

Polymorphisms in *NOS3*, *MTHFR*, *APOB* and *TNF- α* Genes and Risk of Coronary Atherosclerotic Lesions in Iranian Patients

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Background: Atherosclerosis is a complex multifocal arterial disease involving interactions between multiple genetic and environmental factors.

Objectives: In the present study, we investigated the possible association between *NOS3* (rs1799983), *MTHFR* (rs1801133), *APOB* (rs5742904) and *TNF- α* (rs361525) polymorphisms and the risk of coronary atherosclerotic lesions in Iranian patients.

Patients and Methods: In the case-control study, 108 patients with coronary atherosclerosis disease and 95 control subjects with no family history of cardiovascular disease were enrolled. Genotypes for *NOS3*, *MTHFR*, *APOB* and *TNF- α* polymorphisms were identified using polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP).

Results: We specifically detected the *NOS3* TT genotype in 12 patients (11.1%) and did not find the same genotype in any of the controls. The frequencies of T allele in patients and the controls were 24% and 17.8%, respectively. The prevalence of the *MTHFR* TT genotype was 16.7% in patients and 2.2% in control groups. The prevalence of the *APOB*-100 (R3500Q) mutation in this patient population was 0%. The frequency of the A allele in the *TNF- α* gene was 11.1% and 11% in patients and controls, respectively, and the AA genotype was undetected.

Conclusions: Our results show a significant association of *NOS3* and *MTHFR* gene polymorphisms with coronary atherosclerotic lesions. Therefore, these variants might influence the risk of coronary artery disease, specifically in the Iranian population.

Keywords: Atherosclerosis; Polymorphism; PCR-RFLP; *NOS3*; *MTHFR*; *APOB*; *TNF- α*

1. Background

Atherosclerosis initiates early in life and clinical coronary heart disease manifests in middle age and later. Several risk factors affect the appearance of coronary heart disease in adults (age, serum lipoprotein cholesterol levels, and smoking) and are also associated with the extent and severity of atherosclerotic lesions in young males (15 - 34 years of age) (1-3). Genetic variations are known to influence the occurrence of coronary atherosclerotic lesions (4).

Atherosclerosis results from an altered production of nitric oxide (NO) and a defective endothelial function, which is mainly an important endothelium-derived relaxing factor (5). The main enzyme required for vascular nitric oxide (NO) production is endothelial nitric oxide synthase 3 (*NOS3*) (6). Endothelial nitric oxide synthase (NOS) enzymes release NO during the conversion of L-arginine to L-citrulline. The common single nucleotide polymorphism (SNP) of the *NOS3* (GenBank ID 4846) gene in various populations is G894T polymorphism (rs1799983), where glutamic acid is substituted at codon 298 for aspartic acid in the seventh exon (Glu298Asp) (7, 8).

Methylenetetrahydrofolate reductase (*MTHFR*) is a regulatory enzyme of homocysteine (Hcy) metabolism, and is also necessary in the metabolism of tetrahydrofolate, as well as in the synthesis of purine, DNA and RNA. Elevated total plasma homocysteine concentration is now widely accepted as a major independent risk factor for cardiovascular and neural tube defects, colon cancer, acute leukemia, and peripheral vascular disease (9).

A potential candidate gene is the methylenetetrahydrofolate reductase (*MTHFR*) gene. The *MTHFR* gene has been mapped to the chromosomal region 1p36.3. A common C to T transition at nucleotide 677 (C677T) of the *MTHFR* gene-coding sequence leads to the substitution of alanine by valine in the protein structure. This genetic polymorphism is located in exon 4 of the *MTHFR* gene at the folate-binding site (10). The presence of this mutation was shown to associate with increased *MTHFR* thermolability and reduced specific activity. The T/T genotype was, therefore, considered a common risk factor for cardiovascular disease (CVD). It has been shown to predispose to

premature atherosclerosis coronary artery disease, and diabetic retinopathy (11, 12).

