Advances in Bioresearch
Adv. Biores., Vol 6 (1) January 2015:96-101
©2015 Society of Education, India
Print ISSN 0976-4585; Online ISSN 2277-1573
Journal's URL:http://www.soeagra.com/abr.html
CODEN: ABRDC3
ICV 7.20 [Poland]

Advances in Bioresearch

ORIGINAL ARTICLE

Investigation Common Polymorphism 6p24 Loci in Coronary Artery Disease (CAD) Patients Compared with Controls in Iranian Population

Laleh Heidari[1] , Sayyed Mohammad Hossein Ghaderian[2] , Haleh Akhavan Niyaki[1] , Seyed Alireza Salami[3] , Maryam Mafi Golchin[1] , Rozita Jalaliyan[4]

1. Department of Medical Genetics, Babol University of Medical Sciences, Babol, Iran
2. Department of Medical Genetics, Shahid Beheshti University of Medical Sciences, Tehran, Iran
3. Department of Biotechnology, University of Tehran, Iran
4. Department of Medical Sciences, Babol University of Medical Sciences, Babol, Iran

ABSTRACT

Coronary Artery Disease (CAD) is a Multifactorial and Heterogenic disease. During this disease atherosclerosis plaques have been formed in internal wall of coronary artery. This event results to limitation of blood reaching to myocardium .Reduction of blood flow may lead to appearance of some problems such as Ischemia, sudden thrombosis veins and Myocardial Infarction (MI). Several environmental and genetic factors involved in the appearance of this disease that the most important of them Include Hypertension, age, mellitus diabetes, family history of early-onset heart disease and smoking. We collected blood samples from 100 CAD patient and 100 healthy persons as control from Mazandaran province .In this study, association of rs12526453 and rs13191496 polymorphism in PHACTR1 gene with CAD was evaluated by TaqMan Probe Real Time PCR technique .The rs12526453 polymorphism shows significantly associated with CAD but there isn't direct association between rs13191496 variant with risk of coronary artery disease in Iranian population.

Key words*: Coronary Artery- atherosclerosis plaques- polymorphism-PHACTR1 gene*

Abbreviations and Acronyms*: CAD = coronary artery disease; MI = myocardial infarction; SNP = single nucleotide polymorphism; BMI = body mass index; HDL=High-density lipoprotein; LDL = low-density lipoprotein; SBP= Systolic _Blood _Pressure; DBP= Diastolic _Blood _Pressure*

Received 01/10/2014 Accepted 23/12/2014 ©2015 Society of Education, India

How to cite this article:
Laleh H , Sayyed M H G , Haleh A N , Seyed A S , Maryam M G , Rozita J. Investigation Common Polymorphism 6p24 Loci in Coronary Artery Disease (CAD) Patients Compared with Controls in Iranian Population. Adv. Biores. Vol 6 [1] January 2015: 96-101**.** DOI: 10.15515/abr.0976-4585.6.1.96101

INTRODUCTION

Coronary artery disease (CAD) is one of the most important causing agents of death worldwide [1]. CAD is a complex and multifactorial disease which is influenced by several environmental and genetics background such as lifestyle, age, male sex, diabetes mellitus, hypercholesterolemia, smoking and family history [2, 3, 4].

Different approaches like candidate gene approach, genome-wide linkage analysis, functionality analysis approaches and genotyping by chips in a genome-wide association (GWA) platform had been used to determine the genetics of CAD, however, the reproducibility and the power of these strategies was different at all [5].

A well-known genetic polymorphism which was thought to be associated with myocardial infarction or coronary artery disease was found in the gene encoding angiotensin-converting enzyme (ACE), [6]. They found out that there is an association between higher levels of circulating ACE in patients with MI [6]. Then after, several genetic polymorphisms explored which are thought to be associated with CAD, e.g. 17q23A, 17q21.32, 5q23-31, 4q28, 8p12-p11.2, 1p36.3, 17q11.2-q21.1 etc. However, with several reasons such as poor reproducibility and weakness in discovering de novo variants, the candidate gene approach resulted in only limited success in the elucidation of genetic risks for CAD [5].

The same, genome-wide linkage analysis had some limitations. Although, this approach is thought to be able to detect the novel genetic determinants, however, the reproducibility of associations was always questionable. On the other hand, there are just a few loci (2q21-22, Xq23-26, MEF2A and LTA4H) which were associated with CAD [7,8, 9]. Furthermore, using such a strategy to detect a multifactorial disease like CAD seems to face some limitations.

