Iran . J . Immunol
ISSN 1735-1383

Iran. J. Immunol. December 2007, 4 (4), 227–235

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Article Type: Research

ICAM-1 Polymorphisms (G241R, K469E), in Coronary Artery Disease and Myocardial Infarction

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ABSTRACT

Background: Inflammation plays a critical role in atherogenesis. The initial step in atherosclerosis is the adhesion of leukocytes to activated endothelial cells mediated by ICAM-1, an inflammatory protein. Several polymorphisms for Intracellular adhesion molecule -1(ICAM-) gene have been described. **Objective:** To determine the possible role of G241R and K469E polymorphisms in development of coronary artery disease and MI. **Methods:** G241R polymorphism was investigated in 303 patients with angiographically documented CAD, including 151 patients with acute or chronic myocardial infarction (MI), and a control group consisting of 141 healthy subjects with normal coronary angiogram. K469E polymorphism was investigated in 309 patients with CHD, including155 patients with MI, and compared with 150 healthy subjects without CHD as the control group. Finally, G241R and K469R polymorphisms were assessed concurrently in 300 patients with CHD including 152 patients with MI and 140 healthy normal subjects without coronary heart disease (CHD). **Results:** Although the frequency of GR and RR genotypes were higher in the control group compared to the CHD patients, the difference was not statistically significant (7.09% vs. 5.6% and 1.4% vs. 0%, p=0.27and p=0.24, respectively). Despite the higher frequency of KK genotype in the CHD group, the difference was not significant (29.1% vs. 24.6%, p=0.62). KKGG genotype was more frequent in the CHD group, however the difference was not significant (31.1% vs. 27.3%, p=0.66). **Conclusion:** No strong relation was found between G241R and K469E polymorphisms and occurrence of CHD and MI in the studied population from Fars province, Iran.

Keywords: ICAM-1, CHD, MI

INTRODUCTION

Atherosclerosis as manifested by coronary, cerebral and peripheral vascular arterial disease is the leading cause of morbidity and mortality in developed and many developing countries. Of the total cardiovascular deaths, nearly half result directly from coronary artery disease (1, 2).

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Our understanding of the process of atherogenesis has evolved from the epidemiological identification of cardiac risk factors to an increasing understanding of the molecular basis of vascular pathobiology. Evidence for role of chronic inflammation in atherogenesis has been accumulating over the last decade and intracellular adhesion molecule-1(ICAM-1), a transmembrane glycoprotein, consisting of 505 amino acids with 5 extracellular domains, plays a key role in binding of circulatory leukocytes (primarily monocytes) to vascular endothelium in the initial step of the atherosclerotic process (3- 5).
Several studies have reported increasing levels of the soluble form of ICAM-1 in patients with various coronary artery disease processes (6-9). Increased expression of local membrane bound ICAM-1 on endothelial plaque in CHD is also noticeable and the serum level of ICAM-1 is increased in individuals a few years before the occurrence of MI (10- 12).
Correlations between several polymorphisms in ICAM-1 gene and other inflammatory diseases such as Behcets (13), inflammatory bowel disease (14) and multiple sclerosis have been reported.
In this study we investigate two common polymorphisms of ICAM-1 gene; G241R and K469E in coronary artery disease and the potential role of these polymorphisms in genetic susceptibility to CHD and MI in an Iranian subpopulation in Fars province.