Arg3500Gln (R3500Q) mutation in the apolipoprotein B-100 (*APOB*-100) gene causes defected binding to the low-density lipoprotein (LDL) receptor and hypercholesterolemia (13). This is one of the most common inherited mutations, causing abnormality of the lipid metabolism is the mutation, and increased risk of atherosclerosis (14).

An important cytokine in the inflammation process of atherosclerosis is tumor necrosis factor (TNF) and is also involved in lipid metabolism (15). The -238A/G polymorphism in the promoter region of TNF gene has been reported to be associated with TNF production and with susceptibility to inflammatory diseases (16).

We have demonstrated recently that $\epsilon 3/\epsilon 3$ and $\epsilon 3/\epsilon 4$ genotypes of apolipoprotein E (*APOE*) gene are predisposing factors, which in combination with environmental factors, may trigger the degree of luminal narrowing in Iranian coronary atherosclerosis patients (17). Zamani et al. (18) observed two sequence variations in the hemoxygenase 2 (*HMOX2*) gene in 13 Iranian patients with atherosclerosis. They showed a significant association between A to G mutation in codon K89E of hemoxygenase 2 gene and the risk of atherosclerosis.

2. Objectives

Most researches have looked at relations between polymorphic changes in candidate genes and atherosclerosis. Based on this information, we aimed to determine the distribution of the *NOS3*, *MTHFR*, *APOB* and *TNF- α* polymorphisms in Iranian coronary atherosclerotic patients.

3. Patients and Methods

3.1. Participants

This research was performed on 108 patients (61 men and 47 women) selected from 350 cases who referred to cardiac centers in Afshar hospital (Yazd, Iran) during the period between 2012 and 2014, due to symptoms of myocardial infarction. Patients had significant lesions (> 50%

narrowing of luminal diameter) in one, two, or three vessels (left anterior descending artery (LAD), left circumflex (LCX) and right coronary artery (RCA)) that were candidates for coronary artery bypass graft (CABG).

Atherosclerotic patients were diagnosed according to the coronary angiography criteria. The imaging of carotid arteries was performed by ultrasonography to assess the extent of carotid atherosclerosis. The protocol of ultrasound examination involved scanning of the right and left common carotid artery and the area of the carotid sinus (bulb) as high up as possible (19).

Coronary arterial disease (CAD) was considered to be present when up to 50% blockage induced by stenotic lesions was observed in the major epicardial coronaries and their branches. Subjects without any pathological findings formed the control group. We also chose 90 unrelated healthy individuals (mean \pm SD: 53.6 \pm 7.8) that matched for age, sex, and ethnicity to obtain normal or near-normal angiography reports (no lesion greater than 30%) were considered as the control group (Table 1). All the patients and controls were informed of the aims of the study and gave their informed consents for the genetic analysis.

3.1. DNA Studies

Whole blood samples (3 - 5 mL) were collected in an EDTA tube. DNA was isolated by the salting out method. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was performed on 3 SNPs in the *NOS3*, *MTHFR* and *APOB* genes. The primer sequences and PCR parameters are listed in Table 2. PCR was performed in a 25- μ L total reaction volume using 40 ng genomic DNA. The specific DNA fragment was amplified for 30 cycles at 94°C for 30 seconds and 57 - 62°C for 30 seconds, with a final extension at 72°C for 10 minutes. The digested PCR products were run on a 2% agarose gel and stained with ethidium bromide and on 8% polyacrylamide gel and stained with silver. The primer sets, the sizes of PCR products and their corresponding digested products are listed in Tables 2 and 3, respectively.

