Original Article

Increased Risk of CHD in the Presence of rs7865618 (A allele): Tehran Lipid and Glucose Study

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Abstract

Background: Recent genome-wide association studies (GWAS) in European populations have indicated that the rs12526453 polymorphism located in phosphatase and actin regulator 1 gene (*PHACTR1*), mapping to chromosome 6p24 and rs7865618 polymorphism in the cyclin-dependent kinase inhibitor B antisense RNA 1 gene (*CDKN2B-AS1*) on 9p21.3 are associated with coronary heart disease (CHD). This study was carried out to investigate the association of these polymorphisms and CHD in an Iranian population.

Methods: In the present case-control study, 420 patients with CHD events were recruited from the population of the Tehran lipid and glucose study (TLGS); 407 healthy controls matched for age and sex were selected from the same population. The SNPs rs12526453 and rs7865618 were genotyped using amplification refractory mutation system-polymerase chain reaction (ARMS-PCR).

Results: The allele frequency of both SNPs deviated from Hardy–Weinberg equilibrium. The C allele frequency of the rs12526453 (68.5%, *P* = 0.11) and A allele of the rs7865618 (68.8%, *P* = 0.09) were the most prevalent alleles in both the case and control groups. The results indicated a significant association between the presence of risk alleles of rs7865618 and CHD in the TLGS population (*P* = 0.03; OR: 1.73; CI95%: 1.04 – 2.88)

Conclusion: Due to the importance of chromosome 9p21 region and its relation with cardiovascular disease, the allelic pattern of its variation should be studied in different populations. The relation between this polymorphism and cardiovascular disease in the studied population confirms the importance of this region.

Keywords: ARMS-PCR, *CDKN2B-AS1*, coronary heart disease, *PHACTR1*

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Introduction

Coronary heart disease (CHD) plays a significant role in mortality and morbidity in developed countries, specifically among the elderly.[1,2] It has been reported that CHD may rank as the most prominent cause of death by 2020.[3,4] Due to the high prevalence of CHD risk factors reported in Iranian adult populations, CHD is one of the most crucial health problems in this country.[5]

Environmental and genetic factors are two main contributing factors to the incidence of CHD as a multifactorial disease. Although lifestyle modifications (healthy diet and adequate physical activity) reduce most CHD risk factors, genetic susceptibility remains unaffected and interacts with environmental factors.[1] In recent years, the significant influence of genetic disorders on susceptibility to atherosclerotic vascular disease has been confirmed by advanced molecular genetics techniques. Atherosclerosis is the main cause of CHD in which a complex series of lesions occur in the arterial wall. These events, including foam cell formation and smooth muscle cell recruitment, are created by multiple biological pathways and genes.[6,7] Recent studies have discovered a large number of candidate genes, genetic polymorphisms, and susceptibility loci associated with atherosclerotic disease. Genome-Wide Association Studies (GWAS) have revealed several common variants in new candidate genes which seem to take part in the susceptibility and pathogenesis of CHD. The *CDKN2B/ANRIL* gene cluster on 9p21.3 and *PHACTR1* on chromosome 6p24.1,[1,2] are two loci which have been related to CHD.

The 9p21.3 chromosomal locus is identified as one of the most influential genomic markers for CHD and a hotspot for disease-associated polymorphisms through various genome-wide association studies.[8] One of the common single nucleotide polymorphisms (SNP) in the 9p21 locus is rs7865618 located in proximity of *CDKN2B-AS1 (ANRIL)*.[8] Susceptibility to CHD increases by 1.23 and 1.57 folds in the presence of heterozygote (AG) and homozygotes (AA) rs7865618 risk allele, respectively.[9] Several studies have investigated the association between *CDKN2B-AS1* and CHD in different populations. An association between rs7865618 and myocardial infarction (MI) has been reported in European populations.[10] Another study pointed out that the haplotype of *CDKN2B-AS1* gene polymorphisms (rs7044859, rs1292136, and rs7865618) is associated with coronary artery disease.[9]

