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Association between T-786C polymorphism of endothelial nitric oxide synthase gene and level of the vessel dilation factor in patients with coronary artery disease

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A B S T R A C T

Various polymorphisms on endothelial nitric oxide synthase (*eNOs*) gene cause reduced production of NO, the endothelial relaxing factor, and may accelerate the process of atherosclerosis. The study designed to investigate the frequency of T-786C polymorphism of the *eNOs* gene in patients suffering from coronary artery disease (CAD) in north-west of Iran. One hundred twenty subjects including 60 patients with angiographically diagnosed CAD and 60 age and sex matched CAD-free subjects as control were studied. The levels of nitric oxide in the samples were measured with the Griess Method. The genotype studies were carried using allele specific PCR. Comparing with the control, reduced levels of NO were noticed in the patient group (P<0.05). Statistical analysis showed that the genotype TC associated with risk of CAD (OR=2.50, 95% CI: 1.13-5.50, P=0.023). The prevalence of C allele was significantly higher in patients compared to control group (OR=2.04, 95% CI: 1.16-3.57, P=0.013). The low levels of NO and increased frequency of T-786C polymorphism might be a risk factor in progression of coronary artery disease in the studied subjects.

Keywords: Coronary artery disease; Endothelial nitric oxide synthase gene; T-786C polymorphism; Nitric oxide

INTRODUCTION

Coronary artery disease (CAD) is the major cause of mortality and morbidity in most countries. From among many traditional risk factors for CAD development positive family history are now being considered as significant and novel risk factor. Atherosclerosis, an essential factor for the development of CAD, results from a defective endothelial function, which is ascribed mainly to an altered production of nitric oxide (NO), an important endothelium-derived relaxing factor [1]. NO is synthesized via a reaction that includes the conversion of L-arginine to L-citruline catalyzed by endothelial nitric oxide synthase (eNOS),

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which is one of the three isoforms of the enzyme [2]. eNOS is the product of eNOS gene, which is 21kb in size and consists of 26 exons [3]. Additionally, promoter region of the eNOS gene harbors several transcription factor binding sites, regulating gene expression [4].

Nitric oxide (NO) is a vasoactive substance and a major mediator of endothelium-dependent vasodilatation, which is synthesized and also released in the vascular endothelium [5]. Nitric oxide diffuses from the endothelium to the vascular smooth muscle cells, where it increases the concentration of cyclic guanosine monophosphate (cGMP) by stimulating soluble guanyl cyclase, leading to vascular relaxation [5].

Nitric oxide (NO) is a free radical molecule that plays an essential role in numerous physiological actions, including vaso regulation, inhibition of platelet aggregation, and immunological reactions [6]. Endothelial nitric oxide synthase (eNOS), an isoform of NO-producing enzymes that is fairly specific to endothelial cells, has been found to play a prominent role in both angiogenesis and vasculogenesis [7].

Recently, the eNOS gene (MIM: 163729, gene bank accession number: NC_000007.13) has been studied extensively for genetic polymorphisms to elucidate its genetic role in cardiovascular diseases. As a result, a single-nucleotide polymorphism (SNP) in the promoter region, T-786C, has been found to modify the promoter activity in vitro [8].

The eNOS is encoded by a gene (NOS3) located on chromosome 7q35-q36 [9]. A polymorphism in the 5' flanking region of the NOS3 gene (T-786C) has been associated with coronary spasm among Japanese (rs. 2070744) [8, 10]. It is believed that these mutations might result in altered NO metabolism and impaired NO release, leading to increased vascular tone and elevation in blood pressure.

The aim of the present study was to determine the prevalence of T-786C polymorphism of *eNOS* in CAD patient and control. Because of the significant effect of diabetes and smoking on the NO metabolism the samples were obtained from non-diabetic and non-smoking subjects.

MATERIALS AND METHODS

Subjects: The CAD group included 30 females and 30 males with a mean age of 58 years, ranging from 40-78 years. They had various degrees of stenosis in one or more of the main branch of coronary artery documented by coronary angiography. Patients with diabetes mellitus, renal disease, chronic obstructive pulmonary disease, hepatitis and smoker were excluded from the study group. The controls included 30 females, 30 males with a mean age of 57 years, ranging from 40-76 years. There was no difference between two groups for age distribution (P>0.05). The subjects proved to be healthy by health screening and had no obstructions in the coronary artery by angiography.

