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Association between Coronary Artery Disease and rs10757278 and rs1333049 Polymorphisms in 9p21 Locus in Iran

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Abstract

Background: Coronary arteries disease (CAD) has been recognized as one of the most common causes of death worldwide, with an estimated seven million deaths annually.

Methods: Two hundred blood samples from Iranian CAD patients and normal healthy controls were collected. CAD and the 9p21 locus variants *rs1333049* and *rs10757278* were analyzed for potential associations.

Results: No significant differences in *rs10757278* and *rs1333049* polymorphisms were found between patients and controls, but a significant relationship was found between *rs10757278* and *rs1333049* in CAD patients at the genotype level (p= 0.0323). At the haplotype level and on the basis of diplotype analysis, a significant relationship was found between patients and controls (OR= 5.16, p= 0.047, 95% CI: 1.02-26.0). In CAD patients, *rs10757278* and *rs1333049* were associated at locus 9p21.

Conclusions: The inconsistency between the results of this and other studies on different CAD populations may be due to high population, different ethnicities, low prevalence of some alleles in populations, and interactions of different genes.

Keywords: Coronary artery disease (CAD), Polymorphism , *rs1333049*, *rs10757278*, 9p21.

Introduction

Coronary artery disease (CAD) is one of the most common causes of death worldwide. An estimated 3.8 million men and 3.4 million women die each year from CAD (1). The prevalence of CAD in the Iranian population is responsible for almost 50 percent of all deaths each year (2). Hence, detecting high-risk patients is important. Fortunately, CAD risk can be predicted based on family history, the level of adipose cells, blood pressure, and smoking status (3). Of these, family history is most important. Recent studies have identified a major role for genetic factors (4). Hence, more than 100 genes are effective in the emergence of CAD by producing atherosclerosis plaques (5). Correlation studies have identified several genetic variants that are effective in the emergence of multifactorial diseases such as CAD. One of the loci identified as a hotspot for CAD is 9p21.3 in various populations (6).

The 9p21.3 locus has been found to be associated with CAD in Caucasian, Italian, American, Spanish, Southeast Asian, Indian, and Pakistani populations (7). No protein-coding gene has been thus far detected in the 9p21.3 locus, however this 58 kb region harbors the CDKN2B-AS (inhibitor 2B antisense RNA cyclin-dependent kinase) gene, which encodes an antisense noncoding RNA (8). CDKN2B-AS is located near the CDK inhibitor genes CDKN2A (cyclin dependent kinase inhibitor 2A), and CDKN2B, which both inhibit CDK4 and regulate cell growth (9). It has been reported that the

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rs1333049 variant in the INK4 locus (ANRIL), near the cell cycle regulating genes, plays a role in CAD risk (10).

In addition, it has been documented that the r*s10757278* variant is located in one of the identified enhancers in the 9p21 locus. The risk allele of this variant disrupts the binding site of STAT1 protein and alters the cellular response to inflammation, angiogenesis, and atherogenesis (11).

Genetic variants at the 9p21.3 locus increase the CAD risk; hence, the present study was designed to investigate a possible association between the *rs1333049* and *rs10757278* variants in Iranian CAD patients.

Materials and methods

Subjects

This case-control study was conducted on 100 CAD patients. Patients with coronary artery occlusion and plaque, who referred to the angiography and CT-angiography wards in the Tehran Clinic, were recruited under the supervision of a cardiologist. The control group included 100 healthy individuals with no signs of coronary artery plaque in angiography. The participants were informed about the study goals, and written consent forms were obtained.

PCR and sequencing

Three ml of peripheral blood were collected from each subject and stored in EDTA-containing tubes. Genomic DNA was extracted from peripheral blood cells according to the manufacturer's instructions (Favorgen, Taiwan). Specific tetra-ARMS PCR primers were designed for genotyping of each variant using Primer1 software (12) (Table 1). The PCR programs for each variant are shown in Tables 2 and 3.

PCR products were then electrophoresed on 3% agarose gels and stained with green viewer dye (Parstous, Iran). To optimize the tetra-ARMS PCR, each genotype of both variants for a few samples was directly sequenced. Sequences were analyzed using BioEdit software (13). For genotyping, in each PCR run, three control samples with known genotypes were amplified alongside the unknown samples.

The FAMHAP software program was used for Hardy-Weinberg testing, genotype and allele frequency calculation, and haplotype/diplotype analysis.

Table 1. Genotypic distribution of the *rs1333049* and *rs10757278* polymorphisms among CAD cases and healthy controls.