Table 1. The Summary of the Clinical of Coronary Atherosclerosis Patients ^a

| | Patients (n = 108) | Controls (n = 90) | P Value |
|------------------------------------|--------------------|-------------------|---------|
| Male, % | 61 | 57 | 0.383 |
| Age, y | 53.2 \pm 7.6 | 53.6 \pm 7.8 | 0.51 |
| Smokers, % | 25 | 20 | 0.737 |
| Body mass index, kg/m ² | 25.1 \pm 2.2 | 24.7 \pm 1.7 | 0.0017 |
| Cholesterol, mg/dL | 212.6 \pm 52.3 | 172.6 \pm 37.5 | 0.001 |
| LDL-C, mg/dL | 126.2 \pm 44.5 | 116.8 \pm 45 | 0.132 |
| HDL-C, mg/dL | 46.7 \pm 8.6 | 45.6 \pm 10.7 | 0.243 |
| TGs, mg/dL | 202.4 \pm 108.5 | 156.5 \pm 94.8 | 0.019 |

^a Abbreviations: HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; and TGs, Triglycerides.

Table 2. Primers and Parameters Used for PCR Amplification of Polymorphisms

| SNP ID | Gene | Primer Sequences | Size of PCR Products, bp | Annealing Temperature, °C |
|-----------|---------------|----------------------------------|--------------------------|---------------------------|
| rs1799983 | NOS3 | F: TCACGGAGACCCAGCCAATGAG | 292 | 61.5 |
| | | R: TCCATCCCACCCAGTCAATCCC | | |
| rs1801133 | MTHFR | F: TGAAGGAGAAGGTGTCTGCGGGA | 197 | 60 |
| | | R: AGGACGGTGCGGTGAGAGTG | | |
| rs5742904 | APOB | F: CCAACACTTACTTGAATCCAAGAGCACCC | 334 | 61.5 |
| | | R: TCTTCTGGTTCTTAGTGTTAGCAT | | |
| rs361525 | TNF- α | F: AGAAGACCCCTCGGAAcC | 155 | 57.5 |
| | | R: ATCTGGAGGAAGCGGTAGTG | | |

Table 3. Determination of the SNP Genotypes Using PCR-RFLP Assay

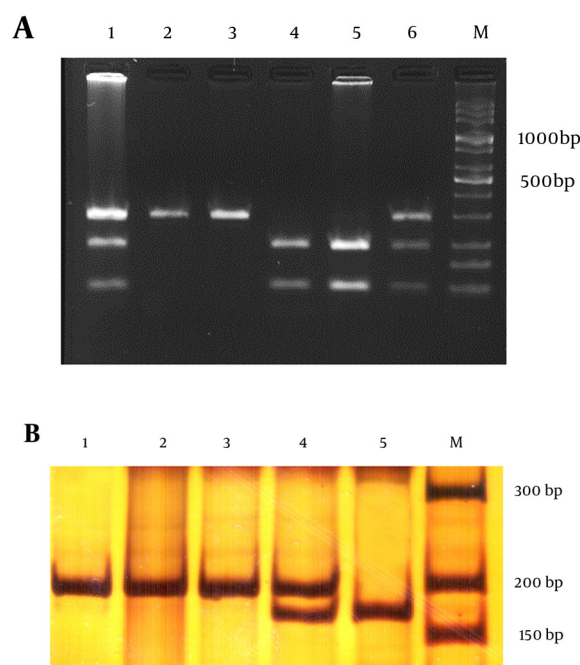
| SNP ID | Gene | Restricted Enzyme | Fragments, bp | Genotype |
|-----------|---------------|-------------------|---------------|---|
| rs1799983 | NOS3 | MboI | 292 | GG: 197 bp + 95 bp; GT: 292 bp + 197 bp + 95 bp; TT: 292 bp |
| rs1801133 | MTHFR | HinI | 198 | CC: 198 bp; CT: 198 bp + 178 bp + 20 bp; TT: 178 bp + 20 bp |
| rs5742904 | APOB | MspI | 334 | GG: 313 bp + 21 bp; GA: 334 bp + 313 bp + 21 bp; AA: 334 bp |
| rs361525 | TNF- α | MspI | 155 | GG: 136 bp + 19 bp; GA: 155 bp + 136 bp + 19 bp; AA: 155 bp |