SUBJECTS AND METHODS

The studied population was categorized into the case group, i.e. patients with coronary artery disease (CHD), and the control group. The case group was further subdivided into CHD patients with myocardial infarction (MI) and those without MI. Patients with a history of inflammatory, metabolic, autoimmune and malignancy diseases or with a family history of such maladies were excluded from the study. A population of 720 subjects underwent coronary angiography (Judkins method, with Siemens coroscope 1997) and 7 ml of whole blood was collected from each participant. Angiograms were studied by two cardiologists who were not aware of the purpose of the study. The control group consisted of subjects with normal coronary angiograms, who underwent coronary angiography for the evaluation of coronary arteries, prior to non-coronary heart surgery or non-cardiac surgeries. . Patients who underwent coronary angiography due to chest pain were excluded from the study. The case group consisted of patients with a 50% or more stenosis involving at least one coronary artery. In the case group, patients with a history of acute or chronic MI were further subclassified into a smaller group (CHD with MI). MI diagnosis was based on the characteristic ECG findings and cardiac specific enzymes, using diagnostic criteria of the American College of Cardiologists. The case and the control groups were matched according to demographic characteristics and presence of common risk factors for coronary artery disease as: Hypertension (BP>160/90 at time of cardiac angiography or medical therapy for HTN), diabetes mellitus (FBS>126 prior to cardiac angiography or medical therapy for DM), hyperlipidemia (according to ATP III definition), smoking and body mass index (BMI).
After matching the patients and the controls, a total of 464 subjects were enrolled in the study. 459 subjects were studied for K469E polymorphism, 444 for G241R polymorphism and 440 were studied for both polymorphisms. All participants were residence of Fars province, Iran, who underwent coronary angiography in the affiliated hospitals of Shiraz University of Medical Sciences (Namazee Hospital and Shahid Faghihi Hospital)

according to ACC/AHA manual, from December 2004 to December 2006.Informed consent was taken from each participant.

Blood Sampling and DNA Extraction. Seven milliliters of blood was collected in EDTA tubes from each participant after angiography. The blood samples were kept at 4°C until DNA extraction. DNA extraction was performed using the proteinase K method. Sample preservation, DNA extraction and PCR methods were all performed at the Institute for Cancer Research (ICR), Shiraz University of Medical Sciences, Shiraz, Iran.

PCR and Genotyping

Amplification of exon 4 of the ICAM-1 gene. An allele specific PCR method (ARMS) was used to amplify a 137 bp fragment of ICAM-1 gene exon 4 using the appropriate primers. The primer sets consisted of Primer G: 5GTGGTCTGTTCCCTGGACG3; Primer R: 5GTGGTCTGTTCCCTGGACA3 and a common primer: 5GCGGTCACACTGACTGAGGCCT3. For each individual two distinct PCR reactions were performed, one with primers G and the common primer and another with primers R and the common primer. Approximately 300 ng genomic DNA was amplified in a total volume of 25 µl of the reaction mixture containing 2.5 µl of 10X PCR buffer, 0.75 µl of 10 µM dNTPs, 0.75 µl of 50 µM MgCl2 (CinnaGen, Tehran, Iran), 1 µl of 20 ρM primers (TIB Molbiol, Berlin, Germany), and 2 unit of Taq DNA polymerase. PCR amplification was done using touch-down method including an initial denaturation at 94 °C for 5 min and two loops of amplification. Loop one included 10 cycles of 94 °C for 20 s, 69→64C ° for 20 s, 72 °C for 20 s, and loop two included 20 cycles of 94 °C for 20 s, 61.5 °C for 20 s, and 72 °C for 20 s. Final extension was performed at 72 °C for 5 min. The amplified PCR products of 137 bp were run on 2% ethidium bromide stained agarose gel and visualized under UV transilluminator. (Figure 1)

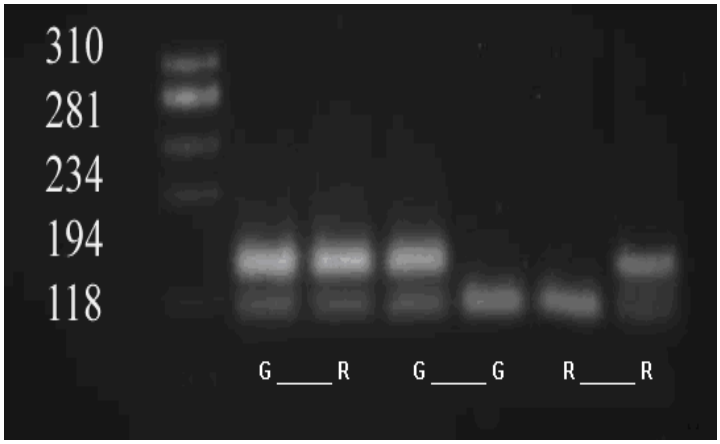

Figure 1. PCR products of codon 241 G to R substitution in ICAM-1 gene. A 100 bp internal control fragment was amplified in each reaction. After DNA size marker in the left hand side there are samples from a heterozygote case in lane 1 and 2, G/G homozygote case in lanes 3 and 4, and R/R homozygote case in last two lanes.

