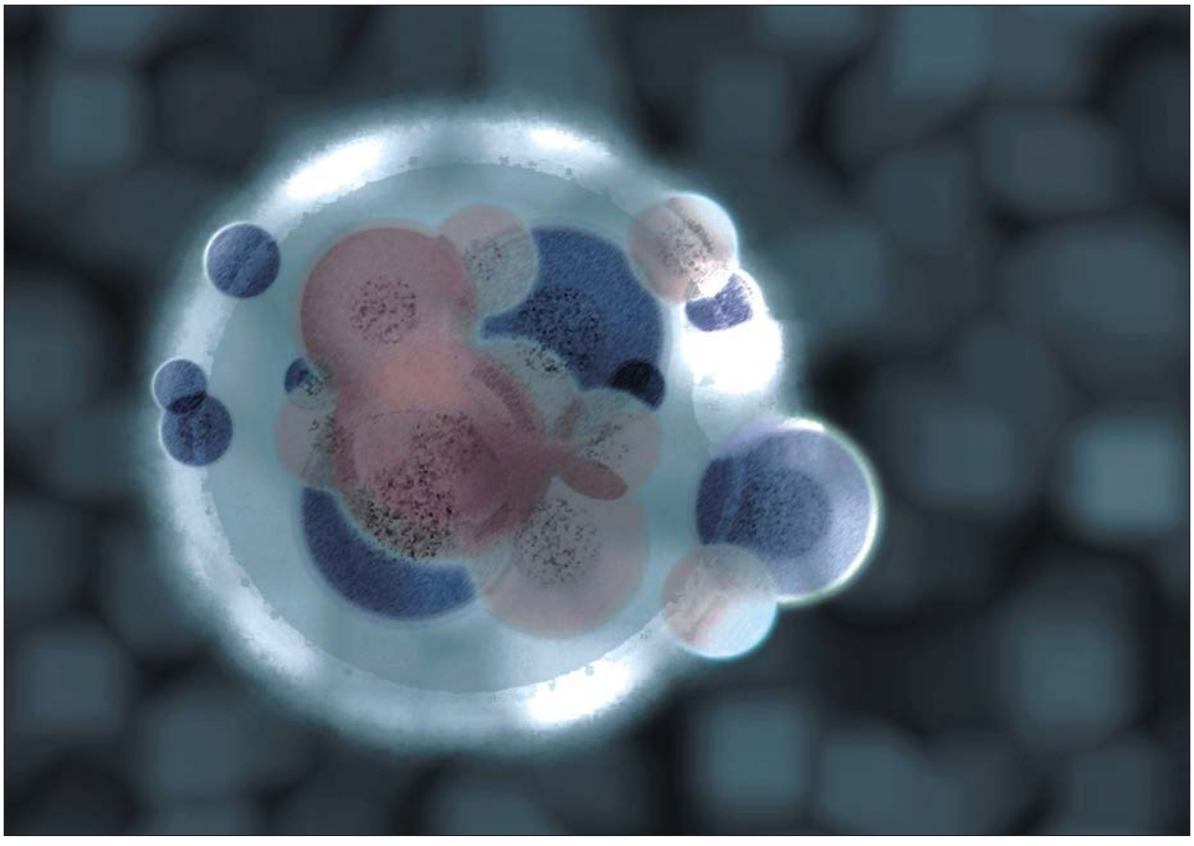

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Lack of Evidence for Association Between Endothelial Nitric Oxide Synthase Gene Polymorphisms (T-786C AND G894T) and Early-Onset Coronary Artery Disease

[1]Nurten Kara, [2]Osman Yesildag, [1]Serbulent Yigit, [2]Serkan Yuksel, [1]Hasan Bagci and [3]Yuksel Bek
[1]Department of Medical Biology and Genetics,
[2]Department of Cardiology,
[3]Department of Biostatistics,
Faculty of Medicine, Ondokuz Mayis University, 55139, Samsun, Turkey

