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GENETIC VARIANTS OF CYTOCHROME b-245, ALPHA POLYPEPTIDE GENE AND PREMATURE ACUTE MYOCARDIAL INFARCTION RISK IN AN IRANIAN POPULATION

GENETIČKE VARIJANTE GENA CITOHROM b-245, ALFA POLIPEPTID I RIZIK OD PREVREMENOG AKUTNOG INFARKTA MIOKARDA U IRANSKOJ POPULACIJI

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Summary

Background: Oxidative stress induced by superoxide anion plays critical roles in the pathogenesis of coronary artery disease (CAD) and hence acute myocardial infarction (AMI). The major source of superoxide production in vascular smooth muscle and endothelial cells is the NADPH oxidase complex. An essential component of this complex is p22phox, that is encoded by the cytochrome b-245, alpha polypeptide (CYBA) gene. The aim of this study was to investigate the association of CYBA variants (rs1049255 and rs4673) and premature acute myocardial infarction risk in an Iranian population.

Kratak sadržaj

Uvod: Oksidativni stres izazvan superoksidnim anjonom ima važne uloge u patogenezi koronarne arterijske bolesti (KAB) a time i akutnog infarkta miokarda (AIM). Glavni izvor produkcije superoksida u ćelijama vaskularnog glatkog mišića i endotelnim ćelijama je kompleks NADPH oksidaza. Važna komponenta ovog kompleksa je p22phox, koji kodira gen citohrom b-245, alfa polipeptid (CYBA). Cilj ove studije bio je da se ispita povezanost varijanti CYBA (rs1049255 i rs4673) sa rizikom od prevremenog akutnog infarkta miokarda u jednoj populaciji Iranaca.

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Methods: The study population consisted of 158 patients under the age of 50 years, with a diagnosis of premature AMI, and 168 age-matched controls with normal coronary angiograms. Genotyping of the polymorphisms was performed by the polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP).
Results: There was no association between the genotypes and allele frequencies of rs4673 polymorphism and premature acute myocardial infarction ($P>0.05$). A significant statistical association was observed between the genotypes distribution of rs1049255 polymorphism and AMI risk ($P=0.037$). Furthermore, the distribution of AA+AG/GG genotypes was found to be statistically significant between the two groups ($P=0.011$).
Conclusions: Our findings indicated that rs1049255 but not rs4673 polymorphism is associated with premature AMI.

Keywords: acute myocardial infarction, p22phox, polymorphism, rs1049255, rs4673

Metode: Proučavanu populaciju činilo je 158 pacijenata mlađih od 50 godina sa dijagnozom prevremenog AIM, i 168 kontrolnih ispitanika odgovarajuće starosne dobi sa normalnim koronarnim angiogramima. Genotipizacija polimorfizama je obavljena pomoću reakcije lančane polimeraze i polimorfizma dužine restrikcionih fragmenata (PCR-RFLP).
Rezultati: Nije utvrđeno postojanje veze između genotipova i učestalosti alela polimorfizma rs4673 i prevremenog akutnog infarkta miokarda (P>0,05). Značajna statistička povezanost je uočena između distribucije genotipova polimorfizma rs1049255 i rizika od AIM (P=0,037). Štaviše, distribucija genotipova AA+AG/GG pokazala se kao statistički značajna između dve grupe (P=0,011).
Zaključak: Naši nalazi ukazuju na to da polimorfizam rs1049255 jeste, ali rs4673 nije povezan sa prevremenim AIM.

Ključne reči: akutni infarkt miokarda, p22phox, polimorfizam, rs1049255, rs4673

Introduction

Acute myocardial infarction (AMI) is one of the leading causes of morbidity and mortality in the world. The most common cause of AMI is coronary artery disease (CAD) that is a multifactorial disease, resulting from genetic and environmental factors' interaction (1). Evidence suggests that the elevated levels of reactive oxygen species (ROSs), known as oxidative stress, are the major contributor to pathologic cardiovascular states such as CAD (2, 3).

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (Nox) represent a class of transmembrane hetero-oligomeric enzymes including five Nox isoforms (Nox1, Nox2, Nox3, Nox4 and Nox5) and two related enzymes (Duox1 and Duox2). The primary function of these enzymes is the production of reactive oxygen species (ROSs) such as superoxide anion ($O_2^{\cdot}$) in many cells particularly endothelial and vascular smooth cells (4, 5). Coupling components such as p22phox, p47phox, p67phox, p40phox and Rac are necessary for the activity and stabilization of these isoforms. All Nox appear to have an essential requirement for p22phox which is a heme binding protein that is located in the membrane. p22phox is composed of the α subunit of cytochrome b-245 and acts as an electron transfer element of NADPH oxidase. This subunit is encoded by the *CYBA* gene that is located on chromosome 16q24 and spans 8.5 kb (6 exons and 5 introns) (6).