Another common locus for CHD identified by GWAS is

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6p24.1. Its high frequency in different ethnicities such as European, Asian, and Middle Eastern populations highlights its importance.[11] Although it is clear that there is an association between *PHACTR1* in locus 6p24 and CHD, the nature of this association in the pathophysiology of CHD needs further investigation. In fact, *PHACTR1* regulates protein phosphatase 1 (PP1) which is a regulator enzyme for endothelial nitric oxide and is known as an important modulator of CHD. In addition, it has been shown that the activity of PP1 increases in patients with end-stage heart failure. The rs12526453, a variant in the 3rd intron of the *PHACTR1* gene is associated with CHD and early-onset MI, according to reports by the MI Genetics consortium;[1] this association was directionally consistent in a study including three different populations.[12]

Despite the data available, there is still lack of evidence regarding the relation between these SNPs and CHD in other populations, especially Iranians. Therefore, we aim to investigate the potential association of rs7865618 of *CDKN2B-AS1* gene and rs12526453 of *PHACTR1* gene and risk of CHD in a cross-section of Iranian patients selected among the participants of the Tehran Lipid and Glucose Study (TLGS).

Materials and Methods

Data collection

This case-control study was carried out in a subpopulation of the TLGS cohort. TLGS, in brief, is an ongoing large scale community-based study with a population of 15,005 people initiated in 1999 in district 13 of Tehran, the capital of Iran.[13] Details of study participants and design have been published before.[14] Written informed consent was obtained from each participant and this study was approved by the research council of the Endocrine Research Center of the Shahid Beheshti University of Medical Sciences.

Samples were selected from participants with CHD outcome during follow-up. Outcome was measured in TLGS during an annual follow-up by telephone call for any medical event which occurred during the past year. In the case of any related event during the previous year, a nurse and/or a physician visited the patient to collect complementary data and in the case of mortality, more data were gathered from hospital or death certificate. Finally, collected data were evaluated by outcome committee. Specific diagnosis for every event was assigned according to the international classification of diseases (ICD-10) criteria and American heart association (AHA) for cardiovascular events.[15] Selected events included myocardial infarction (MI), positive coronary angiography or heart scan, unstable angina (UA), mortality from ischemic heart disease and sudden cardiac death (SCD). Among the 830 cases of CHD diagnosed by 2010, 420 cases (age range 45 – 80 years in men, 55 – 80 years in women) were selected who had genomic samples. Healthy TLGS participants (n = 407) (same sex and age ± 5 years) without any sign of cardiovascular disease, diabetes mellitus and metabolic syndrome were selected as controls.

Laboratory Measurements

Genomic DNA was extracted from peripheral blood leukocytes using salting out protocol as described before.[15] The ARMS-PCR technique was used to amplify a 387-bp fragment for rs12526453C>G in the *PHACTR1* gene and 432-bp for rs7865618A>G in the *CDKN2B-AS1* gene using the oligonucleotide primers as shown in Table 1. PCR was carried out using the following program: After initial denaturation at 95°C for 5 min, each cycle with 94°C (30 s), 60°C (35 s) for rs12526453C>G or 59°C (30 s) for rs7865618A>G, and 72°C (30 s) for 30 cycles. The final extension at 72°C (5 min) (ABI Applied Bio systems 2720 PCR thermal cycler).

Using electrophoresis, the fragments were separated on 1.5% agarose gels and DNA fragments were also visualized by gel documentation (Opticom). Genotype data was confirmed using direct sequencing after initial set-up and also for some of the subjects in different groups. Figure 1 shows ARMS-PCR and direct sequence of the amplified segment in two polymorphisms of rs12526453 and rs7865618, respectively.

Statistical Analysis

Deviation from Hardy–Weinberg equilibrium and allele and genotype frequency were calculated using power-marker v.3.25 software *(Available from: URL: http://statgen.ncsu.edu/powermarker/)*. Statistical Package for the Social Sciences V.20 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.) was utilized to perform statistical analyses. Continuous variables were expressed as mean ± standard deviation (SD) for parameters with normal distribution. Categorical variables were evaluated by the $\chi 2$ test and expressed as number (percentage). The differences in means across groups were examined using one-way ANOVA (Tukey's post hoc test). *P*-values less than 0.05 were considered significant.

