

Research Article

Evaluation of Relationship Between Single-Nucleotide Polymorphism in TNF-Gene Promoter and Susceptibility to Atherosclerosis in Fatemeh Zahra Hospital

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Abstract

Introduction: Tumor necrosis factor alpha is a proinflammatory cytokine that initiates a polyvalent initial response of inflammatory cells, which is facilitated by coronary atherosclerosis. Further, it appears that the polymorphism and susceptibility to atherosclerosis are related to the TNF- α gene promoter. Aim: To assay single nucleotide polymorphisms of the TNF- α gene promoter in two sites (863 and 308) in atherosclerotic patients referred to Fatemeh al-Zahra Hospital.

Materials and Methods: This case-control study was conducted on 120 patients (with stenosis greater than 50%) and 120 healthy individuals (with stenosis lesser than 10%). Genomic DNA was extracted using the Phenol-chloroform procedure from their white blood cells. Genotypes of these individuals and TNF- α gene polymorphisms were analyzed by the RFLP-PCR method. Genotype frequencies, the Hardy-Weinberg equilibrium test, and chi-square analysis were conducted using the SPSS software version 22.

Results: Genotype frequencies of GA, GG, and AA in position -308 of the TNF- α gene of patients were 12.5%, 75%, and 12.5%, respectively; in healthy subjects, they were 7.5%, 21.7%, and 70.8%, respectively. Allele A to G allele increased the risk of disease by 12.716%. The genotype frequencies of AC, CC, and AA in position -863 of the TNF- α gene of patients were 3.3%, 69.2%, and 27.5%, respectively; in healthy individuals, they were 2.5%, 11.7%, and 85.8%, respectively. Allele A to C allele increased the risk of disease by 16.373%. Statistical analysis revealed a significant correlation between the risk of atherosclerosis with single nucleotide polymorphisms in the TNF- α gene-863 at C < A and in gene -308 at G < A ($P < 0.05$).

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Conclusion: According to this study, mutations in the promoter region of the TNF- α gene possibly increased susceptibility to atherosclerosis. Therefore, genotype determination of individuals in these areas can help to identify patients with high risk for cardiac disease.

Key;words: Atherosclerosis, Inflammatory cytokines, PCR-RFLP, Polymorphism, TNF- α

1. Introduction

Cardiovascular diseases are the most important causes of disability and premature deaths worldwide. Atherosclerosis (coronary artery disease) is a common type of cardiovascular disease and is a usual cause of heart attacks, strokes, and peripheral vascular disease [1, 2]. Atherosclerosis initiates with damage to the endothelium and is related to high blood pressure, smoking, and high cholesterol. This damage leads to plaque formation. After transfer of bad cholesterol or LDL through the damaged endothelium, cholesterol enters the walls of the artery and causes white blood cells to stream in to digest the LDL. Over years, the accumulation of cholesterol and inflammatory cells create a plaque in the artery wall [1]. Recent studies have shown that inflammation is a poor prognosis at all stages of atherosclerosis and coronary artery disease [2, 3].

Recent studies have revealed that cytokines, such as tumor necrosis factor alpha (TNF- α), have an important role in the initiation and progression of atherosclerosis [4, 5]. TNF- α is a primary pro-inflammatory cytokine that has different biological functions [6]. TNF- α stimulates endothelial cell activation and expression of adhesion molecules that attract inflammatory cells from the bloodstream to the vessel wall [7]. It also stimulates the production of IL-6, which is a chemoattractant cytokine. These cytokines play an important role in atherosclerosis [8]. In addition, TNF- α has a significant effect on increasing insulin resistance and lipid metabolism. These changes increase the probability of cardiovascular risks [9, 10]. High concentrations of serum TNF- α play an important role in inflammation underlying cardiovascular disease, such as atherosclerosis [11, 12]. Prospective studies suggest that TNF- α levels are associated with the first occurrence of cardiovascular disease [13, 14], as well as a marker for recurrence of coronary events after a heart attack [11]. Environmental stimuli, such as smoking, alcohol consumption, and genetic differences, lead to individual differences in the levels of TNF- α , which can be associated with cardiovascular diseases [15]. Smoking and alcohol consumption are considered as strong risk factors for the development of CHD (Coronary Heart Disease). Further, the difference in TNF- α protein levels in healthy subjects may be a high risk