Sequence 5—3	Product	Annealing Temperature
OF CGAAGTAGAGCTGCAAAGATATTTGGAA OR GGGCTCATAATTGCTGAATAAAACAGAA IF CCTCATACTAACCATATGATCAACAGATG IR CTTACCTCTGCGAGTGGCTGCTTATG	For G 214 bp for C 263 bp two outer 422 bp	62 °C
OF TCAGCAAACCACAATCCCACATTTTAAGG OR GGGCTCATAATTGCTGAATAAAACAGAA IF AAGTCAGGGTGTGGTCATTCCGGGAA IR ACTACTCTGTCTTGATTCTGCATCGCTTCC	For G 263 bp for A 348 bp two outer 555 bp	62 °C

Table 2. The *rs1333049* PCR thermal cycler program

Stage	Number of repeats	Section	Temperature (˚C)	Time
First	1 cycle	First denaturation	95	5 min
		Second denaturation	95	45 sec
Second	30 cycle	Annealing	62	90 sec
		First extension	72	70 sec
Third	1 cycle	Second extension	12	5 min

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Table 3. The *rs10757278* PCR thermal cycle program

Stage	Number of repeats	Section	Temperature (°C)	Time
First	1 cycle	First denaturation	95	5 min
		Second denaturation	95	30 sec
Second	30 cycle	Annealing	62	45 sec
		First extension	72	30 sec
Third	1 cycle	Second extension	12	5 min

Results

The PCR product for each variant was visualized using agarose gel electrophoresis. Sample genotypes were determined by amplifying them alongside control samples of known genotype (Figs. 1 and 2).

Neither variant was in Hardy-Weinberg equilibrium in either the case or control groups. Allele and genotype analyses found no significant association between either variant and CAD risk (Table 4).

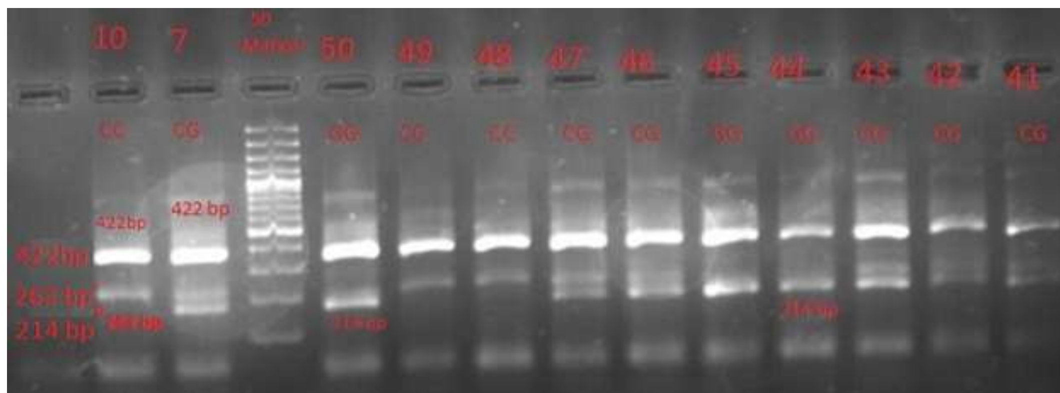

Fig. 1. The results of genotyping for *rs1333049* variant. Lane 10: Normal homozygous Control with CC genotype, Lane 7: Heterozygous control with CG genotype, lanes 41-50: patients samples with the relevant genotype on the top of the gel.

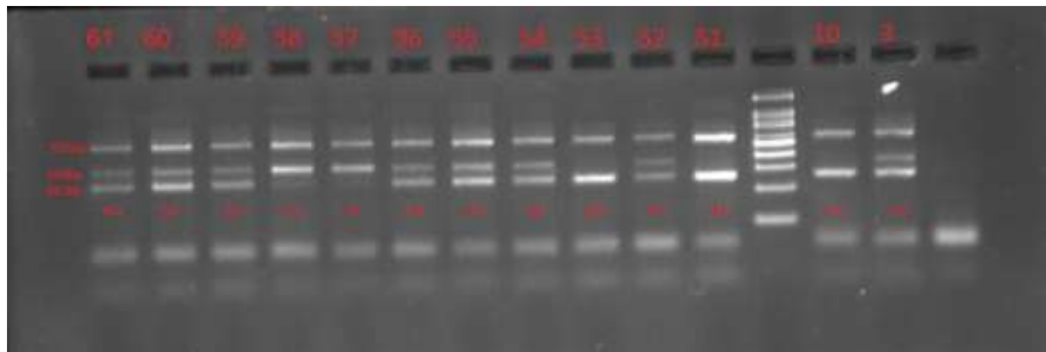

Fig. 2. The results of genotyping for *rs10757278* variant. Lane 10: Normal homozygous Control with GG genotype, Lane 3: Heterozygous control with AG genotype, lanes 51-61: patients samples with the relevant genotype on the top of the gel.

Table 4. Genotype frequency, OR, 95% CI, and P value for both variants among controls and cases.