Results

In the present case-control study, 420 CHD cases and 407 age- and sex-adjusted healthy controls were recruited. Age and sex characteristics of the studied population are presented in Table 2. In genotyping for rs7865618 and rs12526453, some samples failed to be genotyped; therefore, the final genotyped samples

Table1. Primer design information for two studied polymorphisms

SNP	Name	Primer	Length
rs12526453	Reverse-common	ATGGTACCTATGCTGTTTGCTATACTG	27
	Forward-G allele	GGACATATGCCTCTCTAGACTATAATCTG	29
	Forward-C allele	GGACATATGCCTCTCTAGACTATAATCTC	29
rs7865618	Reverse-common	GCCTTCTCTATTCACCTCATATAACTATT	29
	Forward-G allele	GAATATGTTTGTTTAGCTTCTTAATCCG	28
	Forward-A allele	GAATATGTTTGTTTAGCTTCTTAATCCA	29

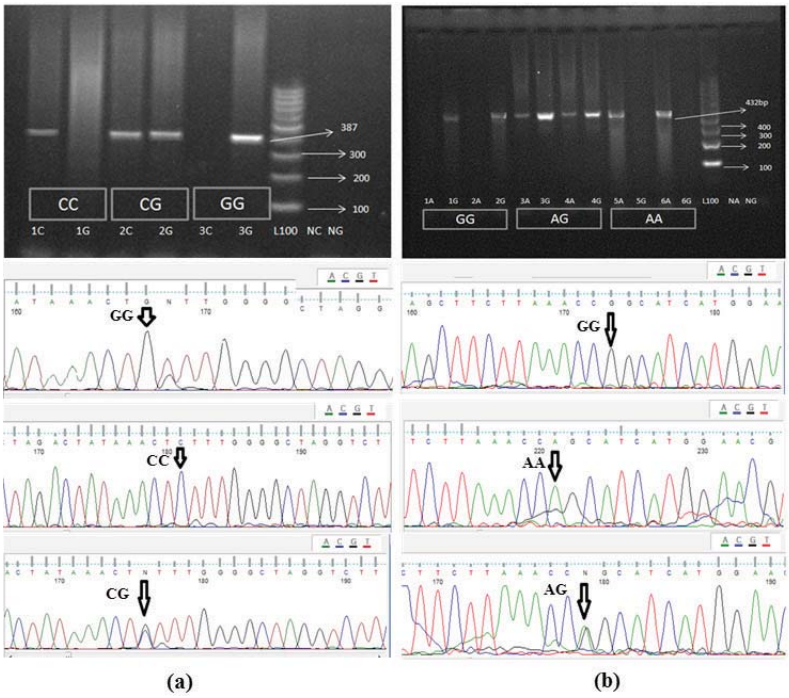

Figure 1. ARMS-PCR and direct sequencing of the amplified segments in **(a)** *PHACTR1* gene rs12526453 and **(b)** *CDKN2B-AS1* gene rs7865618. In both sections a and b, the figures show the labeled genotype for related sequences and electrophoresed on 1.5% agarose. 100 bp DNA ladder was used in both electrophoreses and no band in lanes NG, NA, and NC confirm the lack of contamination during the ARMS-PCR. For the first figure of section a lane 1 homozygote CC, lane 2 heterozygote CG, lane 3 homozygote GG. For the first figure of section b lane 1 and 2 homozygote GG, lane 3 and 4 heterozygote AG, lane 5 and 6 homozygote AA.

