many CAD-associated risk variants have been discovered by several GWAS (30). Based on GWAS results, in a sample of coronary artery disease cases, Willer et al. identified that the T allele of TRIB1 rs2954029 is associated with lipid concentrations and risk of CAD (31). The GWAS results by Teslovich et al. in European ancestry showed that the TRIB1 rs2954029 T allele affects blood lipids and has increased the risk of CAD, which is consistent with our results (32).

Moreover, Different GWAS results showed that TRIB1 rs2954029 affects the concentration of TC, TG, LDL-C, and HDL-C in different populations (28, 32, 33). These findings favor our results about the higher TC and LDL-C in CAD patients than in the control group.

TRIB1, located on chromosome 8 (8q24.13), encodes a G-protein-coupled receptor-induced protein that plays a role in the mitogen-activated protein kinases-(MAPK-) related signaling cascade, which mediates cell proliferation, differentiation, and apoptosis, and can regulate lipid metabolism through this pathway (34, 35). It is also known to be a risk factor for CAD (36). TRIB1 is associated with changes in serum levels of various factors such as TC, LDL-C, HDL-C, TG, and CAD (37). TRIB1 regulates smooth muscle cell proliferation and vascular chemotaxis via the Jun kinase pathway. Its overexpression in human atherosclerotic arteries with chronic inflammation suggests that TRIB1 may play an essential role in CAD and atherosclerosis (38). Recent studies have shown that several SNPs in TRIB1 were associated with one or more lipid parameters and CAD (39).

One possible explanation for the association of TRIB1 locus with lipid metabolism may be the regulatory effects of TRIB1 on mitogen-activated protein kinase that leads to dyslipidemia, but the exact mechanism is still unknown. It has also been postulated that TRIB1 controls chemotaxis and proliferation of smooth muscle cells in the arterial intima, and through this, it may lead to lipoprotein-independent atherosclerosis (27, 28). Rest upon the evidence from the previous human and animal studies and the results of our study; one may conclude that TRIB1 and near SNPs are involved in lipid metabolism and atherosclerosis (14, 27, 32).

Studies with a larger sample size and based on various ethnic groups might be more reproducible. Because lifestyle information, such as eating habits and physical activities,

could not be fully collected, potential gene-environmental interactions could not be eliminated. Lifestyle modification with the guidance of a health care team led by a physician may have a significant positive impact on the future of cardiovascular and vascular events.

For the first time, we found that both TRIB1 adjacent variants rs2954029 and rs6982502 were significantly associated with lipid profile and increased risk of coronary artery disease in Iranian population. Further

studies in more extensive and multiple ethnic populations are needed to confirm the present data.

### Acknowledgements

We are thankful to all participants of this study for their contribution to our study. This study was generously supported by Shahid Beheshti University of Medical Sciences Grant for MSc thesis (ID: 17011). The authors declare no conflict of interest.

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