factor for these diseases [16]. One of the factors affecting the production of TNF- α is the TNF- α transfer gene open G with A at position 308 [17]. Thus, DNA mutations in this gene increase serum concentrations of TNF- α , which has an important pathogenic role in the development of atherosclerosis [18, 19]. Most probably, one of the most important factors for this disease is cytokines encoding gene polymorphisms. Many studies have been conducted to assay effects of TNF- α gene polymorphisms on susceptibility to cardiovascular diseases [20, 21]. Additionally, some findings have shown that the gene polymorphism associated with cytokine production, especially single-nucleotide polymorphisms (SNPs), plays an important role in inflammation and thrombosis [23, 24].

Multiple single-nucleotide polymorphisms in the promoter region of TNF α have been found. It seems that some of these genetic polymorphisms, particularly single-nucleotide polymorphisms at positions -308 G<A and -238 G<A, may play major roles in the expression of the TNF- α gene. It has been reported that polymorphisms in areas -308 G<A and -238 G<A of the TNF- α gene were significantly associated with changes in TNF- α gene activity. Various studies have been performed to evaluate the effect of TNF gene polymorphisms on autoimmune diseases, such as psoriasis, rheumatoid arthritis, multiple sclerosis, asthma, colon cancer, gastric cancer, and some infectious diseases, such as tuberculosis. Most studies have shown relationships between polymorphisms -308 G<A in the TNF- α gene and progression of some inflammatory diseases [25]. Liping Hu and colleagues in 2001 identified seven polymorphisms in the TNF- α gene promoter region and first intron. These polymorphisms are as followings: -863 C\A, -857 C\A, -806 C\T, -376 G\A, -308 G\A, -238 G\A, and 467 G\A. With referring to the NCBI SNP database, species with numbers *rs1800630*, *rs1799724*, *rs4248158*, *rs1800750*, *rs1800629*, *rs361525*, and *rs1800610* were dedicated [26].

Due to controversial information about TNF- α gene polymorphisms and as these studies are new, we have aimed to assay polymorphisms -308 G<A and -863 C<A of the TNF- α gene in patients with atherosclerosis referred to the Mazandaran Cardiac Center (Fatemeh al-Zahra Hospital, Sari).

2. Materials and Methods

This case-control study included 240 individuals (104 women; 136 men) who were referred to CCU center of Fatemeh al-Zahra Hospital, Sari, Iran in 2012–2015.

These people were diagnosed with atherosclerosis, and their age ranges were 35–60 years. Early symptoms of atherosclerosis were defined as stenosis in the heart's main artery (one or more of the arteries) greater than 50% and lesser than 10% in the control group. The patient numbers were 120 (44 females, 76 males, mean age = 53.93), which

TABLE 1: Profile of enzymes and primers used.

SNPs	RS number	Primer	Restriction enzyme	Size created
-863 C>A	1800630	5'GGCTCTGAGGAATGGGTTAC3'	BsaAI	C = 103
		5'CTACATGGCCCTGTCTTCGTTACG3'		A = 23
-308 G>A	1800629	5'AGGCAATAGGTTTGGAGGCCAT3'	NcoI	A = 87
		5'TCCTCCCTGCTCCGATTCCG3'		G = 20

had a history of coronary artery diseases or acute MI but survived. The control group consisted of 120 individuals (60 females, 60 males, mean age = 51.23) without history of coronary artery diseases, chest pain, or ischemic problems.

Structured questionnaires for detailed medical and family history of coronary artery disease, risk factors, such as smoking, and alcohol consumption were designed, and this information was provided by referring to the patient records.

People with congestive heart failure (CHF), peripheral vascular disease (PVD), heart arthritis, cardiopulmonary, chronic kidney, liver, neurodegenerative diseases, and cancer were excluded from the study. To calculate body mass index (BMI), weight and height were measured and recorded.

2.1. Biochemical parameters

After 12 hours of fasting, venous blood was taken. Blood serum was separated and stored at -20°C. Serum total cholesterol (TC), triglycerides (TG), high density lipoprotein (HDL), low density lipoprotein (LDL), and fasting blood glucose (FBG) were measured by an auto analyzer (SK 3003 India).