Abstract: In this study, we investigated the association of two genetic variants of the endothelial nitric oxide synthase (eNOS), T786C and G894T, with CAD. Polymerase chain reaction and restriction fragment length polymorphism analyses were performed to detect the variants of eNOS gene in 102 patients with coronary artery disease and 103 healthy controls under the age 55. The statistical analysis was done by Chi square. The genotype and allele frequencies of CAD patients and controls were compared for the T786C genetic variant of eNOS. No evidence of significant difference was found between the two groups in the frequencies of the genotype [p = 0.810, OR = 0.77(0.25-2.15)] and allele [p = 0.173, OR = 0.72(0.47-1.11)]. In addition, the genotype and allele frequencies of CAD patients and controls were compared for the G894T genetic variant of eNOS. Similarly, no evidence of significant difference were found between the frequencies of genotype [p = 0.627, OR = 0.69 (0.25-1.88)] and allele [p = 0.610, OR = 0.87 (0.56-1.36)]. The present study provides evidence that the T786C and G894T polymorphism of the eNOS gene are not associated with the early-onset coronary artery disease in our population.

Key words: Coronary artery disease, endothelial nitric oxide synthase, gene polymorphism

INTRODUCTION

In the vascular endothelium, Nitric Oxide (NO) is produced by endothelial Nitric Oxide Synthase (eNOS) from L-arginine (Stuehr, 1999) and causes relaxation of Vascular Smooth Muscle Cells (VSMC), the basis for blood flow and pressure regulation (Umans and Levi, 1995). NO also inhibits platelet and leucocyte adhesion to the endothelium and reduces vascular smooth muscle cell migration and proliferation (Stuehr, 1999).

Both animal models and human studies highlight the important role of NO in vascular disease states. Targeted deletion of the eNOS gene in mice results in hypertension (Huang *et al*., 1995) and impairs vascular remodeling (Rudic *et al*., 1998), whereas augmenting vascular NO production by local gene delivery of NOS in animal models improves endothelial function, limits neointimal proliferation and induces regression of atherosclerotic lesions (Channon *et al*., 2000).

The eNOS gene (eNOS) is located on chromosome 7q35-36, comprises 26 exons and spans 21 kb DNA (Marsden *et al*., 1993). Several studies have addressed possible relationships between eNOS polymorphisms and vascular disease states such as coronary artery disease (Hingorani *et al*., 1999; Colombo *et al*., 2002; Colombo *et al*., 2003), myocardial infarction (Hingorani *et al*., 1999; Hibi *et al*., 1998; Shimasaki *et al*., 1998), hypertension (Yasujima *et al*., 1998; Shoji *et al*., 2000) and Behcet disease (Kara *et al*., 2006). In particular, the G to T polymorphism at position 894 of eNOS gene, resulting in a change from Glu to Asp at codon 298, is the only polymorphism identified to date that changes the eNOS protein sequence, leading to speculation that genetic variation, at this site may alter eNOS activity or regulation (Guzik *et al*., 2001). The 298Asp variant has been associated with myocardial infarction (Shimasaki *et al*., 1998) and was identified as a risk factor for coronary artery disease in a Caucasian population (Hingorani *et al*., 1999), whereas some other studies do not support these findings (Granath *et al*., 2001; Wang *et al*., 2001). In addition, a single nucleotide polymorphism (SNP), -786T>C, was identified in the 5' flanking region of the eNOS involving a substitution of Thymine (T) to Cytosine (C) at position 786 base pairs upstream of the eNOS transcription start site (Nakayama *et al*., 1999). Another, a 27 base pair (bp)

Corresponding Author: Dr. Nurten Kara, Department of Medical Biology and Genetics, Faculty of Medicine, Ondokuz Mayis University, 55139, Samsun, Turkey
Tel: +90 362 3121919/3047 Fax: +90 362 4576041

repeat polymorphism in intron 4 of the eNOS gene (eNOS 4a4b), was also reported (Wang *et al*., 1996). Many epidemiological studies evaluated the eNOS polymorphisms in patients with CAD, but the results are often conflicting. Therefore, in the present study, we examined two polymorphisms of the eNOS gene and evaluated the relationship between the polymorphisms and the development of CAD in a Turkish population.

MATERIALS AND METHODS

Study population: We studied 102 patients (90 men and 12 women), consecutively admitted to our hospital with angiographically proven CAD and 103 healthy control (51 men and 52 women) subjects. The patients and controls were unrelated individuals. All subjects were Caucasian with Turkish origin and from Northern Turkey.

The patients and healthy controls were interviewed and data on smoking habits, hypertension, diabetes, hypercholesterolemia, family history of CAD and number of diseased vessels were recorded.