NOS3 (SNP ID: rs1799983): The G894T polymorphism (in exon 7 of *NOS3*) results in substitution of amino acid asparagine with glutamic acid at position 298 (N298E) and introduces the restriction site for MboI endonuclease. As shown in Figure 1, undigested fragment (292 bp) was detected in homozygotes for the G894 allele (genotype GG), digested fragments (197 and 95 bp) were detected in homozygotes for T894 allele (genotype TT) and both digested and undigested fragments (292, 197 and 95 bp) were detected in heterozygotes (genotype GT).

MTHFR (SNP ID: rs1801133): C677T polymorphism (exon 4 *MTHFR* gene) results in substitution of amino acid alanine with valine at position 222 (A222V) and introduces the restriction site for HinI endonuclease. According to Figure 1, undigested fragment (198 bp) was detected in homozygotes for C677 allele (genotype CC), digested fragments (178 and 20 bp) were detected in homozygotes for T677 allele (genotype TT) and digested and undigested fragments (198, 178 and 20 bp) were detected in heterozygotes (genotype CT).

APOB (SNP ID: rs5742904): G1059A polymorphism (exon 26 of *APOB*) results in substitution of arginine by glutamine at codon 3500 (R3500Q) and creates an MspI restriction site in the normal allele but not in the mutant allele. Undigested fragment (334 bp) was detected in homozygotes for G1059 allele (genotype GG), digested fragments (313 and 21 bp) were detected in homozygotes for A1059 allele (genotype AA) and both digested and undigested fragments (334, 313 and 21 bp) were detected in heterozygotes (genotype GA).

TNF- α (SNP ID: rs361525): G-238A polymorphism (promoter *TNF- α* gene) results in TNF production and introduces the restriction site for MspI endonuclease. Undigested fragment (155 bp) was detected in homozygotes for A-238 allele (genotype AA), digested fragments (136 and 19 bp) were detected in homozygotes for G-238 allele (genotype GG) and digested and undigested fragments (155, 136 and 19 bp) were detected in heterozygotes (genotype GA).

Figure 1. Results of Gel Electrophoresis Analysis

Gel electrophoresis analysis of PCR-RFLP of rs1799983 in *NOS3* (A) and rs1801133 in *MTHFR* (B), respectively. Image of 2% ethidium bromide stained agarose and 8% silver stained. A) lanes 1 and 6 show heterozygous (GT), lanes 4 and 5 show homozygous wild type (GG), lanes 2 and 3 show homozygous mutant (TT). B) lanes 1 - 3 show homozygous wild type (CC), lane 4 shows heterozygous (CT) and lane 5 shows homozygous mutant (TT).

3.2. Data Analysis

Fisher's exact test was used to determine associated between *NOS3*, *MTHFR*, *APOB* and *TNF- α* gene polymorphisms and the genetic risk of coronary atherosclerosis. Statis-

tical significance was tested using the GraphPad Prism software (GraphPad Software, Inc. USA) and $P < 0.05$ was considered to indicate a statistically significant result.

4. Results

Mean age (mean \pm SD) was 53.4 ± 7.8 and 52.8 ± 7.9 years for patients and controls, respectively. Coronary angiography revealed 108 patients (CAD+ group) with one-vessel (LAD) ($n = 30$), two-vessels (LCX) ($n = 42$), or three-vessels (RCA) ($n = 36$) that were candidate for CABA (coronary artery bypass graft) and 90 patients (CAD- group) with no angiographically identified narrowing.