Amplification of exon 6 of the ICAM-1 gene. PCR amplification of a 223 bp fragment of ICAM-1 gene exon 6 was performed with PCR-RFLP using two forward K: 5GGTGAGATTGCATTAAGGTC3 and reverse E: 5GGAACCCATTGCCCGAGC3 primers. 300 ng genomic DNA was amplified in a total volume of 25 µl of the reaction

mixture containing 2.5 µl of 10X PCR buffer, 0.75 µl of 10 µM dNTPs, 0.75 µl of 50 µM MgCl2 (CinnaGen, Tehran, Iran), 1 µl of 20 ρM primers (TIB Molbiol, Berlin, Germany), and 2 U of Taq DNA polymerase. PCR was carried out with an initial denaturation at 95 °C for 7 min and 35 cycles of 95 °C for 35 s, 57 °C for 45 s, and 72 °C for 45 s with a final extension of 5 min at 72 °C. Subsequently, 10 µl of PCR products were treated with 3 U of Bsh1236I (FnuDII) in a total volume of 15 µl with 1X buffer R+, incubated for 16 hours at 37 °C for optimum digestion. The amplified fragment of 223 bp from the E469 allele was divided into two fragments of 87 and 136 bp but that of the K469 did not. The fragments were analyzed by 3% ethidium bromide stained agarose gel electrophoresis. (Figure 2)

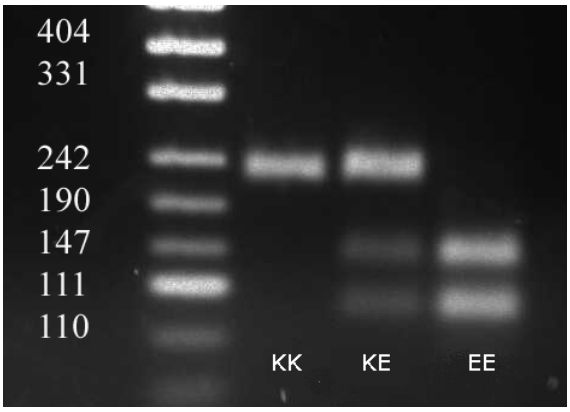

Figure 2. PCR-RFLP results of codon 469 K to E substitution in ICAM-1 gene. After treatment with Bsh1236I restriction enzyme, the original 223 bp PCR product was digested into 87 and 136 bp fragments when E allele existed. Lane one from the left represents DNA size marker and other lanes indicate three different genotypes.

Statistical Analysis. SPSS program version 13.0 was used for statistical analysis. ANOVA and unpaired Student t-tests were used to compare the means of continuous variables. Alleles and genotype frequencies in the case and control groups were compared using Chi-square and Fischer exact tests. Multivariable logistic regression analysis was performed to determine effects of unmatched variables on the case and control groups. Alleles of each polymorphism were said to be in Hardy-Weinberg equilibrium if the frequency of homozygotes observed did not differ significantly from the expected frequencies, when χ^2 test was used ($p>0.05$). P values less than 0.05 were considered as statistically significant.

RESULTS

G241R Polymorphism

Comparison of Population Characteristics and Coronary Risk Factors. G241R polymorphism was investigated in 444 subjects in two groups: the control group and the case group (CHD), The CHD group was further subdivided into: CHD with MI and CHD without MI. The case and the control groups were matched in respect to their age, but the percentage of female participants was greater in the control group (60.3% vs. 47.9%, p=0.015).There were no statistically significant difference between the control

group and the case group (including both subgroups) in regard to the prevalence of the coronary risk factors except for the history of cigarette smoking which was significantly higher in the case group (35.3% vs. 25.5%, p=0.04) (Table1).