Blood specimens were collected between 2002 and 2004 years after approval by the Ondokuz Mayis University Research Ethics Committee and after obtaining informed consent. All patients underwent coronary angiography. Coronary stenosis was considered significant if the luminal diameter of at least one epicardial coronary artery was narrowed by >50%. For coronary risk factors, the following definitions were used: individuals were defined as hypertensive if their blood pressure was >140/90 mmHg or if they were receiving any antihypertensive treatment; individuals with a history of diabetes or those receiving any antidiabetic medication were considered to be diabetic; individuals were accepted dyslipidemic when their total cholesterol concentration was >200 mg dL^{-1}, their triglyceride concentration was >200 mg dL^{-1}, or they were receiving lipid-lowering drugs. Smoking history was coded as never and current smoker. The family history was considered positive for CAD if at least one first-degree relative was diagnosed with CAD male or female at various ages.

DNA analysis: Genomic DNA was extracted from samples of whole blood by standard salting-out method (Miller *et al*., 1998).

The eNOS genotyping for -786T>C mutation was performed as described by Alvarez *et al.* (2001). The primers used were 5′-TGG AGA GTG CTG GTG TAC CC CA-3′ (forward) and 5′-GCC TCC ACC CCC ACC CTG TC-3′ (reverse). The following program was used for PCR reactions: 95°C for 2 min, 95°C for 30 sec and 63°C for 60 sec, 72°C for 60 sec, 35 cycles with 5 min final elongation step. PCR amplifications were performed in total volume of 25 μL. The amplification product (180 bp) was digested by *MspI* (Fermentas). Fragments of 40 (constant) and 140 bp (-786 T) or 90 and 50 bp (-786 C) were visualized on 2.5% numicropore agarose (Prona) gel.

The coding sequence variant was G→T substitution at position 894 in exon 7, which changes Glu to Asp in the eNOS protein. Genotyping of all subjects was performed as described by Hingorani *et al.* (1999). The primers used were 5'- CAT GAG GCT CAG CCC CAG AAC-3' (forward) and 5'-AGT CAA TCC CTT TGG TGC TCAC-3' (reverse). The following program was used for PCR reactions: 95°C for 3 min, 94°C for 45 sec, 60°C for 45 sec, 72°C for 60 sec, 35 cycles with 5 min final elongation step. PCR amplifications were performed in total volume of 25 μL. PCR products were digested with *Mbo*I (Fermentas) restriction enzyme overnight at 37^0C. The 206 bp PCR product is cleaved into 119 bp and 87 bp fragments in the presence of a T at nucleotide 894 which corresponds to Asp. The digestion products were analyzed by electrophoresis on a 2% numicropore agarose gel (Prona).

Statistical analysis: Statistical analysis was done by using the SPSS 13.0 for Windows, release 13.01 (license code 9071653). The distribution of the genotypes for healthy control and coronary artery disease was checked for Hardy-Weinberg equilibrium.

The frequencies of the alleles and genotypes in patients and controls were compared by Chi-square analysis. Odds ratios and the 95% confidence intervals (95% CI) were calculated. A probability value of $p<0.05$ was considered to be significant for genotype, allele frequencies and composite genotypes. In addition, multiple logistic regression analysis was used for comparison of demographic characteristics and distribution of risk factors in patients and control subjects. A probability value of $p<0.01$ was considered to be significant for demographic and clinical characters

RESULTS

All of the patients were younger than 55 years. The mean (±SD) ages of the CAD group and controls were 49.8±4.2 and 48.9±5.8 years, respectively. The mean age was higher in CAD patients than in the control group, but the difference was not statistically significant. Some atherogenic risk factors (male sex, total cholesterol, cigarette smoking, family history of CAD) were significantly higher in early CAD patients ($p<0.01$) (Table 1).