During the last decade whole genome analysis (WGA) and genome-wide association (GWA) helped researchers to overcome the limitations of candidate gene approach and genome-wide linkage analysis. Data mining and data surfing in single nucleotide polymorphism (SNP) databases and performing high-throughput genotyping using Chip-based genome association studies allowed scientists to find genetic variants related to CAD [10,11]. These strategies take the advantages of bias-free associations between SNPs and the diseases and therefore, make the possibility of obtaining novel and unbiased information to better understand the pathophysiology of the CAD.

Several loci were found to be associated with CAD and MI (11,12,13,14,15,16), including SNPs on chromosomes 1p13.3, 1q41, 10q11.21, 9p21, 3q22.3, 6p24, 2q33, 21q22 and 6q26-27, which chromosome locus 9p21.3 being the most often replicated one. Numerous SNPs associated with coronary heart disease risk have been identified in this region. Also, all three chip-based GWA studies have been conducted proved the significant association between CAD and SNPs on chromosome 9p21. However, 9p21.3 explains only about 2.9% of the genetic variability of the disease [17].

Most of the these findings were identified and confirmed in the European ancestry populations, Yoruba people in Nigeria and some Asian populations as International HapMap Project [18,19,20, 21], while a comparatively no comprehensive study on genetic loci associated with CAD have not been reported in Iranian population. Whereas, according to the published data, CAD and MI diseases cause 38-40 percent of death in Iran.

Consequently, we designed the current study in which we aimed to use novel genetics tools to assess risk factors involved in CAD particularly genetics. Two SNPs included rs13191496, rs12526453 in PHACTR1 (protein phosphatase and actin regulator 1) gene were designed to be further investigated in an Iranian population. This candidate gene was selected by reviewing literatures, in which they were reported to be involved in CAD and MI.

Chr6p24.1 is the second most often identified GWAS hit for CHD and MI. The locus was found in European, Asian, and Middle Eastern populations and therefore appears to be relevant across ethnicities. For PHACTR1, a role in cell migration, motility and invasiveness of breast cancer, and melanoma tumor cells was described [22]. Moreover, PHACTR1 is expressed in endothelial cells and involved in regulation of endothelial tubulogenesis and apoptosis. In summary, even though PHACTR1 is an obvious candidate gene at Chr6p24.1, current data on its function is scarce and its mechanism in atherogenesis is still unclear [22].

PHACTR-1 is likely to be a key regulator of endothelial cell function properties. Because of its central role in the control of tube formation and endothelial cell survival [23].

Identification of variants that affect the risk of CAD will improve our understanding of how environmental and genetic factors interplay together to lead to CAD and therefore help us to define the right protection and cure programs at the proper time.

MATERIALS AND METHODS

In current study, we carried out a wide association analysis for CAD in an Iranian population. The study was designed according to WHO indices. Totally, 103 patients and 103 healthy non-CAD individuals were selected. All samples were collected from hospitals North Iran during 2013-2014 and they were randomly selected and assigned to discovery and replication panels.

All CAD patients,(43% males and 57% females, mean age 60.12± 10.02 years), underwent diagnosis of coronary angiography; CAD was defined as ≥50% stenosis in one or more major coronary artery, medical records of percutaneous coronary angioplasty. All control participants, males and females older than 40 years old, showed no signs of CAD, and no signs of risk factors such as hypertension, diabetes mellitus, body fat and body mass index (BMI), cholesterol, drinking and smoking habit, or dyslipidemia. Hypertension was defined as a clinical blood pressure of ≥140/90 mmHg or history of medication. Diabetes was diagnosed by a FBS level of 126mg/dl. Relevant data were also collected from all the participants by direct interviews or from medical-case files including age, gender, lipid profiles, history of hypertension, type 2 Diabetes and dyslipidemia. Blood pressure was measured using a standard mercury sphygmomanometer on the left arm after 5 min rest in the sit position. Fasting blood sugar (FBS), total cholesterol (TC), total triglyceride (TG), high density lipoprotein (HDL), low density lipoprotein (LDL) , etc. were analyzed by standard techniques according to WHO.

SNP selections, primer/probe design and DNA extraction

The candidate genes were selected by reviewing literatures, in which they were reported to be involved in CAD and MI. Number of 2 SNPs included rs13191496, rs12526453, were designed as Human TaqMan® Pre-designed SNP Genotyping Assay to be further investigated in an Iranian population.

Blood samples (5 mL) were drawn from study participants and genomic DNA was isolated using QIAamp DNA Micrro Kit (Qiagen Co.) according to the manufacturers. Quantity and quality of extracted genomic DNAs were tested using Nano Drop ND-1000 (Thermo Co. USA) and on 1% agarose gel electrophoresis.

End-point genotyping using TaqMan® Real-Time PCR

Genotyping of 103 patients and 103 controls was performed in a Real-Time PCR format using Pre-designed SNP Genotyping TaqMan probes and TaqMan Genotyping Master Mix (ABI Inc., USA). All the samples were tested in duplicate.