Table1. Characteristics of study population for G241R polymorphism

Variable	Control (n141)	CHD (n303)	CHD without MI(n149)	CHD with MI (n154)	P value
Age (year)	57.34±8.2	56.61±9.20	57.44±9.32	55.83±9.05	0.404
Sex (F/M)	85/56	145/148	70/79	75/79	0.015
BMI (kg/m2)	23.37±5.8	23.19±2.72	23.32±2.69	23.06±2.75	0.657
Diabetes mellitus (%)	13.5	20.5	22.1	18.8	0.076
Hypertension (%)	43.3	45.9	47.7	44.2	0.60
Hypercholesterolemia (%)	34.0	38.6	40.3	37.0	0.353
Smoking (%)	25.5	35.3	31.5	39.0	0.04

The age and BMI values are presented as mean ± S.D. BMI, body mass index; CHD, coronary heart disease; MI, myocardial infarction

Distribution of Alleles and Genotype Frequencies. The R and G allele prevalence in the studied population were 96.5% and 3.5%, respectively. Specifically, prevalence of R and G alleles was as follows: in the control group, 95.1% and 4.9% and in the case group 97.1% and 2.9%, respectively. In the CHD subtype groups the frequency of R and G alleles were: 96.7% and 3.3% in CHD group with MI and 97.6% and 2.4% in CHD group without MI. Although prevalence of R allele was greater in the control group (4.9%) than the case group (2.9%) but it was not statistically significant (p=0.094). The prevalence of GG, GR and RR genotypes in the studied population was 93.4%, 6.1% and 0.5%, respectively. In the control group the prevalence of GG, GR and RR genotypes were 91.5%, 7.09% and 1.41%, respectively, while it was 94.34%, 5.66% and 0 in CHD group. The prevalence of GG, GR and RR genotypes in two subgroups of CHD patients were as follows: 93.5%, 6.5% and 0 in the CHD group with MI and 95.3%, 4.7% and 0 in CHD group without MI. Although GR and RR genotypes were more common in the control group, however the difference was not statistically significant (7.09% and 1.41% vs. 5.66% and 0, p=0.27 and p=0.24).

Multiple Variable Logistic Regression Analyses. Gender and smoking which were different in two groups had no independent influence on CHD or MI occurrence in the studied population. (p=0.075 for sex and p=0.164 for smoking).

K469E Polymorphism

Comparison of Population Characteristics and Coronary Risk Factors. A total of 459 subjects were investigated for K469E polymorphism in two groups: the control group and the case (CHD) group, the CHD group was further divided into CHDs with MI and CHDs without MI. Females comprised 60% of subjects in the control group while the percentage of female patients in the case group was 48.5% p =0.02).The rate of cigarette smoking was much higher in the CHD group than the control, (35.6% vs. 26.0%, p =0.039). The control and case population were matched with respect to other characteristics (Table2).

Distribution of Alleles and Genotype Frequencies. We found that the prevalence of E and K alleles in the studied population were 52.2% and 47.8%, respectively. In the control group, the E allele was seen in 51% and the K allele had a prevalence of 49%, while in the case group the prevalence of E and K alleles were 46.3 and 53.7%, respectively.

The frequency of E and K alleles in the two CHD subgroups were as follows: in CHD patients with MI, E allele was seen in 47.8% of the subjects and K allele in 55.1%.