The association between CAD risk and several polymorphic sites of the *CYBA* gene including C242T, C549T, A640G and promoter polymorphisms was investigated in previous studies (6). C242T (rs4673) is located in position 273853 of the *CYBA* gene's exon 4. In this single nucleotide polymorphism (SNP) the ancestral allele (T) is substituted by a (C) allele. This substitution causes a missense mutation, resulting in the replacement of a histidine by a tyrosine at the residue of 72 (7). Although there is supporting evidence which suggests that C242T can attenuate the oxidative function of NADPH oxidase, its actual role in CAD pathology remains to be elucidated (8, 9).

The A640G polymorphism (rs1049255) is located in the 3 untranslated region of *CYBA*, with no amino acid substitution. It has been assumed that A640G modifies the stability of p22phox mRNA and translational activity of *CYBA*. A few studies have investigated the relationship between the A640G polymorphism and CAD, but controversy still exists (10, 11).

The present study aimed to investigate the possible association between C242T (rs4673) and A640G (rs1049255) variants of the *CYBA* gene and premature acute myocardial infarction risk in an Iranian population.

Materials and Methods

Study population

Patient and control subjects were recruited from the Shahid Rajaei Cardiovascular Center, Tehran, Iran. The study population consisted of 158 patients under the age of 50 years with a diagnosis of premature AMI, and 168 age-matched controls who had all undergone coronary angiography and had normal coronary angiograms. Diagnosis of AMI was confirmed according to the new criteria of the American College of Cardiology and the European Society of Cardiology definition (12). Clinical information including MI type (STEMI or NSTEMI), MI biomarkers (troponin and creatine kinase-MB) were obtained through medical records. The study was approved by

the Iran University of Medical Sciences' Ethics Committee and written informed consent was obtained from all subjects.

Biochemical parameters

Blood samples were collected after fasting for 12 h. Serum levels of total cholesterol, triglyceride and HDL-cholesterol were measured by routine methods. LDL-cholesterol was estimated using the Friedewald equation.

DNA extraction

Total genomic DNA was extracted from ethylene diamine tetraacetic acid anticoagulated whole blood by a salting out method (13, 14).

rs1049255 and rs4673 genotyping

Genotyping of rs4673 and rs1049255 variants was performed by the PCR-RFLP technique. For rs1049255, PCR amplification was done by Fast start Taq polymerase (Roche) using a thermal cycler (Corbet Research) in a final volume of 25 μL by the following primers: 5′-AGATCGGAGGCACCATCAAG-3′ (forward) and 5′-AGCTGTCAAGGGAGGACTCT-3′ (reverse). The cycling conditions were: 95 °C for 4 min followed by 30 cycles comprising 95 °C for 30 s, annealing time at 62 °C for 45 s and extension at 72 °C for 45 s with a final extension time of 7 min at 70 °C. For the determination of rs1049255 genotypes, PCR product (484 bp) was digested by 10 U of DraIII restriction enzyme (New England Biolab) at 37 °C for 16 h. The resulting fragments were separated on 2% agarose gel and visualized under a UV light after staining with SYBR Green (CinnaGen DNA safe Stain). These included a 484 bp fragment for the GG homozygote, 484 bp, 295 bp and 189 bp fragments for the AG heterozygote and 295 bp and 189 bp fragments for the AA homozygote.

Amplification of the DNA fragment containing the rs4673 was performed using the forward 5′-GTGTGTTTTGTGGGAGGAAAGA-3′ and reverse 5′-TCCTCGGATTTGGAGTGGATC-3′ primers. DNA was amplified for 30 cycles, each cycle including denaturation at 95 °C for 30 s, annealing time at 59 °C for 45 s and extension at 72 °C for 40 s. For the determination of rs4673 genotypes, the PCR product (408 bp) was digested with 7 units RsaI (Fermentase) and products were separated on 2% agarose gel. Three possible genotypes were identified: subjects with the TT genotype were identified by the presence of tow products of 282 bp and 126 bp and those with the CC genotype by the presence of one product (408 bp). Heterozygous subjects were identified by the presence of three products of 408 bp, 282 bp and 126 bp.