4.1. NOS3

Allelic frequencies of *NOS3* polymorphism among the patients with coronary atherosclerotic lesions and controls were 76% and 82.2%, respectively, for allele G (wild type), and 24% and 17.8%, respectively, for allele T (mutant). No association between allele T and the occurrence of CAD was found ($P = 0.14$). A significantly different between the patients (G/G: 63%; G/T: 26%; TT: 12%) and the control group (G/G: 64.5%; G/T: 35.5%; T/T: 0.0%) ($P = 0.0006$).

4.2. MTHFR

Allelic frequencies of *MTHFR* polymorphism among the patients with coronary atherosclerotic lesions and

controls were 66.7% and 82.8%, respectively, for allele C (wild type), and 33.3% and 17.2%, respectively, for allele T (mutant). An association between allele T and the occurrence of CAD was found ($P = 0.0003$). A significantly different between the patients (C/C: 50%; C/T: 33.3%; TT: 18%) and the control group (C/C: 67.7%; C/T: 30%; T/T: 2.2%) ($P = 0.0006$).

4.3. APOB

Allelic frequencies of *APOB* polymorphisms among the patients with coronary atherosclerotic lesions and controls were 0% for allele A (mutant).

4.4. TNF- α

Allelic frequencies of *TNF- α* polymorphism among the patients with coronary atherosclerotic lesions and controls were 88.9% and 93.8%, respectively, for allele G (wild type), and 11.1% and 11%, respectively, for allele A (mutant). No association between allele A and the occurrence of CAD was found ($P = 0.1087$). Also no significantly difference found between the patients (G/G: 77.7%; G/A: 22.3%; A/A: 0.0%) and the control group (G/G: 87.7%; G/A: 12.3%; A/A: 0.0%) ($P = 0.0915$).

The results of statistical analysis of the *NOS3* (rs1799983), *MTHFR* (rs1801133), *APOB* (rs5742904) and *TNF- α* (rs361525) polymorphisms and the risk of coronary atherosclerotic lesions were summarized in Table 4.

Table 4. Genotype Counts and Allele Frequencies Variants in Patients and Controls

| SNP ID | Patients (n = 108, 216 Alleles) ^a | Controls (n = 90, 180 Alleles) ^a | P Value | Odds Ratio (95% CI) |
|--|--|---|---------------------|---------------------------|
| <i>NOS3</i> (rs1799983) genotypes | | | 0.0006 ^b | 0.04265 (0.0024 - 0.7314) |
| GG | 68 (63) | 58 (64.5) | | |
| GT | 28 (26) | 32 (35.5) | | |
| TT | 12 (11.1) | 0 | | |
| <i>NOS3</i> (rs1799983) alleles | | | 0.14 | 0.6819 (0.4163 - 1.117) |
| G | 164 (76) | 148 (82.2) | | |
| T | 52 (24) | 32 (17.8) | | |
| <i>MTHFR</i> (rs1801133) Genotypes | | | 0.0006 ^c | 0.1136 (0.256 - 0.5054) |
| CC | 54 (50) | 61 (67.7) | | |
| CT | 36 (33.3) | 27 (30) | | |
| TT | 18 (16.7) | 2 (2.2) | | |
| <i>MTHFR</i> (rs1801133) alleles | | | 0.0003 | 0.4161 (0.2576 - 0.6721) |
| C | 144 (66.7) | 149 (82.8) | | |
| T | 72 (33.3) | 31 (17.2) | | |
| <i>APOB</i> (rs5742904) genotypes | | | - | - |
| GG | 108 (100) | 90 (100) | | |
| GA | 0 | 0 | | |
| AA | 0 | 0 | | |
| <i>TNF-α</i> (rs361525) genotypes | | | 0.0915 | 0.4873 (0.2240 - 1.060) |
| GG | 84 (77.7) | 79 (87.7) | | |
| GA | 24 (22.3) | 11 (12.3) | | |
| AA | 0 | 0 | | |
| <i>TNF-α</i> (rs361525) alleles | | | 0.1087 | 0.5207 (0.2477 - 1.095) |
| G | 192 (88.9) | 169 (93.8) | | |
| A | 24 (11.1) | 11 (6.2) | | |

^a Values are presented as No. (%).