Table 2. Anthropometric and biochemical characteristics of the studied population

Studied variable (unit)	Control (n = 407)	Case (n = 420)
Male		
Age, year	58.05 ± 8.95*	59.9 ± 7.95
Number (%)	320 (78.6%)	289 (68.9%)
Female		
Age, year	62.83 ± 5.04	63.34 ± 4.73
Number (%)	87 (21.4%)	131 (31.1%)
Systolic blood pressure (mmHg)	125 ± 21	129 ± 19
Diastolic blood pressure (mmHg)	76 ± 11	77 ± 11
Fasting glucose (mg/dL)	94 ± 8	121 ± 46
Total cholesterol (mg/dL)	190 ± 37	180 ± 42
Triglyceride (mg/dL)	103 ± 47	150 ± 69
HDL-C (mg/dL)	52 ± 12	46 ± 10
LDL-C (mg/dL)	118 ± 32	104 ± 37

*Characteristics data are expressed as mean value ± standard deviation. All biochemical characteristics were different significantly between case and control ($P < 0.05$).

included 417 cases and 407 healthy controls for rs7865618 and 419 cases and 406 healthy controls for rs12526453. Power marker analysis demonstrated that genotype distribution of these polymorphisms does not comply with the Hardy–Weinberg equilibrium ($P < 0.05$). Also, in Table 2, the clinical and biochemical characteristics of the study subjects are presented for both case and control groups.

C allele was present in 1130 (68.5%) chromosome in rs12526453 C>G and A allele in 1135 (68.8%) chromosome in rs7865618 A>G. Allelic and genotype distribution of rs7865618 and rs12526453 did not reveal any significant difference between CHD cases and healthy controls (rs7865618 A>G; OR = 1.19, CI95%; 1.18 – 1.20, rs12526453 C>G; OR = 0.84, CI95%: 0.84 – 0.85) (Table 3).

Risk allele carriers were compared with non-carriers using dominant and recessive models for each polymorphism in cases and controls. Subgroup analyses were performed to compare different models between those with MI *vs.* non-MI event, MI *vs.* healthy controls and those with definite diagnosis *vs.* healthy controls.

The presence of A allele (AA or AG genotype) in rs7865618 was associated with increased incidence of CHD ($P < 0.03$) compared to healthy controls (OR: 1.73; CI95%: 1.04 – 2.88) (Table 4).

Table 3. Genotype and allele frequency of two SNPs on chromosome 9p21 and 6p24 with CHD cases and controls in the TLGS population

	All, N (%)	Case, N (%)	Control, N (%)
rs7865618			
Genotype			
AA	379 (45.9)*	199 (47.7)	180 (44.3)
AG	377 (45.8)	192 (46.1)	185 (45.4)
GG	68 (8.3)	26 (6.2)	42 (10.3)
Allele			
A	1135 (68.8)	590 (70.7)	545 (66.9)
G	513 (31.2)	244 (29.3)	269 (33.1)
rs12526453			
Genotype			
CC	414 (50.2)	212 (50.5)	202 (49.8)
CG	302 (36.6)	152 (36.4)	150 (36.9)
GG	109 (13.2)	55 (13.1)	54 (13.3)
Allele			
C	1130 (68.5)	576 (68.8)	554 (68.2)
G	520 (31.5)	262 (31.2)	258 (31.8)

*The data are presented as number (percent)

Table 4. Subgroup analysis to compare different models between those with CHD *vs.* non-CHD

SNP	Model	Genotype	CHD, N (%)	Without CHD, N (%)	OR (CI- 95 %)
rs7865618	Recessive	GG/AG	199 (48)*	180 (44)	1.15 (0.87–1.51)
		AA	218 (52)	227 (56)	
	Dominant	AA/AG	391 (94)	365 (90)[ɪ]	1.73 (1.04–2.88)
		GG	26 (6)	42 (10)	
rs12526453	Recessive	GG/CG	212 (51)	202 (50)	1.03 (0.78–1.35)
		CC	207 (49)	204 (50)	
	Dominant	CC/CG	364 (87)	352 (87)	1.01 (0.67–1.51)
		GG	55 (13)	54 (13)	

CHD: coronary heart disease, CI: confidence interval, OR: odds ratio; *Data are N (%); [ɪ]*P*-value less than 0.05.