We genotyped 102 patients and 103 healthy control subjects from the same region of Northern Turkey for

Table 1: Clinical and demographics of the study population

Characteristics	CAD (n = 102)	Controls (n = 103)	p-value
Mean age years	49.8±4.2	48.9±5.8	0.140
Male sex n (%)	90 (88.2%)	51 (49.5%)	<0.001
Hypertension n (%)	28 (27.5%)	16 (15.5%)	0.056
Diabetes n (%)	10 (9.8%)	3 (2.9%)	0.049
Total cholesterol>200 mg dL^{-1} n (%)	38 (37.3%)	3 (2.9%)	<0.001
Smoking n (%)	75 (73.5%)	7 (6.8%)	<0.001
Family history of CAD	54 (52.9%)	9 (8.7%)	<0.001
Number of diseased vessels			
One vessel	37.0	-	
Two vessel	34.0	-	
Three vessel	31.0	-	

Table 2: Distribution of eNOS genotypes and allele frequencies of coronary artery patients and controls

Genotypes and alleles	Patients (n = 102)	Controls (n = 103)	p-value	Odds ratios	95% CI
Genotypes n (%)					
-786T>C					
TT	58 (56.9)	47 (45.6)			
TC	37 (36.3)	47 (45.6)	0.756		
CC	7 (6.8)	9 (8.8)			
Dominant model n (%)					
CC+TC	44 (43.1)	56 (54.4)	0.141	0.64	0.35-1.15
TT	58 (56.9)	47 (45.6)			
Recessive model n (%)					
TT+TC	95 (93.1)	94 (91.2)	0.810	0.77	0.28-2.15
CC	7 (6.9)	9 (8.8)			
Alleles					
T allele	153.0	141.0	0.173	0.72	0.47-1.11
C allele	51.0	65.0			
894G>T (glu298asp)					
GG	60 (58.8)	58 (56.3)			
GT	35 (34.3)	35 (34.0)	0.274		
TT	7 (6.9)	10 (9.7)			
Dominant model n (%)					
TT+GT	42 (41.2)	45 (43.7)	0.82	0.90	0.50-1.63
GG	60 (58.8)	58 (56.3)			
Recessive model n (%)					
GG+GT	95 (93.1)	93 (90.3)	0.627	0.69	0.25-1.88
TT	7 (6.9)	10 (9.7)			
Alleles					
G allele	155.0	151.0	0.610	0.87	0.56-1.36
T allele	49.0	55.0			

Table 3: Composite genotype analysis of the eNOS gene polymorphisms -786T>C and 894G>T

Composite genotypes (%)	Patients (n = 102)	Controls (n = 103)	p-value	OR	95% CI
-786T>C/894G>T					
TT/GG	40 (39.2%)	36 (35.00%)	0.626	1.2	0.681-2.118
TC/GT	16 (15.7%)	20 (19.34%)	0.604	0.77	0.375-1.591
CC/TT	0 (0.0%)	3 (2.90%)	0.248*		

variants the -786T>C and Glu298Asp in the eNOS gene. The genotype and allele frequencies in CAD patients and controls of the -786T>C and Glu298Asp polymorphisms are shown in Table 2. The distribution of the eNOS-786T>C and 894G>T genotype and allele frequencies in patients and controls was compatible with Hardy-Weinberg's equilibrium (HWE; χ^2 = 0.11, p = 0.946; χ^2 = 0.366, p = 0.833, respectively).

Differences in prevalences of the -786T>C genotypes (TT, TC, CC) between CAD and controls were not statistically significant (p = 0.756). According to dominant and recessive model of inheritance, respectively, there was no significant association between the polymorphism of -786T>C and CAD (p = 0.41, OR = 0.64; 95% CI: 0.35-1.15), (p = 0.810, OR = 0.77; 95% CI: 0.28-2.15) (Table 2). In addition, allele frequencies did not differ significantly between CAD and controls (p = 0.173, OR = 0.72; 95% CI: 0.47-1.11) (Table 2).

Differences in prevalences of the 894G>T genotypes (GG, GT, TT) were not statistically significant between CAD and controls (p = 0.274) (Table 2). According to dominant and recessive model of inheritance, respectively, there was no significant association between the polymorphism of 894G>T with CAD (p = 0.82, OR = 0.90; 95% CI: 0.50-1.63), (p = 0.627, OR = 0.69; 95% CI: 0.25-1.88) (Table 2). Allele frequencies did not differ

between CAD and controls (p = 0.610, OR = 0.87; 95% CI: 0.56-1.36) (Table 2). In addition, analysis of composite genotypes did not show association a significant between genotype and CAD (p = 0.626 for TT, GG; p = 0.604 for TC, GT; p = 0.248 for CC, TT genotype) (Table 3).

