Int Cardiovasc Res J.2020;**14**(2):59-64.icrj.100140

International Cardiovascular Research Journal
www.ircrj.com

Investigation of PCSK9 Gene Polymorphisms in Two Iranian Ethnic Groups with Coronary Artery Disease in Fars Province

Mohammad Javad Zibaeenezhad [1], Majid Yavarian [1,*], Mohsen Fathzadeh [1], Mohammad Heydari Kamrodi [1], Mostafa Fattahi Mofrad [1], Hajar Khazraei [2], Zahra Daneshvar [1]

[1] Cardiovascular Research Center, Shiraz University of Medical Sciences, Shiraz, IR Iran
[2] Colorectal Research Center, Shiraz University of Medical Sciences, Shiraz, IR Iran

ARTICLE INFO

Article Type:
Research Article

Article History:
Received: 13 Dec 2019
Revised: 12 May 2020
Accepted: 7 Jun 2020

Keywords:
Coronary Artery Disease
PCSK9
Polymorphism

ABSTRACT

Background: Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9), a serine protease, plays an important role in the regulation of cholesterol metabolism. PCSK9 interacts with Low Density Lipoprotein Receptor (LDLR) on the surface of hepatocytes, promoting its lysosomal degradation, thus leading to the accumulation of cholesterol outside the cells followed by hypercholesterolemia.

Objective: This study aimed to assess three polymorphisms (rs662145, rs505151, and rs562556) of the PCSK9 gene in two Iranian ethnic groups (Turk and Lur) with Coronary Artery Disease (CAD) in Fars province.

Methods: In this cross-sectional study, 114 Turk and 73 Lur patients with CAD were selected based on the clinical examination by a cardiologist. The three polymorphisms were assessed by Real-Time Polymerase Chain Reaction (PCR) method using specific primers and probes. Chi-square test was used to compare the two groups regarding the qualitative variables. All analyses were performed using the SPSS 18 software and $P < 0.05$ was considered to be statistically significant.

Results: The results revealed no significant difference between the Turk and Lur groups regarding the three polymorphisms ($P > 0.05$). In addition, no significant relationship was observed between the rs662145 polymorphism and clinical characteristics like family history of CAD, diabetes, Myocardial Infarction (MI), Body Mass Index (BMI), and High Density Lipoprotein (HDL) level ($P > 0.05$). However, a significant relationship was found between the rs505151 polymorphism and MI ($P = 0.03$) and Low Density Lipoprotein (LDL) level ($P = 0.04$) in Turk patients. Nonetheless, no significant correlation was detected between the rs505151 polymorphism and clinical parameters ($P > 0.05$). The results also showed no significant relationship between the rs505151 polymorphism and clinical parameters in Lur patients ($P > 0.05$). Analysis of the rs562556 polymorphism also revealed no significant relationship between this polymorphism and clinical characteristics in both Turk and Lur samples ($P > 0.05$).

Conclusion: rs662145 polymorphism was associated with LDL and Triglyceride (TG) levels in both Turk and Lur samples. Besides, rs505151 polymorphism was correlated to LDL level and MI in Turk patients. Therefore, in addition to other predisposing genes to CAD, analysis of PCSK9 mutations can be used for predicting hypercholesterolemia and premature CAD.

1. Background

Coronary Artery Disease (CAD, also known as atherosclerotic heart disease) is a prevalent type of cardiac disease and the main cause of heart attacks mediated by plaque formation along the inner walls of the heart vessels (1). Various studies have demonstrated the role of genetic factors in CAD. Evaluation of sequence changes in genes that increase the risk of diseases has the potential to clarify the biological pathways involved in diseases, resulting in further improvement in their diagnosis and treatment (2).

Genome wide linkage study in families as well as in various populations has demonstrated more than 12 genetic loci linked to the disease (2). PCSK9, previously

*Corresponding author: Majid Yavarian, Cardiovascular Research Center, Mohammad Rasoul-allah Research Tower, Mollasadra Street, Shiraz, Iran, Tel: +98-7136122238, E-mail: majid.yavarian@gmail.com.