Table 2. Characteristics of study population for K469E polymorphism

Variable	Control (n150)	CHD (n309)	CHD without MI(n154)	CHD with MI (n155)	P value
Age (year)	57.15±8.42	56.65±9.37	57.42±9.63	55.88±9.0	0.581
BMI (kg/m2)	23.43±5.72	23.17±2.75	23.28±2.70	23.05±2.8	0.507
Sex (F/H)	90/60	150/159	73/81	77/78	0.021
Diabetes mellitus (%)	13.3	20.7	22.1	19.4	0.055
Hypertension (%)	44.0	46.9	48.7	45.2	0.555
Hypercholesterolemia(%)	34.0	38.8	46.6	36.1	0.315
Smoking (%)	26.0	35.6	32.5	38.7	0.039

The age and BMI values are presented as mean ± S.D; BMI, body mass index; CHD Coronary heart disease; MI, myocardial infarction

In CHD patients without MI, the E and K alleles were seen in 44.9% and 55.1% of the subjects, respectively. Although the K allele was more prevalent in the CHD population compared to the control group, this difference was not significant (53.7% vs. 49%, p value=0.41).The prevalence of KK, KE and EE genotypes in the studied population was 27.9%, 48.6% and 23.5%, respectively. In the control group the prevalence of KK, KE and EE genotypes were 24.67%, 48.66% and 26.66%, respectively, while it was 29.5%, 48.5% and 22% in the CHD group. In two subgroups of CHD patients, the prevalence of KK, KE and EE genotypes were as follows: 27.1%, 50.3% and 22.6% in the CHD group with MI and 31.8%, 46.7% and 21.5% in CHD group without MI. Although KK genotype was more frequent in the CHD group compared to the control group but the difference was not statistically meaningful (29.5% vs. 24.67%, p =0.62).

Multiple Variable Logistic Regression Analyses. Data analyses showed that gender and cigarette smoking were not independent factors and had no effect on the occurrence of CHD and MI. (p =0.23 for sex and p=0.136 for smoking).

G241R and K469E Polymorphisms

Comparison of Population Characteristics and Coronary Risk Factors. Both G241R and K464E polymorphisms were investigated in 440 subjects. The subjects were categorized into two groups (control and CHD group. Comparing the two groups, no statistically significant difference existed between the case and the control group in regard to the individual characteristics and coronary risk factors except for the history of cigarette smoking which was significantly higher in the CHD group (34.3% vs. 25%, p=0.03) (Table3).

Table 3. Characteristics of study population for both GR241 and KE 469 polymorphisms

Variable	Control (n140)	CHD (n300)	CHD without MI(n148)	CHD with MI (n152)	P value
Age (year)	54.4±8.26	56.51±9.13	57.32±9.28	55.82±9.10	0.228
Sex (F/H)	84/56	144/156	70/78	74/78	0.062
BMI (kg/m2)	23.38±5.88	23.18±2.69	23.31±2.70	23.08±2.76	0.793
Diabetes mellitus (%)	13.6	20.7	22.3	19.1	0.156
Hypertension (%)	42.9	44.4	47.3	44.1	0.734
Hypercholesterolemia (%)	33.6	38.65	40.5	36.8	0.472
Smoking (%)	25	34.3	31.8	39.5	0.03

The age and BMI values presented as mean± S.D.; BMI, body mass index; CHD, coronary heart disease; MI, myocardial infarction

Distribution of Genotypes and Frequencies. Combination of G241R and K469E polymorphisms results in 9 different genotypes (KKGG, KKGR, KKRR, KEGG, KEGR,

KERR, KEGG, EEGG, EEGR, EERR). Of these, KERR and KKRR polymorphisms were not seen in the studied population. The most common genotypes observed were: KEGG (43.2%), KKGG (27%) and EEGG (19.5%). The latter three polymorphisms were compared between the case and the control groups. Although KKGG genotype was more prevalent in the CHD than the control group, the difference was not significant (30.0% vs.25.0%, p=0.66) (Table4).

Table 4. Genotype frequencies of the combination of both polymorphisms

Variable	Control (n140)	CHD (n300)	CHD without MI(n148)	CHD with MI (n152)
Genotype n (%)				
EEGG	28(20.0)	58(19.3)	29(19.6)	29(19.1)
EEGR	5(3.6)	6(2.71)	4(2.7)	4(2.6)
EERR	2(1.4)	0	0	0
KEGG	65(40.4)	135(45)	64(43.2)	71(46.7)
KEGR	4(2.9)	9(3.0)	3(2.0)	6(3.9)
KKGG	35(25.0)	90(30.0)	48(32.4)	42(27.6)
KKGR	1(0.7)	0	0	0

CHD, coronary heart disease; MI, myocardial infarction. * P values were not significant

Multiple Variable Logistic Regression Analysis. Although the rate of cigarette smoking was higher in the CHD group, but its role as an independent risk factor in the occurrence of CHD or MI was not significant (p=0.11).