Statistical analysis

Statistical analysis was performed by Statistical Software Package for the Social Sciences (SPSS 18.0, Chicago). The quantitative parameters in groups were expressed as mean±SD and compared by Student's t-tests. Compatibility of genotype frequencies with Hardy–Weinberg equilibrium expectations was checked by chi-square goodness-of-fit test with one degree of freedom. Moreover, the association between categorical variables, such as genotype distributions and premature AMI was determined with the χ^2 test. The odds ratios (ORs) and 95% confidence intervals (CIs) were calculated as a measure of the association of rs4673 (C/T) and rs1049255 (A/G) variants with AMI. Logistic regression analysis was performed to find the significant predictors among sex, family history of CAD, smoking, hypertension, LDL-cholesterol, triglyceride, total cholesterol and *CYBA* gene variants for CAD development risk. *P* values which were less than 0.05 were considered to be significant.

Results

Baseline characteristics of patient and control subjects (158 patients and 168 controls) are summarized in *Table I*. Data showed that the male sex was significantly associated with premature AMI ($P<0.001$). There were no significant differences in the serum HDL-cholesterol level ($P=0.06$) and BMI ($P=0.7$) between the groups whereas LDL-cholesterol ($P=0.001$), total cholesterol ($P=0.001$) and triglyceride ($P=0.000$) levels were significantly higher in patients when compared to controls.

The genotype distribution and allele frequencies of rs1049255 and rs4673 are presented in *Table II*. The distributions of the CYBA genotype and allele frequencies in patient and control groups were compliant with the Hardy–Weinberg equilibrium (all $P>0.05$). Genotype distributions of rs4673 and its allele frequencies had no significant differences ($P>0.05$). Moreover, we did not find a significant difference in rs4673 TT+CT versus CC between the two groups ($P>0.05$) (*Figure 1*). Significant statistical association was observed between the genotype distributions and allele frequencies of rs1049255 polymorphism between patient and control subjects (*Table II*). Furthermore, the difference of AA+AG/GG genotype was found to be statistically significant between the two groups ($P=0.011$) (*Figure 1*). Our study did not confirm the association between the two variants and AMI risk factors such as hypercholesterolemia and hypertension ($P>0.05$).

Logistic regression analysis demonstrated that male sex, hypertension and rs1049255 are significant predictors for AMI risk. Our results showed that there is no significant association between the other studied predictors such as rs4673, smoking, hyperlipidemia and serum lipid profile (*Table III*).

Table I Demographic and clinical characteristics of the study population.

Parameter	Control group (n=168)	Case group (n=158)	*P*
Sex (male/female)	68/100	122/36	0.000
Age (years)	44.7±6.8	46.32±5.2	0.07
Body mass index (kg/m^2)	25.58±3.43	26.57±5.45	0.07
STEMI	–	115	–
NSTEMI	–	43	–
Family history of CAD	26	48	0.001
Hypertension	23	41	0.005
Hyperlipidemia	48	67	0.009
Smoking (yes/no)	56/112	77/81	0.001
LDL-cholesterol (mmol/L)	5.19±1.50	5.77±1.71	0.001
HDL-cholesterol (mmol/L)	2.24±0.49	2.14±0.51	0.06
Triglyceride (mmol/L)	8.20±3.31	10.31±5.96	0.000
Total cholesterol (mmol/L)	9.25±2.07	10.09±2.48	0.001

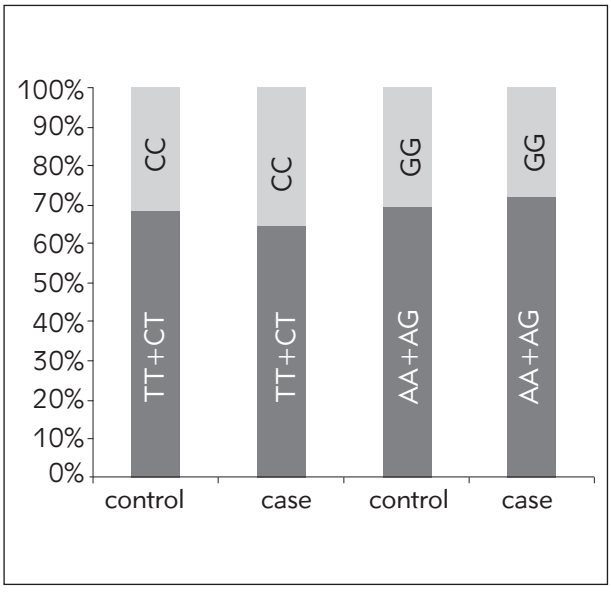

Figure 1 Genotype distribution for rs1049255 (AA+AG/GG) and rs4673 (TT+CT/CC). AA+AG/GG was significantly higher among controls (P=0.011; OR 1.916; CI 1.157–3.174) whereas TT+CT/CC distribution was not significant between two groups (P>0.05).