Discussion

The present study investigates the association of two SNPs in different loci on 9p21 *(CDKN2B-AS1)* and 6p24 *(PHACTR1)* with susceptibility to CHD for the first time in an Iranian population. A strong association was identified between the presence of risk allele of rs7865618 and CHD in dominant model. One of the most common manifestations of atherosclerosis is coronary heart disease, a chronic inflammatory disease that has been regarded as the major cause of morbidity and mortality in many countries, including Iran.[16] The prevalence of CHD and coronary risk factors in Iran has been increasing steadily compared to Western countries.[5] A review of previous studies shows that in some cases, there is an association between genetic risk of CHD at *CDKN2B-AS1* with (rs7865618) as well as an association between *PHACTR1* with (rs12526453). The 9p21.3 region was the first genetic risk variant of CHD discovered by GWAS. Different studies have illustrated that this region is linked to platelet reactivity and high platelet reactivity might clarify the association between it and CHD. Other studies have shown how SNPs in the 9p21.3 region affects inflammatory signaling and vascular cell proliferation. Since *CDKN2B-AS1* gene is located in the 9p21.3, it can be hypothesized that SNPs in this gene may influence susceptibility to CHD. However, the molecular mechanisms of predisposition to CHD need further definitive evidence.[17]

The *PHACTR1* gene that is a molecule expressed in different parts of body like brain, lung, testis, and heart has been recently associated with increased risk of CHD through the SNP rs12526453 which is located in the 6p24.1 locus. It was identified that rs12526453 CC homozygotes are associated with increased risk of CHD. This could be attributed to inflammatory response, cell mediated immune response, and cellular development. In spite of the several suggested hypotheses for the mechanisms of the association between *PHACTR1* polymorphism and CHD, the underlying mechanisms remain unclear.[18]

However, according to our findings, there was no significant relationship between the presence of risk alleles of rs12526453 polymorphism and prevalence of CHD in the TLGS population. This lack of association indicates that the effects may be mediated through undiscovered pathways. Our findings support the results of recent studies which report a significant association between *CDKN2B-AS1 (ANRIL)* gene rs7865618 polymorphism and CAD/MI in some populations (Table 5).[1,9,10,12,19,20] Table 5 provides an overview of relevant studies related to two studied polymorphisms. According to this table, there is ample evidence supporting the association between specific SNPs at *CDKN2B-AS1* and *PHACTR1* genes and coronary heart disease. These results show that the incidence rates of CHD increased significantly with presence of A allele in rs7865618.

To conclude, the results demonstrated by this study indicate an

Table 5. SNPs associated with CAD/MI in the observed association with heart disease in discovery studies and fine mapping work

SNP	Study	Disease	Case Number	Control Number	OR (95% CI)	*P*-Value
rs7865618	Samani, et al. (2007)	CAD/MI	1926	2938	1.26 (1.18–1.35)	1.9×10^{-11}
	Koch, et al. (2011)	MI	3657	1211	1.34 (1.22–1.47)	9.5×10^{-10}
	Xie, et al. (2011)	CAD	2335	1078	0.85 (0.72–1.00)	14×10^{-2}
rs12526453	MIGen (2009)	MI	2967	3075	1.10 (1.06–1.13)	6.5×10^{-10}
	Qi, et al. (2011)	CAD/DM	1076	1430	1.25 (1.10– 1.41)	2×10^{-4}
	Hager, et al. (2012)	CAD	1210	792	1.15 (1.05– 1.22)	8.9×10^{-4}

MI: myocardial infarction; CAD: coronary artery disease; DM: diabetes mellitus; CI: confidence interval; OR: odds ratio.

association between *CDKN2BAS1* gene rs7865618 polymorphism and CHD in the studied population from Iran. However, further studies are recommended to elucidate the role of other polymorphisms near the *CDKN2B-AS1* gene in the pathogenesis of the CHD. Functional studies should be considered to clarify to what degree the *CDKN2B-AS1* gene is involved in occurrence of coronary heart disease. The results of this study can be generalized by considering a database with larger sample sizes.

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