known as NARC-1, is a serine protease highly expressed in hepatocytes and the small intestine and is synthesized in the kidney, skin, and brain in minor amounts (3). This protein is firstly expressed as a proprotein (72kDa) in the endoplasmic reticulum and becomes active to 63kDa after autolytic cleavage in FAQ152↓SIP position. The final protein consists of a signal sequence (1 - 30 amino acids), prodomain (31 - 152 amino acids), catalytic domain (153 - 425 amino acids), and carboxy-terminal domain (425 - 692 amino acids). The important role of PCSK9 in the regulation of cholesterol level is mediated by the Low Density Lipoprotein (LDL) receptor. Regulation of cholesterol metabolism and hypercholesterolemia occurs by the LDL receptor and lysosomal degradation (4). In humans, the PCSK9 gene is located on the 1p32.3 chromosome and consists of 12 exons (3, 4). In 2003, mutation in the PCSK9 gene and its relationship with Alcohol Dehydrogenase (ADH) expression was detected in two French families for the first time (5). To date, more than 50 types of various mutations in the PCSK9 gene have been identified in humans, which modulate the plasmacholesterol level. These mutations are in accordance with increased activity (gain of function) and decreased activity (loss of function) in the PCSK9 gene, which increases the plasma cholesterol level and decreases the risk of cardiovascular disease, respectively (6-11). The rs662145 polymorphism is located on 1:55064155 in the PCSK9 gene and the Minor Allele Frequencies (MAFs) are C = 0.09434/20 (Vietnamese), C = 0.188333/133 (Northern Sweden), C = 0.240561/892 (TWINSUK), C = 0.250649/966 (ALSPAC), C = 0.258929/1160 (Estonian), C = 0.275759/1381 (1000Genoms), C = 0.321515/40372 (TOPMED), and C = 0.322503/10095 (GnomAD). The rs662145 polymorphism in PCSK9 is associated with increased Triglyceride (TG) levels, leading to hypertriglyceridemia (12). The rs562556 polymorphism is located on 1:55058564 in the PCSK9 gene and the MAFs are G = 0.001623/1 (Vietnamese), G = 0.13099/656 (1000Genoms), G = 0.136667/82 (Northern Sweden), G = 0.143326/36006 (GnomAD_exomes), G = 0.145565/17657 (ExAC), G = 0.14861/11687 (PAGE_STUDY), G = 0.161161/722 (Estonian), G = 0.164755/20688 (TOPMED), G = 0.168818/5283 (GnomAD), G = 0.178803/663 (TWINSUK), G = 0.182224/2370 (GoESP), and G = 193046/744 (ALSPAC). The results of a meta-analysis indicated a relationship between rs562556 and lower serum cholesterol levels (13). The rs505151 polymorphism is located on 1:55063514 in the PCSK9 gene and the MAFs are G = 0.032093/119 (TWINSUC), G = 0.032953/127 (ALSPAC), G = 0.041667/25 (Northern Sweden), G = 0.052288/32 (Vietnames), G = 0.052302/13098 (GnomAD_exomes), G = 0.056716/6849 (ExAC), G = 0.059598/267 (Estonian), G = 0.101038/506 (1000Genomes), G = 0.105978/3322 (GnomAD), G = 0.106643/13391 (TOPMED), and G = 0.110564/1438 (GoESP). A study showed that the females carrying the rs505151 polymorphism exhibited higher total cholesterol, TG, High Density Lipoprotein (HDL), and LDL levels compared to the male group carrying the same genotype ($P < 0.05$) (14).

Based on what was mentioned above, the PCSK9 gene can be mentioned as one of the important modulators of plasma cholesterol level. Considering the effect of this gene on the balance of the risk of coronary atherosclerosis disease, it is necessary to study different genotype sequences among healthy controls and CAD patients. Furthermore, mutations in this gene make it more active or deactivate.

2. Objectives

The present study aims to investigate rs562556, rs505151, and rs662145 polymorphisms in CAD among Turk and Lur groups in Fars province.

