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Polymorphisms of *LPA* gene, rs1801693 and rs7765781, are not associated with premature myocardial infarction in the Iranian population

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Original Article

Abstract

BACKGROUND: Myocardial infarction (MI) is one of the leading causes of mortality globally. Although it is most prevalent in the elderly, it may occur in young adults (men ≤ 55 years or women ≤ 65 years) as premature MI (PMI). As awareness of genetic risks may lead to effective prevention of PMI, we aim to investigate the association of two susceptible single nucleotide polymorphisms (SNPs) in the *LPA* gene with PMI in the Iranian population, rs1801693 and rs7765781, identified in previous genome-wide association studies (GWAS).

METHODS: A total number of 85 patients with PMI and 85 healthy controls were recruited from December 2015 to March 2016 from Isfahan, Iran. Peripheral blood samples were collected from all individuals. Deoxyribonucleic acid (DNA) was extracted and genotyped at rs1181693 and rs7765781 polymorphisms, using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Results were statistically analyzed to find any possible association of the two polymorphisms with PMI by SPSS software and P-values less than 0.05 were considered to be statistically significant.

RESULTS: Statistical analysis displayed no significant difference between rs1801693 (P = 0.815)/rs7765781 (P = 0.746) alleles in patients with PMI and healthy control subjects.

CONCLUSION: There is no meaningful association between rs1801693/rs7765781 and PMI incidence in the Iranian population.

Keywords: Apolipoproteins; Myocardial Infarction; Single Nucleotide Polymorphism

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Introduction

Cardiovascular diseases (CVDs) are considered as the leading cause of mortality and morbidity worldwide. As World Health Organization (WHO) reports, 31% of the total mortality in 2016 (equal to 17.9 million deaths) were due to CVD, mostly related to developing and low-income countries. As an instance, 43% of Iranian population mortalities in 2016 were due to CVD, which makes Iran one of the leading countries in the CVD death rate.[1] Within the CVD category, coronary artery disease (CAD) or, more specifically, acute myocardial infarction (AMI) is one of the most prevalent causes of mortality.[2] The AMI occurs as a result of coronary bloodstream obstruction, which is mostly due to the presence of atherosclerotic plaques in arteries (atherosclerosis) and a thrombotic event.

North Africa and Middle East (including Iran) have the highest proportion of young adults ≤ 40

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years experiencing AMI, which is defined as premature myocardial infarction (PMI) (men under 55 and women under 65 years old).[3]

Genetic hereditary, dyslipidemia, and high smoking rates can be mentioned as significant risk factors of PMI.[4-6] Investigations demonstrate that PMI is strongly related to a family history of CAD/myocardial infarction (MI). As the number of first- and second-degree families involved with MI increases, the strength of PMI risk increases.[4,7,8] The disease does not fit in a specific inheritance pattern as its multifactorial development. Many genes, loci, and polymorphisms may be related to disease progression in addition to many environmental factors.

PMI causes a heavy burden on societies. Therefore, multiple studies were conducted to find responsible genes for CAD, understand the pathogenesis of atherosclerosis, and design prophylactic strategies. Genome-wide association studies (GWAS) identified multiple single nucleotide polymorphisms (SNPs) in loci and genes associated with CAD and MI, such as the 9p21 genomic locus (which contains the *CDKN2A/2B* gene), *QKI* locus, *CXCL12*, *CELSR2/PSRC1/SORT1*, *PCSK9*, *MIA3*, *PHACTR1*, *LDLR*, *SLC5A3*, *MRPS6*, *KCNE2*, *WDR12,* and *LTA* genes. GWAS also implicated the 6q26-27 locus susceptible to be associated with CAD. This locus includes *SLC22A3*, *LPAL2*, and *LPA* genes.[9-13]

LPA gene encodes a large glycoprotein named apolipoprotein A (apo-A) which, along apolipoprotein B100 (apo B-100), binds covalently to low-density lipoprotein cholesterol (LDL-C)-rich particles and forms Lipoprotein(a) [Lp(a)].[14] This particle's physiological function is uncertain, but it was shown that it had roles in CAD progression by participating in inflammation, foam cell formation, and thrombosis, which all may lead to MI. Lp(a) concentration in blood is affected by polymorphisms within the *LPA* gene, and it is an independent risk factor for CAD.[15-18] *LPA* gene polymorphisms which increase apo-A size, lower plasma Lp(a) concentration and decrease CAD risk, and vice versa.[19]

Two previous studies identified 16 SNPs within the *LPA* gene, associated with CAD. From which, rs10455872 and rs3798220 are the ones showing the strongest correlations with CAD and AMI.[20,21] There are other susceptible variants that have not been investigated in the Iranian population yet, including rs1801693 and rs7765781. Thus, in a case-control study, we aim to find the association between two SNPs in the *LPA* gene, rs1801693 and rs7765781, and PMI in the Iranian population.