Blood sampling: Blood samples were collected in the morning by venipuncture after an overnight fast and were allowed to clot at room temperature for about 1 hour. Sera were separated from cells by centrifugation at 1500xg for 10 min and kept at -80°C and blood samples were stored at -20°C until analysis.

Measurement of serum NO: The levels of NO in serum were determined colorimetrically by Griess method [11]. In this method, NO undergoes a series of reactions

with several molecules present in biological fluids including O_2^-, O_2 and NO_2. The *in-vivo* final products of NO are nitrite (NO_2^-) and nitrate (NO_3^-). The relative proportion of NO_2^- and NO_3^- is variable and cannot be determined with certainty. Thus, the best index of total NO production is the sum of both NO_2^- and NO_3^-. The method used in this study provides an accurate and convenient measurement of nitrate/nitrite concentration in a simple two-step process. The first step is conversion of nitrate to nitrite utilizing nitrate reductase. The second step is the addition of Griess reagent which converts nitrite into deep purple azo compound. Colorimetric measurement of the absorbance due to this azo chromophore accurately determines nitrite concentration.

Molecular analyses: DNA was extracted using standard phenol-chlorofor procedure. Genotyping of T-786C, a newly developed allele-specific polymerase chain reaction (PCR) was used [12]. The oligonucleotide primers used in the reaction are listed in Figure 1. Artificial mismatches were included in the 2684T and 2684C primers as indicated in figure 1. Amplification was performed in a total volume of 20 µL containing 100 ng genomic DNA, 5 µM 2684T and 2684C primers, 1.6µM T_0 and C_0 primers and 10 µL Master Mix. After a hot start at 96 ^{C}, amplification was achieved by 36 cycles at 94 ^{C} for 40 seconds, 54 ^{C} for 40 seconds, and 72 ^{C} for 45 seconds. The C and T alleles gave a 176bp and a 250bp product, respectively, with a 387bp common product (Figure 1).
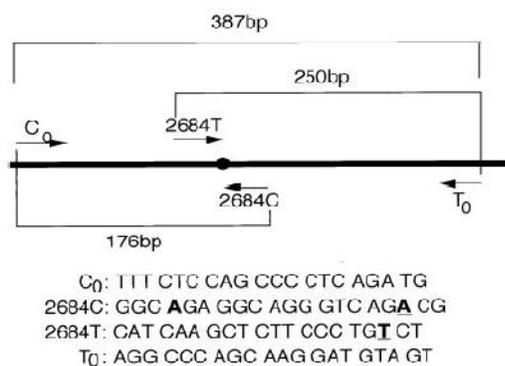

Figure 1: Allele specific PCR for T-786C polymorphism in the *eNOS* gene. Top panel shows the primers used in the reaction and the expected PCR products. Underlining in the primer sequences indicates artificially introduced mismatches. Bottom panel shows a typical result of genotyping. (See figure 2 in result section)

Statistical Analysis: Allele frequencies were calculated from the genotype counts. The observed genotype counts were compared with those expected under Hardy-Weinberg equilibrium with χ^2 test. Data were analyzed using t-test, expressed as mean ± SD and χ^2.
The associations between genotypes and risk of CAD were assessed by calculating odds ratios (ORs) and 95% confidence intervals (CIs). Data were compared in the groups by using SPSS software version 16. P<0.05 was chosen as the level of significance.

RESULTS

No difference was noticed between the family history of CAD in the patient and control groups (41.6% vs 31.6%; P>0.05). The percent of hypertensive subject in the patient group was significantly higher than that of the control group (55.0% vs 36.6%; P=004).

Comparing NO levels in the patient (101.7±38.2 µM/L) and control groups (122.4±56.5 µM/L), significantly low levels of NO were noticed in the patient group (t= 2.35, df=118, P=0.02).

Genotypic and allelic frequencies of T-786C polymorphism among the patient and control groups are shown in Table 1. In patient group, the frequencies of TT, CT and CC genotypes were 38.3, 46.7 and 15.0%, respectively. In the control the frequencies of TT, CT and CC genotypes were 61.7, 30.0 and 8.3%, respectively. In the study we found a marked difference in the frequency of the T-786C polymorphism of *eNOS* gene between patient and control groups. Statistical analysis showed that the genotype TC associated with risk of CAD (OR=2.50, 95% CI: 1.13-5.50, P=0.023). The prevalence of C allele was significantly higher in patients compared to control group (OR=2.04, 95% CI: 1.16-3.57, P=0.013).