^b Calculation was performed for GG and GT versus TT.

^c Calculation was performed for CC and CT versus TT.

5. Discussion

Atherosclerosis develops as a result of complex interaction between multiple genes and environmental factors (such as nutrition, smoking, obesity and physical exercise) (20). Considerable amount of evidence has accumulated evaluating the role of the *NOS3* gene not only in cardiovascular disease but also in other complex disorders (21-23). Philip et al. recently showed enhanced vasoconstriction in response to phenylephrine in Asp298 homozygotes that may be ascribed to an impaired endothelial NO modulation of adrenergic vasoconstriction (24). Previous studies from Japan and the UK have suggested a role for Asp298Glu polymorphism in the development of coronary atherosclerosis (25). In this study, we investigated the relationship between Asp298Glu missense variant, which results from G to T mutation in exon of the *NOS3* gene, and CAD in the Iranian population. Our data show that this missense variant of *NOS3* confers and increased risk of developing coronary atherosclerosis in Iranian patients.

Our results showed that homozygosity frequency of the 677 C > T variant in *MTHFR* gene was 16.7% and, allele frequency of T was 33.3%. These results demonstrated an association 677 C > T variant and increased risk of developing coronary atherosclerosis in Iranian patients. The 677 C > T variant is extremely common in North America, with homozygosity frequency of 11% - 15% and, allele frequency of approximately 35% while in Europeans, the homozygous mutant genotype ranges from 5% to 23% (26, 27).

The frequencies of *APOB* gene variants are different between populations. The frequency of *APOB*-100 (R3500Q) mutation is estimated as (1/200) in central Europe (e.g. Switzerland), decreasing gradually in Mediterranean or Northern European populations (28). The *APOB*-100 (R3500Q) mutation could not be found in Lebanon (29), Russia (30) and Turkey (31). Mutations in the *APOB* gene were not detected in any of our patients. Similar results were also obtained in previous studies in Iran (32, 33). Our finding about R3500Q mutation is quite expected to the geographic distribution of this mutation.

It seems that *TNF- α* has an important role in the development of the myocardial ischemia and infarction and the stenosis of the arteries (16, 34, 35). Cho et al. (36) analyzed *TNF- α* -238 G/A polymorphism in Korean CAD patients. They established that the wild-type *TNF- α* -238 GG genotype occurrence is 14.2%, the heterozygote GA is 58.8%, while the mutant AA is 0.0 %. Our results showed that at atherosclerotic patients *TNF- α* -238 GG genotype is found with a frequency of 77.7% compared to its frequency in the control group (87.7%). We did not find significantly difference found between the patients and the control group.

In conclusion, our results demonstrate the 894 G > T polymorphism of *NOS3* gene and the 677 C > T variant of *MTHFR* gene influenced the risk of CAD specifically in the Iranian population. However, the study of other polymorphisms of these genes might be clinically useful as markers to increasing the risk of CAD.

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Authors' Contributions

Study concept and design: Mohammad Mehdi Heidari; Acquisition of data: Mehdi Hadadzadeh, Mahbobeh Kazemi, Sahar Mahamed, Pegah Malekzadeh, Massomeh Mirjalili; Analysis and interpretation of data: Mehri Khatami; Drafting of the manuscript: Mohammad Mehdi Heidari, Mehri Khatami; Critical revision of the manuscript for important intellectual content: Mohammad Mehdi Heidari; Statistical analysis: Mohammad Mehdi Heidari; Administrative, technical, and material support: Mohammad Mehdi Heidari, Mehdi Hadadzadeh, Mahbobeh Kazemi, Sahar Mahamed, Pegah Malekzadeh, Massomeh Mirjalili; Study supervision: Mohammad Mehdi Heidari, Mehri Khatami.

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