Materials and Methods

Study population: The study sample consisted of 85 Iranian patients with PMI and 85 Iranian healthy control subjects selected from Isfahan, Iran. Subjects were recruited during their angiography, myocardial revascularization, or coronary artery bypass grafting (CABG) in the tertiary hospitals of Isfahan, Nour and Chamran. Subjects were evaluated for PMI incidence based on WHO criteria, a cardiac troponin rise accompanied by typical symptoms, pathological Q waves, ST elevation or depression, or coronary intervention in male subjects ≤ 55 years or female subjects ≤ 65 years old.

Control subjects were selected from the individuals in the same age range, displaying no stenosis in their angiography results. The subjects' recruitment started in December 2015 until the following March of 2016.

Data collection: Informed written consent was taken from all subjects and an interview was handled by qualified health professionals using a questionnaire. Demographic data, medical history, smoking habits, and physical activity data were collected. Fasting blood samples were taken from each participant and biochemical laboratory measurements were performed. Measurement of anthropometric parameters was carried out and the body mass index (BMI) was calculated.

Inclusion and exclusion criteria: The inclusion criteria for case patients were the incidence of PMI regardless of their family history. All subjects with a history of severe systemic illnesses, hematologic, neoplastic, renal, liver, or thyroid diseases, and mental retardation, as well as pregnant women and breastfeeding mothers were excluded from the study.

Deoxyribonucleic acid (DNA) extraction: Genomic DNA was extracted from whole peripheral blood by standard salting-out method, from 85 patients with PMI and 85 control subjects. All DNA samples were dissolved in water and stored at -20 °C until use.[22]

Genotyping

Restriction enzyme selection for polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP): Restriction maps of rs1801693 and rs7765781 located regions (NC_000006.12:g.160548597A>G,C;160586464G>C) were determined by Gene Runner (version 6.5) software. From all restriction enzymes having recognition site at rs1801693 and rs7765781 locations, *Nco*I and *Alu*I were selected, respectively.

They were used for allele detection of the mentioned SNPs by PCR-RFLP. Adjacent nucleotides of the desired SNPs, located in *Nco*I and *Alu*I cut sites, were checked in the NCBI SNP database for any other SNP interfering with digestion. Both rs1801693 and rs7765781 had adjacent SNPs located in *Nco*I and *Alu*I cut sites that could interfere with digestion, but they were temporarily ignored as their low frequencies.

Primer design and PCR-RFLP: polymerase chain reaction (PCR) primers were designed and checked by MFE (version 3.0) online software (Table 1). All PCR reactions were carried out by Bio-Rad T100™ thermal cycler, in a total volume of 30 µl, containing 1X Amplicon PCR Master Mix, approximately 50 ng of DNA sample, and 0.2 µM forward and reverse primers. Amplifications were done according to standard PCR protocol: one cycle of pre-denaturation at 95 °C for two minutes followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 30 seconds, extension at 72 °C, and one final extension step at 72 °C for five minutes.

PCR products were digested in a total volume of 20 µl reactions, by *Nco*I for rs1801693 and *Alu*I for rs7765781 allele detection separately. Digestion reactions contained 1X enzyme buffer, 4 U of the related enzyme, and 15 µl of PCR product, incubated at 37 °C for 16 hours, and were kept at -20 °C until the next step.

Digested fragments were electrophoresed in 2% agarose gel, stained by safe DNA stain, and alleles of both SNPs were detected in each DNA sample.

It was assumed that indigestion was due to rs1801693 and rs7765781 variations, but since besides studied SNPs, other SNPs were located that could interfere with digestion and cause false undigested results, and also as rs1801693 had three types of variants (A/C/G), one-third of the samples were randomly selected, amplified with the same primers, and sequenced by Sanger sequencing to check the accuracy of PCR-RFLP results.