Table 1: T-786C genotype frequencies in patient and control groups

Genotypes	Patient	Control	OR	95% CI	P-Value
TT	23	37	1.0	-	-
TC	28	18	2.50	1.13-5.50	0.023
CC	9	5	2.89	0.86-9.71	0.085
T allele	74	92	1.0	-	-
C allele	46	28	2.04	1.16-3.57	0.013

DISCUSSION

Nitric oxide (NO) plays an essential role in regulating vascular tone and hemodynamic. NO stimulates endothelial proliferation and angiogenesis, thereby playing an important role in wound healing and microcirculation. In addition, NO inhibits the release of endothelin-1 (a vasoconstrictor) [13]. Besides being an endothelium derived vasodilator molecule, NO also has important physiological and pathological effects. It can be synthesized in most tissue and cells. Its most prominent roles in cardiovascular system are blood pressure regulation, inhibition of thrombocyte aggregation, leukocyte adhesion, smooth muscle cell proliferation, and LDL oxidation. The decreases in production and bioavailability are associated with events that accelerate the development of atherosclerosis such as vasoconstriction, thrombocyte aggregation, migration of monocytes to the vascular wall, oxidized LDL and foam cell production. The main hypothesis of our study was that increased oxidative stress could reduce NO synthesis. Although patients with CAD had increased MDA levels, no

significant correlations were determined between MDA and NO. NO levels were tended to be higher than those of the control group [14].

NO levels showed a significant relation with higher BMI and hypertension in coronary artery disease. It was suggested that adipose tissue contains NO synthetase enzyme, and is thus a potential NO source [14]. In a study of healthy individuals at adolescent age, it was demonstrated that serum NO levels highly correlated with BMI and that NO levels were significantly higher in obese individuals. In our study, serum NO levels were found significantly lowers in patients.

Scribner and et al. [15] reported that, NO levels were significantly lower when compared to those without hypertension. Again in the same study, NO levels of the coronary artery patients without hypertension were similar to those of the control group [15]. In this study, serum levels of NO in patient groups were meaningfully higher than those of control and our observations confirm data from previous studies.

Recently, several studies revealed that there are various polymorphisms on *eNOS* gene and these mutations might be a risk factor for CAD. The polymorphisms differ largely among races. In this study, we investigated the relationship between T-786C mutation of *eNOS* gene and CAD specifically in the Iranian population. To our knowledge, this polymorphism has never been investigated in this population. The results demonstrated an association between C allele and CAD in the Iranian population. The polymorphism is a result of a thymidine being replaced by a cytosine at nucleotide -786 (T-786C).

Interaction between smoking *eNOS* genotype and CAD has been reported [16]. Smoking is known to induce oxidative stress, which is a potent suppressor of eNOS activity. Also others found an association between genetic variation in the *eNOS* gene and diabetes in CAD, therefore in this study all subjects selected were non-smoker and non-diabetic.

This polymorphism of *eNOS* gene was also investigated by several studies for the association with hypertension [16], the presence and severity of CAD [17], and MI [18]. Rossi et al. reported that they found a significant association between the T-786C mutation and CAD. Colombo et al. showed a positive association between the polymorphisms and the extent of CAD in the Italian population [19]. T-786C polymorphism was also reported as being related to coronary spasm in the Japanese by Nakayama et al. [20]. In this study high frequency of CC genotype of T-786C was noticed in the patient group. The frequency of C allele was significantly higher in patient group. The results are in agreement with those reported by others.

According to Nakayama et al. [8], the -786C allele would be 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. The coronary spasm might be more severe and prolonged in CC homozygotes, increasing the risk of CAD [21]. The T-786C mutation in the promoter region of eNOS resulted in the reduction of eNOS promoter transcription rate [8], leading to the reduced NO production in blood vessels and endothelial dysfunction [22].

It was concluded that the presence of the eNOS mutant allele reduces endothelial production of NO and may predispose the patients carrying the mutant allele to coronary spasm, hypertension and coronary artery diseases.

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