Statistical Analysis

SNPs were analyzed using SNP Analyzer software. SNPs genotypes were tested for Hardy-Weinberg equilibrium among patients and controls using χ^2 test (SPSS, version 18.0). Multivariate analysis was performed by incorporating age, sex, and hypertension covariates by using multivariate logistic regression (SAS, version 8.1). P values less than 0.05 were considered statistically significant.

RESULTS

The demographic characteristics of the patients and age and gender-matched controls are summarized in Table 1. It was shown that there were significant associations between these factors such as HDL, LDL , Total cholesterol , systolic and diastolic blood pressure, triglycerides ,FBS , Smoking (P<0.001), except for age , sex and BMI (P>0.05).

Table 1. Characteristics of Patients and Control Subjects (Mean ± SD)

Characteristic	CAD (N=103)	Control (N=103)	P-Value
Sex (Male/Female)	43/60	44/59	0.140
Smoking (Yes/No)	45/58	8/95	<0.001
Age, (year)	59.09±8.78	61.15±11.07	0.888
BMI, (Kg/m2)	27.32 ± 6.61	26.79±4.58	0.504
Total cholesterol, (mg/dl)	171.19±32.24	192.72 ± 27.37	<0.001
HDL cholesterol,(mg/dl)	38.94±7.47	41.80 ± 5.83	<0.001
LDL cholesterol, (mg/dl)	101.02± 23.49	115.10 ±23.26	<0.001
Triglyceride,(mg/dl)	152.57±67.55	181.10 ±53.73	<0.001
FBS,(mg/dl)	140.35±64.49	122.20 ±39.62	<0.001
DBP ,(mm Hg)	83.77±12.83	72.91 ±12.72	<0.001
SBP,(mm Hg)	137.25±26.58	122.52 ±18.82	<0.001

Table 2 shows the prevalence distribution of genotypes and alleles in CAD and control groups in two polymorphisms of PHACTR1 gene. The genotypic frequencies of CC, CG, GG in rs12526453 polymorphism of CAD group were 50%, 49% and 1% and the control group were 69.9%, 30.1% and 0%, respectively (P <0.05). The homozygous GG and the heterozygous CG were frequent in CAD group and there was no homozygous GG in the control group. In addition, CG + GG genotypes were 49.5% in CAD and were 30.1 in % the control group, so that they dramatically increase the risk of CAD (OR = 0.44 (95% CI = 0.25-0.78, P = 0.004).

Table 2. Comparison of genotype and allele frequency of PHACTR1 gene polymorphism between control group and CAD patients.

Chr6P24.1 (PHACTR1)			CAD patients	Controls	OR	(%95 CI)	P value
rs13191496	**Genotype**	AG	18(%17)	18(%17)	Ref.	-	**-**
		GG	85(%83)	85(%83)	1.00	(0.49-2.05)	**1.00**
	Allele	A	18(%9)	18(%9)	.Ref	-	**-**
		G	188(%91)	188(%91)	1.00	(0.54-1.98)	**0.98**
rs12526453	**Genotype**	CC	52(%50.5)	72(%69.9)	Ref.	-	**-**
		CG	50(%48.5)	31(%30.1)	0.45	(0.25-0.79)	**0.01***
		CG+GG	51(%49.5)	31(%30.1)	0.44	(0.25-0.78)	**0.004***
	Allele	C	154(%75)	175(%85)	Ref.	-	**-**
		G	52(%25)	31(%15)	1.90	(1.16-3.12)	**0.01***

Abbreviations: OR, Odds ratio; CI, Confidence interval ; *P<0.05 is significant

The C allele frequency was also significantly different between patients with CAD and controls: 25% versus 15%, OR = 1.90 (95% CI = 1.16–3.12, P < 0.05).However, the rs13191496 polymorphism had similar AG and GG genotype frequencies in CAD and control groups equal to 17% and 83%, respectively, and AA genotype was not observed among the studied participants and the G-allele frequency showed no difference between CAD and control groups (P> 0.05).
The genotypes in rs12526453 polymorphism (χ2 = 0.052, P = 0.819) but not in rs13191496 polymorphism were in HEW (χ2 = 17.78, P < 0.001).
Demonstrated in Table 3, there was no significant difference in the haplotype frequencies between patient group and control group (P>0.05).