Table II Genotype distribution and relative allele frequencies of rs1049255 and rs4673.

Genotypes	Control group (n=168)	Case group (n=158)	*P*
rs1049255			
GG	55 (32.7%)	32 (20.3%)	
AG	76 (45.3%)	82 (51.9%)	
AA	37 (22%)	44 (27.8%)	0.037
Allele frequency			
G	186 (55.35%)	146 (46.2%)	
A	150 (44.65%)	170 (53.8%)	0.019
Rs4673			
CC	53 (31.54%)	56 (35.44%)	
CT	81 (48.22%)	74 (46.83%)	
TT	34 (20.24%)	28 (17.73%)	0.714
Allele frequency			
C	187 (55.65%)	186 (58.86%)	
T	149 (44.35%)	130 (41.13%)	0.408

Table III Logistic regression analysis results.

Logistic regression	P	OR	95% CI
Sex	0.001	7.830	3.853–15.912
Hypertension	0.013	2.403	1.202–4.806
Hyperlipidemia	0.324	1.348	0.745–2.438
Family history of CAD	0.348	1.354	0.719–2.548
Smoking	0 .697	0.878	0.455–1.692
HDL-cholesterol	0.146	0.978	0.949–1.008
LDL-cholesterol	0.242	1.009	0.994–1.024
Triglyceride	0.205	1.002	0.999–1.006
Cholesterol	0.503	1.004	0.993–1.015
rs4673	0.508	0.820	0.455–1.477
rs1049255	0.017	1.747	1.105–2.763

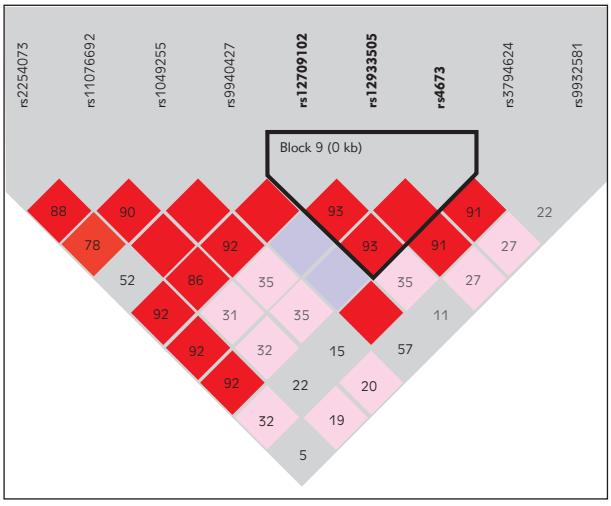

Figure 2 LD plot shows no strong linkage disequilibrium between rs4673 and rs1049255. The plot was created by Haploview using HapMap release 2 data.

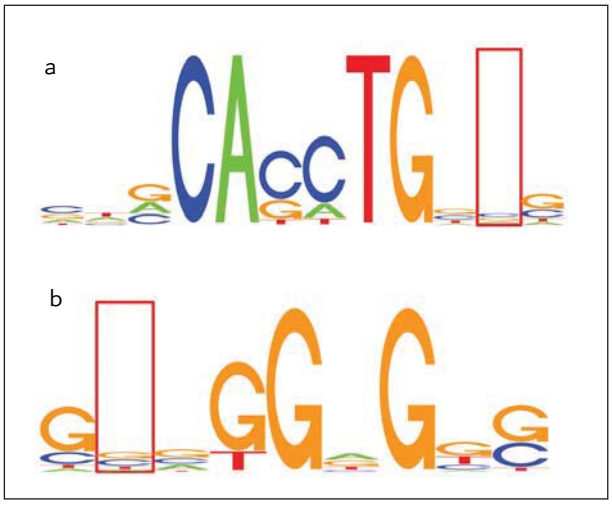

Figure 3 Position weight matrix for Lmo2 complex and WT1 transcription factor binding site. (a) Canonical motif for Lmo2 complex binding. (b) Canonical motif for WT1 binding. The red rectangular in the sequence logo represents rs1049255 position in the relevant motif.