Data statistical analysis: Quantitative variables were reported as mean ± standard deviation (SD), and qualitative variables were expressed as percentages (absolute number). Kolmogorov-Smirnov test was used to check the normality assumption. A comparison of the quantitative variables between case and control groups were performed by Student's t-test or Mann-Whitney U test, where applicable. Categorical variables were compared using chi-square test or Fisher's exact test when required. Logistic regression models were used for evaluating the association between gene and occurrence of PMI. We considered two-tailed P-values of less than 0.05 to be statistically significant. Analyses were conducted using SPSS statistical software (version 22.0, IBM Corporation, Armonk, NY, USA).

Results

Characteristics of the study population: Measured characteristics for the study population are listed in table 2. More than half of the participants were aged under 45 years in both study groups, and no significant difference was observed among PMI case and control groups in age, gender, marital status, living region, physical activity, BMI, smoking status, high-density lipoprotein-cholesterol (HDL-C) and LDL-C levels, and waist circumference (WC) ($P > 0.05$).

The number of subjects with metabolic syndrome and dyslipidemia was significantly higher in PMI cases ($P = 0.008$ and $P = 0.028$, respectively). Hypertension (HTN) and diabetes mellitus (DM) cases were significantly higher in PMI cases ($P = 0.042$ and $P = 0.009$, respectively). PMI cases had significantly elevated fasting blood sugar (FBS) compared to the control group ($P = 0.011$). C-reactive protein (CRP) level, which has roles in inflammation and atherosclerotic plaque formation, displayed significant elevation ($P = 0.016$) in PMI cases. Triglyceride (TG) elevation in PMI cases was close to significant ($P = 0.051$). In PMI cases, systolic blood pressure (SBP) and diastolic blood pressure (DBP) were both significantly higher ($P = 0.018$ and $P = 0.009$, respectively) than control subjects. Our results agree with previous studies in the association of metabolic syndrome, dyslipidemia, HTN, DM, FBS, CRP level, SBP, and DBP with PMI.[6,23-25]

Association of rs1801693 and rs7765781 with PMI: Rs1801693 and rs7765781 were genotyped by PCR-RFLP in all 85 PMI cases and 85 control subjects. Further information on these SNPs is shown in table 3.

Table 1. Primer sequences designed to amplify LPA rs1801693 and rs7765781 surrounding region

Variant	Forward primer	Reverse primer
Rs1801693	GTTGTCCAAGCACATAGAGAGGTA	ACAGAGAGTTAGACAGGTATAGACG
Rs7765781	GGCTGTCCATGACTTCACCTTCGA	TGTTGACAGTTGTTTAGAAGTTGAGGACC

Table 2. Characteristics of study groups

Variables	Controls (n = 85)	Patients (n = 85)	P
Age (year)	43.76 ± 5.14	44.05 ± 5.14	0.701
35-45	51 (60.0)	48 (56.5)	
46-55	33 (38.8)	35 (41.2)	
56-60	1 (1.2)	2 (2.4)	
Gender			> 0.999
Women	38 (44.7)	37 (43.5)	
Men	47 (55.3)	48 (56.5)	
Marital status			0.682
Married	81 (95.3)	83 (97.6)	
Widowed	4 (4.7)	2 (2.4)	
Residency			0.438
Urban	66 (77.6)	71 (83.5)	
Rural	19 (22.4)	14 (16.5)	
Physical activity (minute/week)	1021.2 ± 641.8	1023.6 ± 581.4	0.980
BMI (kg/m^2)	28.00 ± 4.83	27.05 ± 5.01	0.214
Smoking status			0.646
Current smoker	16 (18.8)	21 (24.7)	
Past smoker	3 (3.5)	3 (3.5)	
Never smoker	66 (77.6)	61 (71.8)	
Metabolic syndrome			0.008*
No	42 (49.4)	60 (70.6)	
Yes	43 (50.6)	25 (29.4)	
Dyslipidemia			0.028*
No	3 (3.5)	12 (14.1)	
Yes	82 (96.5)	73 (85.9)	
HTN			0.042*
Normal	54 (63.5)	67 (78.8)	
High	31 (36.5)	18 (21.2)	
DM	14 (16.5)	3 (3.5)	0.009*
FBS (mmol/l)	5.10 ± 1.79	4.54 ± 0.93	0.011*
HDL-C (mmol/l)	1.19 ± 0.28	1.24 ± 0.26	0.288
LDL-C (mmol/l)	3.39 ± 1.19	3.26 ± 1.11	0.458
CRP (mg/l)	3.99 ± 2.69	2.89 ± 1.76	0.016*
TG (mmol/l)	2.67 ± 1.39	2.27 ± 1.27	0.051
SBP (kPa)	16.83 ± 3.28	15.77 ± 2.51	0.018*
DBP (kPa)	10.91 ± 1.88	10.20 ± 1.67	0.009*
WC (cm)	97.49 ± 12.42	96.46 ± 12.70	0.593