Table 3. Comparison of haplotype frequency of PHACTR1 gene polymorphisms between control group and patient group.

rs12526453	rs13191496	Haplotype	CAD (N=103)	Control (N=103)	OR (%95 CI)	P-Value
C	**G**	**CG**	%70	%76	1.00	-
G	**G**	**GG**	%20	%14	055(0.29-1.04)	0.06
C	**A**	**CA**	%3	%8	1.89(0.67-5.33)	0.23
G	**A**	**GA**	%4	%0.3	0.06(0.01-80.5)	0.44

DISCUSSION

Cardiovascular diseases occur by sudden onset and one of the major health problems in the developing countries, i.e. Iran. The category of the diseases has 39% prevalence as the first cause of mortality in Iran [24]. The new treatment methods for the cardiovascular diseases have developed and improved, yet its prevalence is increasing [24]. In this study, various factors have been studied, e.g. paraclinical factors related to CAD and rs12526453/rs13191496 polymorphisms in PHACTR1 gene.
In another study by Hatami et al. (2007), the prevalence of CAD risk factors in the Iranian population was assessed. In the present study, 3000 healthy adult subjects aged over 18 are randomly selected in Tehran for the clinical and laboratory tests, e.g. FBS, total cholesterol, LDL-c, HDL-c and triglycerides. In the present study, it is observed that the prevalence of many risk factors, e.g. total cholesterol, physical inactivity and LDL, are most observed in the Iranian population and afterward triglycerides, smoking, family history of heart disease among first degree relatives, systolic blood pressure, diastolic blood pressure, diabetes, and HDL are observed, respectively [25]. In the present study, there is a significant

relationship between the CAD patients and the risk factors, e.g. systolic blood pressure, diastolic blood pressure, smoking, total cholesterol, triglycerides, LDL, HDL, and diabetes (P <0.001) (Table 1).
The results of the polymorphism genotype frequencies (rs12526453) for CC, CG and GG are 50.5%, 48.5% and 1% in patients with CAD and 69.9%, 30.1% and 0% in the control group, respectively. CG + GG genotype frequencies in patients with CAD were significantly more than the control group (51% vs. 31%, P = 0.004), as G-allele carriers had higher risk for developing CAD than CC homozygous carriers after the age, gender, smoking, hypertension, cholesterol matching (Table 2).
Although the prevalence of genotypic polymorphism (rs13191496) for AA, AG and GG were assessed equal to 0%, 17% and 83% in patients with CAD and control group, respectively, and there was no specific difference and AG + GG genotype frequencies were observed 100% in both groups similar to the other Asian population studies in NCBI. AA genotype was studied in the Iranian population. In order to validate our findings, we examined the haplotype genetic frequencies related to SNPs (Table 3). This analysis shows that there is no significant difference between both patient and control groups (P> 0.05).
Another GWA study in 2009 was conducted for the first time on 2967 patients and 3075 control participants by using SNP detection method via Affymetrix Gene Chip with two loci, including 6p24 locus in PHACTR1 gene and 22q21 locus of MRPS6 gene; in the continued association studies, these loci and their association with cardiovascular disease were examined, as there was a significant relationship between rs12526453 and the disease (CAD OR = 1.13 (95% CI = 1.13-1.18, P = 0.007)(26). The present study showed there is a relationship between rs12526453 and the disease CAD (OR = 0.44 ; 95% CI = 0.78-0.25; P = 0.004).
Trégouët, et al. (2009), studied rs13191496 polymorphism on 1,926 patients and 2,938 controlled participants, showed that this SNP is not associated with CAD patients (P = 3.63)(15). This study is consistent with the results of the present study and there is no relationship between rs13191496 and CAD patients (P> 0.05) (Table 2). The relevant past literature compared the relationship of both polymorphisms in the different years (Table 4).
In summary, in spite of small sample size, we found out a relationship between rs12526453 polymorphism of PHACTR1 gene and CAD in Iranian population. Our results revealed that presence of G nucleotide in position 561 of E-selectin gen may be a genetic risk factor for CAD. In regard of the high prevalence of cardiovascular diseases is determined, particularly CAD patients' genetic susceptibility, as one of the most effective approaches to prevent and treat them.

Table 4. P-value compared to previous studies and the current study in relation to SNPs

SNPs	Study	Association With CAD	Odd ratio (95% CI)	P-value	Reference
rs12526453	Genome-wide association(2009)	Yes	1.13 (1.09-1.17)	0.007	**26**
	Carla Lluís-Ganella (2010)	Yes	1.13 (1.04-1.24)	0.004	**27**
	Schunkert et al, (2011)	Yes	1.12 (1.08-1.17)	0.001	**28**
	Genome-wide association(2013)	Yes	1.10	0.001	**29**
	Current study (2014)	Yes	0.44 -0.25) (0.78	0.0043	*
rs13191496	Tregouet et al, (2009)	NO	-	3.63	**15**
	Current study (2014)	NO	-	1.00	*

ACKNOWLEDGEMENTS
This study was approved and supported by Babol University of Medical Sciences.

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