2.2. Genotyping

In this study, TNF- α gene polymorphisms -308 G>A (Figure 1) and -863 C>A (Figure 2) and the genotypes of the individuals were determined. Genomic DNA from peripheral blood leukocytes was isolated by the standard phenol-chloroform procedure [24, 25]. For genotyping of individuals, PCR-RFLP (polymerase chain reaction and restriction fragment length polymorphism) was used. For the TNF- α polymorphisms, primers pairs were designed (Table 1). Fragments were prepared in a volume of 25 μ L containing 1 μ L of each primer (10 pmol), 2.5 μ L buffer (10X), 2 μ L MgCl₂ (50 mM), 1.5 μ L dNTP, 0.2 μ L Taq polymerase, 2 μ L DNA (100 ng), and 11 μ L of distilled sterile water.



Figure 1: Genotyping results of TNF- α gene polymorphism -308 G<A. From left to right: GeneRuler 50, AA, AA, AG, GG, AA, AG, and GG.

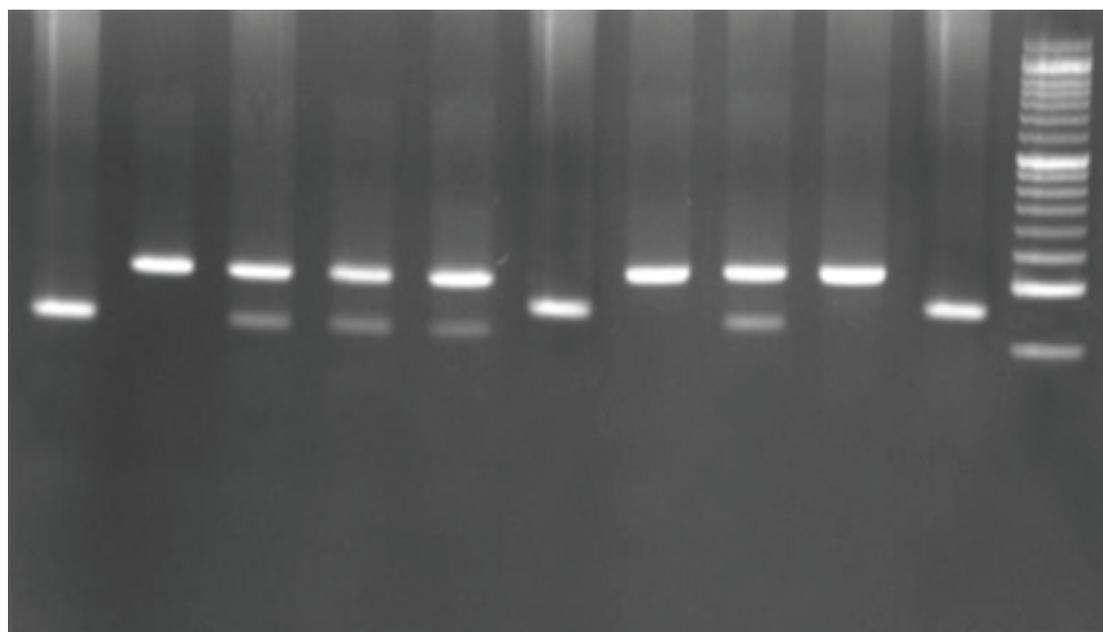


Figure 2: Genotyping results of TNF- α gene polymorphism -863 C<A. From left to right: CC, AA, AC, AC, AC, CC, AA, AC, AA, CC, and GeneRuler 50.

The PCR overall program consisted of three main stages: initial denaturation, amplification, and final elongation. The program used in this study conducted primary denaturing at 94°C for 3 minutes, performed in one cycle. Following, there were 35 cycles of amplification in a denaturing step at 94°C for 30 seconds, annealing at 69°C for 1 minute, and elongation at 72°C in 2 minutes. The final elongation cycle was performed in 5 minutes at 72°C.

To ensure the accuracy of testing, PCR products were analyzed on a 2% agarose gel. Subsequently, PCR products were digested by *NcoI* restriction enzymes at 37°C for 16 hours. Digested products were separated on a 2.5% agarose gel stained with ethidium bromide dye at 80 volts.

PCR products were also digested by restriction enzyme *BsaAI* at 37°C for 16 hours. Restriction enzymes were purchased from Tehran Thermo-Scientific Co.