3. Patients and Methods

This cross-sectional study was conducted on 114 Turk and 73 Lur patients with the clinical presentation of CAD selected by clinical cardiologists in Shiraz during 2015 to 2017. The CAD patients who suffered from inflammatory disorders or other chronic comorbidities than cardiovascular disorders were excluded from the study. At first, written informed consent forms were signed by all patients. Then, blood samples were obtained in EDTA-treated tubes. Genomic DNA of the white blood cells was extracted by the salting out method and proteinase K using standard methods (15). Additionally, the selected Single Nucleotide Polymorphisms (SNPs) in the PCSK9 gene; i.e., rs662145, rs505151, and rs562556, were evaluated by Real-time Polymerase Chain Reaction (PCR) method. Genetic variations were also analyzed by the LC-Green (Idaho Technology, Salt Lake City, Utah, USA) High-Resolution Melting (HRM) curve method following PCR amplification (Qiagen company). PCR cycling conditions included an initial activation step at 95 °C for 5 min followed by 40 cycles of denaturation at 95 °C for 10 s and an annealing/extension step at 55 °C for 30 s to allow for fluorescence data acquisition on the green channel. A 192 bp PCR product was accomplished by HRM analysis in the range of 65-95 °C and 0.1 °C rising at each cycle. All tests were run with the Null Template Control (NTC) and control of the known genotype on each course. A Sanger sequence confirmed sample was used as the control in HRM Real-Time PCR and 20 samples (case and control) were randomly selected and checked by sequencing for re-conformation. To assure the results, 10% of the samples were randomly selected and re-checked by direct sequencing.

After all, the data were entered into the SPSS statistical software, version 18 (SPSS Inc., Chicago, USA). Chi-square test was used to compare the two groups regarding the qualitative variables. $P < 0.05$ was considered to be statistically significant.

4. Results

The patients (114 Turk and 73 Lur) were evaluated and the genotypes of all three SNPs have been presented (Table 1). The mean age of the patients was 61.54 ± 11.16 years. Additionally, their mean age was 62.6 ± 10.87 years in the Turk group and 59.86 ± 11.46 years in the Lur group ($P = 0.100$). Moreover, 59.8% of the Turk patients and 65.8% of the Lur ones were male. The results revealed no significant difference between the two groups concerning sex ($P = 0.420$).

Despite the increase in the frequency of the CC genotype of rs662145 in Turk patients, the difference between the two groups was not statistically significant ($P = 0.14$).

Table 1. Comparison of the Two Groups regarding Allelic Distribution and Frequency of the Three SNPs

SNPs	Alleles and Genotypes	Turk, N (%)	Lur, N (%)	Total	P-value
rs662145	T	120 (53%)	87 (60%)	207	0.31
	C	108 (47%)	59 (40%)	167	
	TT	35 (31%)	24 (33%)	59	0.14
	TC	50 (44%)	39 (53%)	89	
	CC	29 (25%)	10 (14%)	39	
rs505151	A	203 (89%)	127 (87%)	330	0.66
	G	25 (11%)	19 (13%)	44	
	AA	94 (82%)	59 (81%)	153	0.76
	AG	15 (13%)	9 (12%)	24	
	GG	5 (5%)	5 (7%)	10	
rs562556	A	208 (91%)	136 (93%)	344	0.6
	G	20 (9%)	10 (7%)	30	
	AA	96 (84%)	63 (86%)	159	0.52
	AG	16 (14%)	10 (14%)	26	
	GG	2 (2%)	0 (0%)	2	

Table 2. Evaluation of the Relationship between the rs662145 Polymorphism and LDL Level, TG Level, and Blood Pressure in Turk CAD Patients

Parameters	Genotype	Normal	Higher than Normal	P-value
LDL	TT	15 (22%)	20 (44%)	0.01*
	TC	36 (54%)	13 (29%)	
	CC	16 (24%)	12 (27%)	
TG	TT	22 (29%)	13 (34%)	0.003*
	TC	41 (54%)	9 (24%)	
	CC	13 (17%)	16 (42%)	
Blood pressure	TT	14 (26%)	21 (34%)	0.03*
	TC	30 (57%)	20 (33%)	
	CC	9 (17%)	20 (33%)	

Abbreviations: LDL, low density lipoprotein; TG, triglyceride

Table 3. Evaluation of the Relationship between the rs505151 Polymorphism and LDL Level and MI in Turk CAD Patients

Parameters	Genotype	Normal	Higher than Normal	P-value
LDL	AA	51 (76%)	41 (91%)	0.04*
	AG + GG	16 (24%)	4 (9%)	
MI	AA	57 (76%)	36 (91%)	0.03*
	AG + GG	7 (24%)	13 (9%)	

Abbreviations: LDL, low density lipoprotein; MI, myocardial infarction

Table 4. Evaluation of the Relationship between the rs662145 Polymorphism and LDL and TG Levels in Lur CAD Patients