DISCUSSION

In this study, we demonstrated that the eNOS -786T>C and 894G>T polymorphisms are not associated with early-onset CAD. In our population, early CAD is strongly associated with male sex, high total cholesterol, smoking and CAD in family history. According to Cam *et al.* (2005) family history, hypertension, diabetes, smoking, obesity, high total cholesterol, LDL-C triglycerides levels and low HDL-C were increasing the risk of premature CAD.

Recent reports of molecular genetic analysis have suggested that genetic polymorphisms of the eNOS gene may be associated with CAD or Myocardial Infarction (MI). According to Nakayama *et al.* (1999) the -786 C allele is associated with a significantly reduced eNOS promoter activity. The reduced endothelial production of NO in the coronary arteries would predispose carriers of the C allele to coronary spasm (Alvarez *et al.*, 2001).

-786T>C polymorphism in the 5' flanking region of the eNOS3 gene was significantly associated with early CAD in their population. The frequency of the CC genotype was significantly increased (p = 0.039) in patients compared to controls. Colombo *et al.* (2002) studied 201 patients with CAD and 114 controls from Italy and concluded that Glu298Asp polymorphism of the eNOS gene appears to be associated with the presence, extent and severity of angiographically assessed CAD. Tangurek *et al.* (2006) found a statistically significant difference in the -786T>C distribution between CAD and normal individuals (p<0.05). Hingorani *et al.* (1999) studied 298 patients with positive coronary angiograms and 138 unrelated healthy individuals from the United Kingdom and reported that the Glu298Asp variant of the eNOS is a major risk factor for CAD. Still, Colombo *et al.* (2003) showed that Glu298Asp and -786T>C polymorphism were associated with the presence and severity of angiograpically defined CAD in the Italian population and that those individuals carrying both eNOS variants simultaneously might have a higher risk of developing CAD. Cam *et al.* (2005) found a significant association between TT genotype and premature CAD for the polymorphism 894G>T (p = 0.0001). In the same way, Kim *et al.* (2007) studied to influence of eNOS gene polymorphisms (-786T>C, 4a4b, 894G>T) in Korean patients with CAD. They demonstrated that polymorphisms of the eNOS -786T>C and 4a4b are associated with CAD in the Koreans. Fatini *et al.* (2004) showed that was associated with an increased susceptibility to the disease in acute coronary syndromes carrying both of the 894 T and -786C homozygous variants.

The polymorphisms of endothelial nitric oxide synthase gene have been associated with coronary artery disease in some but not all studies. For instance, Granath *et al.* (2001) in a large case-control study, they found no evidence for an association between several eNOS gene polymorphisms (eNOS 4ab, G894T, T-786C) and premature CAD in an Australian Caucasian population. In addition, Wang *et al.* (2001) found no evidence of an association between the G894T variant of the eNOS gene and CAD/MI among Taiwanese. To determine the impact of the mutant Asp298 eNOS allele on the development of premature CAD, Nassar *et al.* (2001) examined the prevalence of this mutation in patients with early-onset CAD and compared with the prevalence in patients manifesting CAD later in life. There was no significant difference in the frequency of the mutant Asp 298 allele between the two groups. Guzik *et al.* (2001) studied the relationship between the G894T polymorphism in eNOS and NO-mediated endothelial function in 104 atherosclerotic patients undergoing routine coronary artery bypass and found that this polymorphism did not have a major direct functional effect on vascular eNOS activity in human atherosclerosis. Aras *et al.* (2002) studied 205 patients with CAD and 117 controls from our Country. They found no association [OR = 1.37(0.87-2.16, p = 0.17] between eNOS gene polymorphism (Glu298Asp) and CAD in middle Anotolian Turkish population. Similarly, we found no evidence of an association between the eNOS gene -786T>C and 894G>T polymorphisms with early CAD in Northern Turkey. In addition, composite genotypes did not show association with early CAD. Findings of eNOS gene polymorphism are shown differently in Turkish Populations. The differing results of genetic associations may stem from different populations and sizes (Ioannidis *et al.*, 2003).

In summary, we have studied the relationship of -786T>C and Glu298Asp variants of endothelial nitric oxide synthase gene with early-onset coronary artery disease in a Turkish population. Present study does not support the hypothesis that homozygosity for -786C and asp298 for early-onset CAD in northern Turkey population. In premature CAD, additional studies on other populations are required to confirm these results.

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