3. Statistics

Statistical analyses were performed using the SPSS software (version 22). The differences in variables between patients and controls were analyzed using the independent Student's t-test, and data were shown as mean \pm SD. Further, differences between variables of classified genotype distribution were analyzed by the Hardy-Weinberg test and χ^2 or Fisher exact test. In order to examine the role of polymorphisms in the development of atherosclerosis, the effects of conventional risk factors, such as age, hypertension (HTN), diabetes, and lipid levels, were adjusted with multivariate logistic regression. The relationship between genotype and risk of atherosclerosis was compared considering the odds ratio (OR) and confidence interval of 95% (CI), ($P \leq 0.05$).

4. Results

Clinical characteristics of populations are shown in Table 2. Two groups of patients and controls were matched for age and sex. Cigarette smokers were specified as people who have smoked at least 100 cigarettes in their lifetime; drinker were those who drank at least 12 times during the year when interviewed.

The average age of patients in the study was 51.23 years. Correspondingly, the age of 51 is almost the emergence age for beginning symptoms of atherosclerosis. Statistical analysis showed a significant difference between the HDL cholesterol level and FBS in the patient and control groups ($P < 0.05$). It was shown that there was no significant difference between the level of serum triglycerides and LDL between the patient and control groups ($P > 0.05$), but differences in traditional risk factors, such as smoking and alcohol use, history of diabetes, hypertension, cardiovascular disease, and high-fat diet, were significant ($P < 0.001$). For BMI and history of hyperthyroidism, there were no significant differences between patients and the healthy control group ($P = 0.05$).

TABLE 2: Clinical characteristics of patients and controls.

Control (n = 120)	Characteristics	Patients (n = 120)	P-value
Gender, M/F, n	60/60	76/44	0.037
Average age, years	53.94±7.8	51.23±7.1	0.005
BMI, kg/m ²	24.30±3.50	25.12±3.58	0.054
Smoking no, n (%)	95	50.8	<0.001
Drinker no, n (%)	94.2	65.8	<0.001
Hypertension no, n (%)	63.3	19.2	0.001
SBP, mm Hg	125.63±17.53	78.44±9.16	
DBP, mm Hg	128.65±20.42	75.05±10.52	
Diabetes no, n (%)	89.2	34.2	<0.001
Hypothyroid no, n (%)	98.3	92.5	0.031
Hyperthyroid no, n (%)	96.7	96.7	1
FBG, mmol/L	108.9±55.4	206.0±87.4	<0.001
TC, mmol/L	212.8±71.4	230.8±66.6	0.045
TG, mmol/L	210.5±92.7	216.1±80.7	0.618
HDL-C, mmol/L	35.7±6.88	35.7±10.2	<0.001
LDL-C, mmol/L	133.4±62.6	145.3±61.4	0.141
Family history of CHD, no (%)	75	53.3	<0.001
High-fat diet, no (%)	28.3	56.7	<0.001
Physical activity, no (%)	37.5	46.7	0.15

Abbreviations: BMI, body mass index. SBP, systolic blood pressure; DBP, diastolic blood pressure; FBG, fasting blood glucose; TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low lipoprotein cholesterol; OR, odds ratio; CI, confidence intervals.

4.1. Association between TNF gene polymorphisms and atherosclerosis

In this study, the frequencies of TNF gene polymorphisms (rs1800629) and (rs1800630) in the promoter region of 240 individuals were assayed using PCR-RFLP. Genotype and allele frequency between patients and control groups were also analyzed. The results are shown in Table 3. A significant deviation from the Hardy–Weinberg equation was not found. For SNP –308 (rs1800629) in the normal group, the genotype frequency for AA was 12.5%, 75.0% for GG, and 12.5% for GA. In patients, the genotype frequency for AA was 70.8, 21.7% for GG, and 7.5% for AG. It was shown that the allele A increased the chance of disease 12.716 times more than the allele G. Further, the AA genotype increased risk 6.626 times more than the GG genotype.

For SNP -863 (rs1800630) in normal individuals, the genotype frequency of AA was 27.5%, 69.2% for genotype CC, and 3.3% for AC. The AA genotype frequency in patients was 85.8%, the CC genotype frequency was 11.7%, and the AC genotype frequency was

TABLE 3: TNF- α genotype and allele distributions of -308 G<A and -863 C<A polymorphisms in the study groups.