Data are presented as mean ± standard deviation (SD) or number and percentage

*Significant difference between patients and controls was observed

BMI: Body mass index; FBS: Fasting blood sugar; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; CRP: C-reactive protein; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; WC: Waist circumference; HTN: Hypertension; DM: Diabetes mellitus; TG: Triglyceride

Rs1801693 genotyping revealed that 9.4%, 45.9%, and 44.7% of PMI cases and 10.6%, 41.2%, and 48.2% of control subjects were A allele homozygote, AG heterozygote, and G homozygote, respectively. Comparing study groups displayed no significant difference. Rs7765781 genotyping also revealed that 29.4%, 48.2%, and 22.4% of PMI cases and 30.6%, 49.4%, and 20.0% of control subjects were G allele homozygote, GC heterozygote, and C homozygote, respectively.

Table 3. Information of rs1801693 and rs7765781 variants

Variant	Location	Alleles	Protein level change
Rs1801693	Chromosome 6, *LPA* gene†,	A>G	NP_005568.2:p.Met1679Arg
NC_000006.12:g.160548597A>G,C	exon 32	A>C‡	NP_005568.2:p.Met1679Thr
Rs7765781	Chromosome 6, *LPA* gene†,	G>C	NP_005568.2:p.Leu1372Val
NC_000006.12:g.160586464G>C	exon 26		

†LPA (NC_000006.12) codes apolipoprotein A (apo-A) precursor; ‡C allele presence was ignored for all study subjects in this study, due to its absence in all randomly sequenced samples, suggesting that this allele's frequency might be ignorable in Iranian population

Table 4. Association of single nucleotide polymorphisms (SNPs) with premature myocardial infarction (PMI) incidence

Variant	PMI cases	Control group	OR (95% CI)	Age and sex-adjusted OR (95% CI)
Rs1801693				
AA	8 (9.4)	9 (10.6)	1.15 (0.68-2.23)	1.17 (0.59-2.31)
AG	39 (45.9)	35 (41.2)	1.11 (0.54-2.43)	1.15 (0.53-2.49)
GG	38 (44.7)	41 (48.2)	1	1
RS7765781				
GG	25 (29.4)	26 (30.6)	1.16 (0.49-2.73)	1.20 (0.53-2.64)
GC	41 (48.2)	42 (49.4)	1.14 (0.52-2.50)	1.18 (0.51-2.85)
CC	19 (22.4)	17 (20.0)	1	1

Data are presented as number and percentage; P-value displayed no significant difference between patients and control subjects in rs1801693 and rs7765781 genotypes
OR: Odds ratio; CI: Confidence interval; PMI: Premature myocardial infarction

The results demonstrated that there was no significant difference between case and control groups, suggesting that none of the investigated SNPs were correlated with PMI in the Iranian population (Table 4).

Results were also adjusted once by age and sex and once by age, sex, FBS, SBP, DBP, and TG, but no significant difference was observed between study groups (Table 5).

Sequencing of randomly selected samples confirmed PCR-RFLP results and demonstrated that adjacent SNPs of rs1801693 and rs7765781, located in *Nco*I and *Alu*I cut sites, had not interfered with digestion in any of the sequenced samples, due to their low frequency. Therefore, they were ignored in all the samples.

Discussion

Both environmental and genetic factors were found to be involved in CAD progression, but exact involved genes are not clear yet. Since in PMI, genetic hereditary is the major risk factor, finding involved genes and loci may help to prepare early prophylactic strategies for risky individuals.