Table IV SNPs are in linkage disequilibrium with rs1049255.

Position (hg19)	r2	D'	Variant	Motif changed	GENCODE gene	db SNP functional annotation
Chr16:88709737	1	1	rs1049255	WT1, Lmo2 complex	CYBA	3'-UTR
Chr16:88710833	0.8	0.92	rs3180279	HNF1	CYBA	intronic
Chr16:88710882	0.8	0.92	rs3794622	AIRE, HNF4	CYBA	intronic
Chr16:88710888	0.8	0.92	rs3794623	AIRE, HNF4	CYBA	intronic

Discussion

The most common cause of AMI is CAD that is a multifactorial disease, resulting from the interaction of genetic and environmental factors (1, 15, 16). Evidence over recent years has indicated that oxidative stress induced by superoxide anion plays critical roles in the pathogenesis of CAD and hence AMI. The major source of superoxide production in vascular smooth muscle and endothelial cells is the NADPH oxidase complex (17). Among the subunits of NADPH oxidase, there has been considerable interest in exploring the possible disease-association of genetic variations in the gene encoding p22phox (18). This subunit is encoded by the *CYBA* gene. Several studies have been published on the association between *CYBA* variants including rs4673 (C242T) and rs1049255 (A640G) and CAD development risk. However, the results are controversial (19).

In the present study, we investigated the association of *CYBA* variants (rs4673 and rs1049255) and AMI in a case-control study. We could not detect a significant effect for rs4673 polymorphism. There are also other studies which showed no association signal for rs4673 in AMI patients (11, 20, 21). The rs4673 relationship with cardiovascular pathologies was first described by Inoue et al. in a Japanese population (22). They studied 402 individuals (201 patients/201 controls) and observed a significantly decreased risk of developing CAD in subjects carrying a T allele of rs4673. Subsequently, this association was reproduced by Lee (23) and He (24) in Korean and Chinese populations, respectively. Overall, the role of rs4673 in AMI is not clear yet and studies with larger sample size are necessary to resolve this controversy (25).

The rs1049255 is located 3.4 kb downstream to rs4673. Although one may think they are linked, a strong linkage disequilibrium could not be found (r^2: 0.09, D': 0.35) (*Figure 2*). A statistically significant association was observed between the rs1049255 polymorphism and AMI. The frequency of rs1049255 G allele was significantly higher in controls than in patients with AMI (OR=1.916; 95% CI: 1.157–

3.174, $P=0.011$) (*Figure 1*). Our results are in agreement with Gardemann et al. study (26). They reported that the G allele had a protective role against coronary artery disease in a German population. There have been more investigations carried out to address the role of rs1049255 polymorphism, but they failed to show a significant association (6, 22, 24).

Under a logistic regression model, our analysis showed that sex ratio is a significant predictor for AMI risk ($P=0.001$). The male sex has an impressively increased chance of developing AMI (OR for men vs. women: 7.830). Furthermore, using a logistic regression model, we also found hypertension (OR: 2.403) and rs1049255 (OR: 1.747) as two additional risk factors for AMI in both men and women.

There are several lines of evidence that support the rs1049255 potential functional relevance. The ENCODE DNase footprinting assay experiments revealed that rs1049255 (chr16: 88709736) located at the 3'UTR of *CYBA* is a part of a canonical binding motif for Lmo2 complex and WT1 transcription factor (*Figure 3*) (27). Alternate substitution of A and G in this site might affect transcription factors binding efficiency. Moreover, it seems that histones H3 and H4 undergo different modifications around chr16: 88709736. The ENCODE chip-seq experiments confirmed that H3 and H4 undergo methylation and acetylation in different cell types, around rs1049255. Variations in base composition at such a location may interfere with the recruitment of epigenetically important DNA-binding proteins and hence contribute to functional relevance (27).

In addition to functional genomics data, population genetics also supports rs1049255 functional relevance. Analysis of 1000 genome projects data revealed that rs1049255 is in strong linkage disequilibrium ($r^2 \geq 0.8$) with three other variants (*Table IV*) which all have the potential to change transcription factors binding motifs (28).

In conclusion, our findings indicate that rs1049255 but not rs4673 polymorphisms are associated with the risk of premature AMI. However, larger studies should be carried out to confirm our results.

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Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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