SNP	Genotype frequency, %			Allele frequency, %		P-value
	G/G	A/A	AG	A	G	
rs1800629						0
Controls	75	12.5	12.5	18.8	81.2	
Patients	21.7	70.8	7.5	74.6	25.4	
rs1800630						0.006
Controls	69.2	27.5	3.3	29.2	70.8	
Patients	11.7	85.8	2.5	87.1	12.9	

SNP, single-nucleotide polymorphism

2.5%. It was shown that the allele A increased the chances of disease 16.3 times more than allele C, and genotype AA increased the chances of disease 6.6 times more than CC.

5. Discussion

Many studies have explored the relationship between TNF- α gene polymorphisms and coronary artery disease or atherosclerosis [19, 27]. In several studies, increased levels of TNF- α protein have been reported in patients with MI; however, the role of TNF- α genotypes in the pathogenesis of atherosclerosis has not yet been clearly described. The aim of this study was to evaluate the possible association between TNF- α gene polymorphism of -308 G<A and -863 C<A with susceptibility to atherosclerosis, especially as TNF- α gene polymorphisms have not been studied in Iranian patients with atherosclerosis.

The results of our study showed significant differences in the distribution of genotypes and alleles of polymorphisms -308 G<A and -863 C<A between patients and healthy controls, and TNF- α gene polymorphisms in these two areas (-308 and -863) were associated with atherosclerosis disease. The results of this study are consistent with a previous study in Japan, which showed that the polymorphism of -863 C<A in the TNF- α gene and its association with CAD [28]. In another study, Vendrell et al reported that polymorphisms of -308 G<A in the TNF- α gene increased the risk of CAD in women with type 2 diabetes in Europe [19]. Conversely, in a study by Herman et al., it was reported that there was no relationship between TNF- α gene polymorphisms and susceptibility to CHD (coronary heart disease) [29]. Further, in some other studies, it was shown that no association existed between the polymorphism -308 G<A and CAD [27, 30]. These controversial results may be due to differences in genetic and environmental risk factors

that influence the differences in selection criteria of patients with coronary artery diseases. In our recent study, for the TNF- α gene polymorphism at -308, the GG genotype in healthy individuals and the AA genotype in patients had the highest frequencies.

In addition, for gene -863, the CC genotype was found to be higher in healthy subjects and AA genotype in patients. It was shown that in the frequency distribution of GA and AA for polymorphism -308 was G<A in patients, and for polymorphism -863, C<A. Previous studies have shown that the frequencies of polymorphisms -308G\A between Japanese, Indian, and European people were quite different [31, 32]. Moreover, some studies have shown that the frequency of allele polymorphism -308G\A in the Chinese population was similar to Japanese individuals [33].

Our study found that the risk of coronary artery disease or atherosclerosis in individuals carrying the allele A increased; therefore, it can be concluded that allele A can be a predictive marker of CAD patients in Iran. The results of this study confirmed previous results of Szalai and colleagues who found an increased risk of myocardial infarction in individuals carrying the allele A of TNF- α gene -308 [31]. Korean researchers have also shown that the polymorphism of TNF- α -238 G<A had a significant association with CAD. Additionally, allele A increased the risk of CAD and can be a predictive marker for sclerosis in Korea [34]. In a study conducted by Lee and colleagues in European patients with lupus showed that the TNF- α gene -308A allele was significantly associated with this autoimmune disease, but this allele in Asian and African people was not associated with CAD disease [34].

Several studies about the TNF- α polymorphism in inflammatory diseases showed a regulatory role of TNF- α and an association between the -308 G<A polymorphism and diseases, such as CHD [31], insulin resistance [35], and Alzheimer's [36]. However, several controversial findings also have been observed for polymorphisms of -308G\A and expression of TNF- α in cardiovascular diseases. Kaluza and colleagues using a luciferase reporter gene assay found that allele A of polymorphism -238 G>A significantly reduced transcriptional activity of TNF- α [21]. Therefore, further studies are needed to clarify the molecular mechanisms underlying the relationship between TNF- α polymorphisms and CHD.

6. Conclusion

The results of this study show a relationship between the TNF- α genotype polymorphism and atherosclerosis in an Iranian population. However, due to some controversial reports in other studies, additional complementary studies, especially on other inflammatory

mediators, are needed to better demonstrate the role of inflammation and cardiovascular disease.

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Conflicts of Interest

The authors report no declarations of conflict of interest.

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