Parameters	Genotype	Normal	Higher than normal	P-value
LDL	TT	4 (9%)	20 (64%)	< 0.001*
	TC	30 (73%)	8 (26%)	
	CC	7 (18%)	3 (10%)	
TG	TT	5 (14%)	19 (50%)	0.002*
	TC	22 (63%)	17 (45%)	
	CC	8 (23%)	2 (5%)	

Abbreviations: LDL, low density lipoprotein; TG, triglyceride

Similarly, no significant difference was observed between the two groups regarding the frequency of the rs662145 polymorphism (P = 0.31). The frequency of the rs505151 polymorphism in AA, AG, and GG genotypes has been presented in Table 1. There was no significance difference between the genotypes and alleles in the rs505151 polymorphism. However, there was a significant relationship between the rs662145 polymorphism and blood pressure (P = 0.03), LDL level (P = 0.01), and TG level (P = 0.003) in Turk patients. As for the Lur patients, there was a significant relationship between the rs662145 polymorphism and LDL (P < 0.001) and TG (P = 0.002) levels.

The results showed a significant relationship between the rs505151 polymorphism and LDL level (P = 0.04) and MI occurrence in Turk patients (P = 0.03), but not in Lur patients. However, no significant correlation was found between the rs562556 polymorphism and clinical characteristics among Turk and Lur patients. The results of the relationships between the rs662145 and rs505151polymorphisms and TG level, blood pressure, LDL level, and MI in Lur and Turk CAD patients have been presented in Tables 2 - 4.

5. Discussion

This study aimed to compare two Iranian ethnic groups with CAD in Fars province with respect to the PCSK9 SNPs, including rs662145 (c.571 T > C), rs505151 (c.2009 A > G or E670G), and rs562556 (c.1420 A > G or V474I). The relationship between the polymorphisms and the clinical characteristics was also determined in the two groups. The results revealed that the rs662145 polymorphism in the PCSK9 gene had a direct relationship with LDL and TG plasma levels in both Turk and Lur patients. There are few studies on the relationship between the rs662145 polymorphism and clinical properties like LDL or TG level in cardiac patients. Chen et al. reported a significant increase in LDL cholesterol level, but no increase in TG level among the atherosclerotic patients with CC genotype (16). In addition, Postmus et al. showed no significant relationship between the rs662145 polymorphism and plasma LDL level (17). However, the present study findings demonstrated a significant relationship between the TT genotype and LDL and TG plasma levels in Turk and Lur cardiac patients. Accordingly, patients with the TT genotype (natural or WT) had higher LDL and TG plasma levels in comparison to those with TC or CC (mutant) genotypes.

rs662145 polymorphism in the 12th exon of the PCSK9 gene in the C-terminal is in the 3′UTR area of the gene and cannot be translated. Different studies have indicated that the 3′UTR area of the genes has an important role in gene expression control in different levels, like transferring to nucleus, adding A sequences (polyadenylation), translating efficacy, and mRNA degradation (18-20). The regulatory structure of gene expression like AU rich sequences (ARE), as protected AUUUA motifs and Iron-Responsive Elements (IRE), and more complication of this region in comparison to the 5′UTR gene area show the importance of the 3′UTR sequence in gene expression (21, 22). Thus, polymorphism in the 3′UTR area of the LDL receptor gene next to the PCSK9 gene has a main role in and a significant relationship with plasma cholesterol level regulation (23, 24). Despite the unknown role of these polymorphisms in PCSK9 protein activity, changes in this area of the PCSK9 gene may act as an important regulatory factor in PCSK9 gene expression similar to the polymorphism in the 3′UTR area of the genes that is related to cholesterol modulation like the LDL receptor. According to the results of the current study, it can be proposed that mRNA stability of the PCSK9 gene may be higher in the patients with the TT genotype in comparison to those with heterozygote and CC genotypes, which results in further degradation of the LDL receptor at the hepatocyte level and causes an increase in plasma LDL level, hypercholesterolemia, and premature coronary vessel diseases. A larger sample of cardiac patients and more studies, especially in other populations, can be useful to identify the importance of this polymorphism in PSCK9 gene activity.