DISCUSSION

To our knowledge, this is the first report on the two commonly known polymorphisms of ICAM-1 gene in patients with coronary artery disease and myocardial infarction. In this study, both G241R and K469E polymorphisms were investigated separately and together at the same time.

In contrast to other studies, subjects with normal coronary angiography were selected as the control group. As we know, the incidence of asymptomatic coronary artery disease (both without clinical symptoms and without findings by non-invasive methods such as ECG and echocardiography) is increasing by age. Therefore to select the control group, participants underwent coronary angiography and only those with normal angiograms were enrolled in this study. The genotype distributions in our studied population are in accordance with Hardy-Weinberg expectation. The case and control groups were identical in respect to demographic and coronary risk factors except that the female ratio was higher in the control group and the case group had a higher rate of cigarette smokers compared to the control group. Using the multiple variable logistic regression analyses, gender and history of cigarette smoking had no independent effect on the incidence of CHD or MI.

G241R polymorphism is due to the substitution of a G with an A nucleotide at codon 241 of exon 4 in ICAM-1 gene, which replaces an arginine by a glycine in the 3rd domain (D3) of the extracellular portion of ICAM-1 molecule. MAC-1 (CD11b) molecule, on the surface of leukocytes, interacts with D3 segment of ICAM-1 molecule. In the absence of D3 segment, ICAM-1 and MAC-1 will not interact. It seems that G→A mutation decreases the affinity of MAC-1 for ICAM-1. The unbound ICAM-1 has inhibitory

effects on the expression of ICAM-1 gene resulting in decreased levels of ICAM-1 in the blood (19).

In a study on children with UTI (20), it was observed that patients with RR genotype had a much lower chance of developing renal parenchymal scar after UTI, than patients with GG genotype. It seems that G→A mutation plays an inhibitory or protective role against inflammation. In our study the prevalence of R allele and RR genotype were also higher in the control group, although the difference was not statistically significant.

K469E polymorphism is due to the substitution of an A with a G nucleotide at codon 469 of exon 6 in ICAM-1 gene which replaces a lysine with a glutamic acid in the 5th domain (D5) of the extracellular portion of ICAM-1. D1 segment of ICAM-1 molecule interacts with LFA-1 (CD11b) on the surface of dendritic cells and leukocytes, while D3-5 segments are required for this interaction. It seems that A→G mutation and replacement of lysine by glutamic acid in D5 segment facilitates and reinforces adherence of ICAM-1 with LFA-1. (19)

The association between K469E genotype and occurrence of coronary artery disease and MI has been investigated before, the results indicated that K allele, KK and KE genotypes are more common in patients with CHD and MI (21,22). But in another study the effects of K469E polymorphism in families with a history of coronary artery disease were investigated and no meaningful relation was found between K469E polymorphism and occurrence of coronary artery disease. (19)

In this study we also found that the K allele and the KK genotype are more common in the patient group, although the difference was not statistically significant.

Although in our study, the KKGG genotype was more common in the patient group compared to the control group, the difference was not also statistically significant.

Common risk factors can influence the incidence of coronary artery disease. Their effects are alleviated or attenuated in genetically susceptible subjects, that is why the prevalence of coronary artery disease varies among different communities irrespective of the effects of common coronary artery risk factors.

In conclusion, no strong relation was found between K469E and/or G241R polymorphisms and the occurrence of CHD and MI in the studied population from Fars province, Iran.

ACKNOWLEDGEMENT

This work was financially supported by a grant (No.85-3096) from Shiraz University of Medical Sciences Shiraz, Iran, and in part by Shiraz Institute for cancer research.

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