In this study, 85 PMI cases and 85 healthy control subjects were genotyped at *LPA* gene rs1801693 and rs7765781 polymorphisms. Statistical analysis demonstrated that the mentioned SNPs did not have any meaningful association with PMI in the Iranian population.

Xu *et al.* also investigated the same SNPs in 521 Han Chinese patients with premature CAD and healthy controls, and they reported no meaningful association.[26] Though Dong *et al.* evaluated the association of rs1801693 with CAD in 831 Han and 829 Uyghur subjects and found this polymorphism associated with CAD in male Han subjects.[27]

In Iranian population, other SNPs also have been evaluated. Rs10455872, rs3798220, and rs10755578 did not show any association with CAD, indicating that more is needed to be done.[28-30]

Plasma Lp(a) level plays an important role in CAD progression. Dai *et al.* investigated the association between plasma Lp(a) level and severity of coronary lesions in Chinese population. The outcome indicated that Lp(a) was associated with CVD and played an important role in Chinese population CVD progression, suggesting that interventional treatment for Lp(a) would be effective in lessening the CVD progression in Chinese population.[31]

Many other clinical studies have demonstrated the role of high plasma Lp(a) level as a risk factor for CAD, especially the premature ones in diverse populations and also in Iranians.[32-36] It appears to be even more causative than LDL-C.[37-40]

The Lp(a) has different plasma levels and sizes in each ethnicity and its heterogenicity in different ethnical populations has been proven. But it seems that regardless of ethnicity, high Lp(a) level is associated with CVD progression.[40]

Table 5. Association of single nucleotide polymorphisms (SNPs) and premature myocardial infarction (PMI) incidence with adjusted variables

Variant	OR (95% CI)†	P†	OR (95% CI)‡	P‡
Rs1801693	1.06 (0.67-1.68)	0.795	1.09 (0.67-1.77)	0.719
Rs7765781	1.08 (0.70-1.66)	0.700	1.04 (0.66-1.62)	0.853

†Age/sex-adjusted; ‡Age/sex/fasting blood sugar (FBS)/systolic blood pressure (SBP)/diastolic blood pressure (DBP)/triglyceride (TG)-adjusted; P-value in both adjustments displayed no significant difference between patients and control subjects
OR: Odds ratio; CI: Confidence interval

Therefore, it might be needed to investigate the involved genetic polymorphisms in each ethnicity and find the causative. But in countries like Iran that different ethnicities exist, it might be better to investigate each ethnic group separately and not mixed with other ethnicities; this has not been done in Iran yet.

It has been shown in previous studies that Lp(a) has affinity to molecules which are found in foam cells, suggesting its important role in foam cell formation and emphasizing the importance of its evaluation.[31,41-43] Overall, it seems that Lp(a) level and *LPA* polymorphisms might be an important target for preventive strategies, and more attention should be paid to their evaluation.

In most of the previous studies, individuals have been genotyped by DNA sequencing or ligation detection reaction (LDR) methods, which are more accurate and sensitive. The more economical and accessible restriction fragment length polymorphism (RFLP) method may not be able to differentiate exact replaced nucleotide in the recognition site, as there were other SNPs located in our selected enzyme recognition site. Thus, in this study, one-third of the samples were randomly sequenced to check the accuracy of the results, confirming the RFLP results and the absence of adjacent SNPs in the recognition site in all randomly selected samples.

Totally, CADs are complex and multifactorial diseases; finding exact involved genes might be difficult. Moreover, heterogenic atherosclerosis locations in coronary arteries were observed, suggesting different sets of genetic loci involved in atherosclerosis progression.[44] Therefore, for better identification of genetic causes, it might be needed to stratify patients with CAD by their phenotypes and ethnicities and then investigate to find the related genes and polymorphisms.

Conclusion

This study demonstrated that there was no meaningful association between rs1801693/rs7765781 and PMI incidence in the Iranian population.

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Conflict of Interests

Authors have no conflict of interests.

Authors' Contribution

MS, MG, and HR participated in the study concept and design. MR, EK, and LS contributed to the collection of data and DNA extraction. MR and HK monitored the process of DNA extraction. MD did the statistical analysis. All authors participated in drafting the manuscript and approved it.

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