Polymorphisms	Controls	Cases	OR	95% CI	p value
rs1333049					
CC	15	16	1.079	0.502-2.322	0.845
CG	71	77	1.367	0.724-2.581	0.333
GG	14	7	0.462	0.178-1.2	0.106
rs10757278					
AA	23	27	1.238	0.652-2.352	0.514
AG	69	62	0.733	0.408-1.316	0.298

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Haplotypes differed significantly between then the two groups (X2= 8.7836, df= 3, p= 0.0323), with the CG haplotype more frequent in the control than in the CAD group, indicating a protective role for this haplotype against CAD (Table 5).

Table 5. Haplotype analysis of *rs1333049* and *rs10757278* variants.

Haplotype (*rs1333049-rs10757278*)	Frequency % (Cases)	Frequency % (Controls)
AC	0.396	0.455
CG	0.148	0.049
GA	0.183	0.119
GG	0.271	0.375

In diplotype analysis which is the combination of haplotypes on each chromosome, combination of CA haplotype of *rs1333049-rs10757278* variants and GA haplotypes of *rs1333049-rs10757278* variants showed increased the risk of CAD (CI= 1.13-5.69, OR= 2.53). While in contrast, combination of CA haplotype of *rs1333049-rs10757278* variants and CA haplotypes of *rs1333049-rs10757278* variants showed a protective role (CI= 0.13-1.14, OR= 0.386) (Table 6).

Table 6. Diplotype analysis of 9p21 variants.

Haplotype 1 (*rs1333049-rs10757278*)	Haplotype 2 (*rs1333049-rs10757278*)	Freq % (Cases)	Freq % (Controls)	OR (95 % CI)
CA	GG	43.8	53	0.693 (0.4-1.2)
GA	CG	4.2	5	0.82 (0.22-3.1)
CA	CA	5	12	0.386 (0.13-1.14)
CG	GG	7	3	2.434 (0.61-9.69)
CA	GA	22	10	2.538 (1.13-5.69)
GG	GG	2	3	0.66 (0.11-4.04)
CG	CG	2	2	1 (0.14-7.24)
GA	GA	0	1	-
GA	GG	5	10	0.474 (0.16-1.44)
CA	CG	9	1	-

Discussion

Accounting for approximately 22.9% of all deaths, CAD is among the most common causes of death worldwide (14). CAD heritability is estimated to be 40-50% according to family studies (15). Genome-wide association studies (GWAS) have shown a significant relationship between the 9p21 nucleotide sequence and CAD risk in various populations. This locus contains ANRIL, a non-coding RNA antisense sequence and no protein coding genes (16), but maps near cyclin-dependent kinase inhibitors MTAP (CDKN2B, CDKN2A), and CDKN2BAS, another non-coding RNA (17).

In this study, the *rs10757278* and *rs1333049* polymorphisms in the 9p21 locus were analyzed for their association with CAD. No significant associations were found between the two polymorphic genotypes and CAD risk (p> 0.05).

Çakmak found a significant association between the *rs1333049* polymorphism and CAD risk in a Turkish population (p= 0.037). The frequencies of GG, AG, and AA in the *rs10757278* polymorphism were 23%, 69%, and 8% in the patient group, and 27%, 62%, and 11% in the control group, respectively (18). In a similar study conducted on 100 patients with CAD in Khuzestan province, the genotypic frequencies of GG. CG. and CC in the *rs1333049* polymorphism were 8%, 67%, and 25% in the case group, and 16.5%, 65.3%, and 18.2% in the control group. The genotypic frequencies of GG, AG, and AA in rs10757274 were 2%,63%, and 35% in the case group, and 33.5%, 58.2%, and 8.3% in the control group. They reported a significant relationship between *rs1333049* and CAD risk in the studied population (19).

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Recently, a similar study revealed a significant relationship between *rs10757278* and CAD risk (p= 0.047), whereas the *rs1333049* polymorphism had no significant association (p> 0.05) (20).

Likewise, *rs1333049* was significantly correlated with CAD risk in Japan, Iraq, Italy, China, and India (p< 0.05). *rs10757278* was also significantly associated with CAD risk in the Caucasus (p< 0.04), while no such association was observed in Poland (p= 0.011) (21).

In the latter study, no significant association was found between *rs1333049* or *rs2383207* and CAD, but haplotype and diplotype analyses demonstrated a significant relationship (22).

The above findings can be explained by different allelic distributions in various populations or low frequencies of some alleles in the target population. Additionally, a variety of ethnic groups live in Iran, each of which can have a specific allelic frequency, affecting the results of such studies.

In addition, CAD is a multifactorial disease influenced by environmental factors. Ultimately, SNPs create a genetic potential for an individual, the expression of which depends on one's genetic background, interactions between other genes, and the environment. Future studies are recommended.

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