Evaluation of the rs505151 polymorphism and clinical characteristics of Turk cardiac patients showed no significant relationship between this polymorphism and clinical characteristics like family history of cardiovascular disease, diabetes, blood pressure, Body Mass Index (BMI), and HDL level. However, there was a significant relationship between this polymorphism and LDL level and MI occurrence in Turk cardiac patients. On the other hand, no significant relationship was detected in this regard among the Lur cardiac patients. The rs505151 SNP is in the 12th exon of the PCSK9 gene, causing A nucleotide to change into G nucleotide and changing glutamic acid to glycin (E670G). Different studies have investigated the relationship between this polymorphism and LDL level. Some of these studies have indicated a relationship between GG genotype and increase in LDL level. However, Hsu et al. showed a more significant decrease in LDL level among the CAD patients with G allele (mutant) compared to those with other alleles (natural allele) (25). In that study, a non-significant decrease in the LDL level was observed among the CAD patients with G allele in comparison to the control group. In the study by Abboud et al., G allele was introduced as an important predictor for Large-Vessel Atherosclerosis (LVA) (26). Nonetheless, some studies have not shown any relationships between this polymorphism and LDL level. Consistent with the results of the research by Hsu et al., the present study findings demonstrated an increase in the frequency of the AA genotype in the Turk cardiac patients with high plasma LDL levels in comparison to those with normal plasma LDL levels. The results also revealed a significant increase in the frequency of the AA genotype in the Turk group with MI compared with those without MI. However, there was no significant relationship between this polymorphism and LDL level and MI occurrence in the Lur cardiac patients. This might be attributed to the small sample size of the Lur group (n = 73) compared to the Turk group (n = 114).

The difference between the two ethnic groups regarding genetic variations in other genes affecting the cardiovascular expression related to this polymorphism was also evaluated in the present study. Nonetheless, the frequency of AG and GG genotypes in Turk and Lur patients was low, which caused limitation in the statistical analysis. Hence, investigation of this polymorphism in larger samples of Turk and Lur patients can lead to more considerable results about the effect of this polymorphism on LDL and TG levels, hypercholesterolemia, and premature coronary vessel disease.

The current study findings revealed no significant relationship between the rs562556 polymorphism and clinical characteristics like familial history of cardiovascular disease, diabetes, blood pressure, BMI, HDL and LDL levels, TG level, and MI occurrence in Turk and Lur cardiac patients. The rs562556 SNP in the 9th exon of the PCSK9 gene makes nucleotide A change into nucleotide G and isoleucine amino acid change into valine amino acid (I474V). A prior study in Japanese population revealed an increase in LDL cholesterol level in the cardiac patients with II genotype in comparison to heterozygote and VV genotypes (27). However, there was no relationship between this polymorphism and MI occurrence in these patients (27). In another study, I474V polymorphism was found to decrease the plasma levels of PCSK9 and LDL (28). In the same line, the present study results demonstrated that the I474V polymorphism was accompanied with a decrease in plasma LDL level in Turk and Lur patients, but the

difference was not statistically significant.

In conclusion, the study results showed that the rs662145 polymorphism in the PCSK9 gene was related to plasma LDL and TG levels in Turk and Lur cardiac patients. Additionally, the E670G polymorphism had a significant relationship with LDL plasma level in Turk cardiac patients. Therefore, evaluation of the PCSK9 gene polymorphism, in addition to other effective genes, was considered to be a predictive factor for hypercholesterolemia and premature CAD. Studies on the relationship between this polymorphism and cardiovascular properties in different populations with larger sample sizes could be more informative. Furthermore, other polymorphisms in the PCSK9 gene, especially those with decreased activity (LOF) like R46L and those in the PCSK9 gene regulatory area (promoter), and their effects on PCSK9 activity and expression could explain the importance of investigation of the changes in the PCSK9 gene as an important genetic factor for prediction of CAD occurrence in different ethnic groups.

5.1. Ethical Approval

The study was approved by the Ethics Committee of Shiraz University of Medical Sciences (No. 87-S4048).

Acknowledgements

There is no acknowledgment.

Authors' Contribution

MJZ: Conceptualization, data handling, supervision, project administration, and final approval. MY: Conceptualization, data handling, writing and reviewing, and final approval. MF: Data analysis, study validation, writing and reviewing, and final approval. HM: Conceptualization, data handling, writing and reviewing, and final approval. MF: Conceptualization, data handling, writing and reviewing, and final approval. HKh: Data handling, writing and reviewing, and final approval. ZD: Data handling, writing and reviewing, and final approval

Funding/Support

The study was financially supported by Shiraz University of Medical Sciences (grant No. 3048).

Financial Disclosure

The authors have no financial interests related to the material in the manuscript.

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