

Full Length Research Paper

Association between nucleotide mutation of eNOS gene and serum level of vessel expansion factor (VEF) in non-smoker patients with vascular heart disease

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Various mutation on endothelial nitric oxide synthase (eNOS) gene cause reduced production of NO, the expansion factor (VEF) and may accelerate the process of atherosclerosis. The study was designed to investigate the frequency of T-786C polymorphism of the gene or nucleotide mutation of eNOS gene in patients suffering from vascular heart disease (VHD) or coronary artery disease (CAD) in North West of Iran. 120 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 out using allele specific PCR. Comparing with the control reduced levels of NO were noticed in the patient group ($P < 0.05$) and significantly high frequency of eNOS -786C genotype was found in CAD patients ($P < 0.05$). 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.

Key words: Vascular heart disease, endothelial nitric oxide synthase gene, TC 786 SNP, vessel expansion factor (VEF), non-smoker.

INTRODUCTION

Vascular Heart Disease (VHD) or coronary artery disease (CAD) is the major cause of mortality and morbidity in most countries. Among many traditional risk factors for CAD development, positive family history is 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 or a vessel expansion factor (VEF) (Davignon and Ganz, 2004). NO is synthesized via a reaction that

includes the conversion of L-arginine to L-citrulline catalyzed by endothelial nitric oxide synthase (eNOS), which is one of the three isoforms of the enzyme (Angeline et al., 2010; Mayer and Hemmens, 1997). eNOS is the product of eNOS gene which is 21 kb in size and consists of 26 exons (Ragia et al., 2010; Marsden et al., 1993). Additionally, promoter region of the eNOS gene harbors several transcription factor binding sites and regulating gene expression (Nishevitha et al., 2009; Karantzoulis-Fegaras et al.,). Nitric oxide (NO) is a vasoactive substance and a major mediator of

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Abbreviations: eNOS, Endothelial nitric oxide synthase; VHD, vascular heart disease; CAD, coronary artery disease; VEF, vessel expansion factor; NO, nitric oxide.

endothelium-dependent vasodilatation, which is synthesized and also released in the vascular endothelium (Kelm, 2006; Moncada and Higgs, 1993). 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 (Kelm, 2006; Moncada and Higgs, 1993). 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 (Gross and Wolin, 1995).

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 (Kimura and Esumi, 2003). Recently, the eNOS gene was 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, was found to modify the promoter activity *in vitro* (Angeline et al., 2010; Nakayama et al., 1999). The eNOS is encoded by a gene (*NOS3*) located on chromosome 7q35-q36 (Esposti et al., 2011; Nadaud et al., 1994). A polymorphism in the 5' flanking region of the *NOS3* gene (T-786C) has been associated with coronary spasm among Japanese (Nakayama et al., 1999; Casas et al., 2006). 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. One of the mutations in the eNOS gene is a result of a thymidine (T) being replaced by a cytosine (C) at nucleotide -786 (T-786C). The aim of this present study was to determine the prevalence of T-786C polymorphism of eNOS in CAD patient and control. Because of significant effect of diabetes and smoking on the NO metabolism, the samples were obtained from non-smoking subjects.

MATERIALS AND METHODS

Subjects

The CAD group included 30 females and 30 males with a mean age of 56 years, ranging from 40 to 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 and 30 males with a mean age of 54 years, ranging from 40 to 76 years. 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 h. Sera were separated from cells by centrifugation at

1500x g 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 (M-Miranda et al., 2001). In this method, NO undergoes a series of reactions with several molecules present in biological fluids including O₂⁻, O₂ and NO₂. The final products of NO *in vivo* are nitrite (NO₂⁻) and nitrate (NO₃⁻). The relative proportion of NO₂⁻ and NO₃⁻ varies and cannot be predicted with certainty. Thus, the best index of total NO production is the sum of both NO₂⁻ and NO₃⁻. 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-chloroform procedure. Genotyping of T-786C, a newly developed allele-specific polymerase chain reaction (PCR) was used (Figure 1) (Esposti et al., 2011; Little, 1995). 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, 1.6 µM T₀ and C₀ primers and 10 µL Master Mix. After a hot start at 96°C, amplification was achieved by 36 cycles at 94°C for 40 s, 54°C for 40 s and 72°C for 45 s. The C and T alleles gave a 176 and a 250 bp product, respectively, with a 387 bp common product (Figure 2).

Statistical analysis

Data were analyzed with t-test, expressed as mean ± SD and χ^2 chi-square. Data were compared in the groups by using SPSS software version 16. P<0.05 was chosen as the level of significance. Allele frequencies were calculated from the genotype counts. The observed genotype counts were compared with those expected under Hardy-wienberg equilibrium with χ^2 test.

RESULTS AND DISCUSSION

The characteristics of the case (CAD) as well as control group are summarized in Table 1. VEF differences were noticed between the mean values of age, sex and family history of CAD in patient of non- smoker and control groups. The percent of hypertensive subject in the patient groups was significantly higher than that of control group (p<0.05) (Table 1). Comparing VEF levels in the patient including non-smoker and control groups, significantly low levels of VEF were noticed in the patients group (p<0.05) (Table 2). Genotype frequencies of T-786C among the patient of non- smoker and control groups are shown in Table 3. In the present study, we found a marked difference in the frequency of the T-786C mutation of eNOS gene between patients of non- smoker and control groups. In total patients group, the frequency

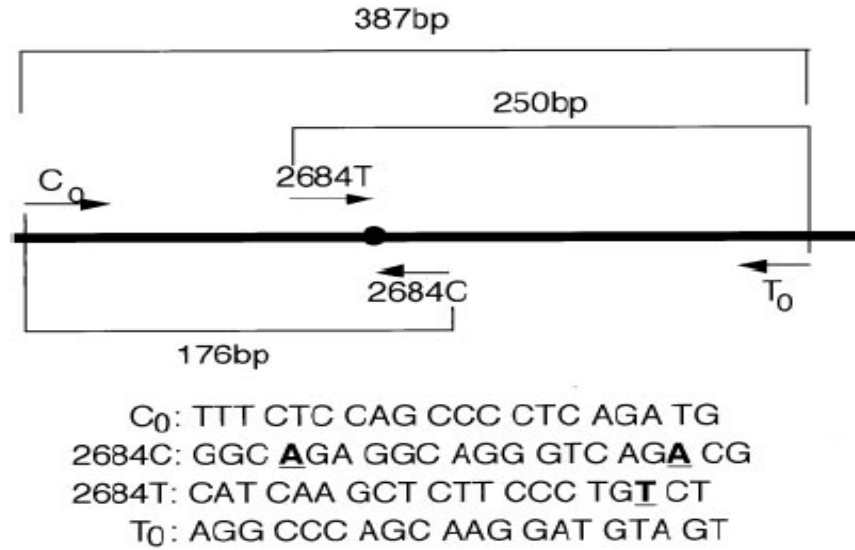


Figure 1. Allele specific PCR for T-786C polymorphism in the eNOS gene. Top panel shows the primers used in the reaction and the expected PCR products. Underlining in the primer sequences indicates artificially introduced mismatches. Bottom panel shows a typical result of genotyping (Figure 2).

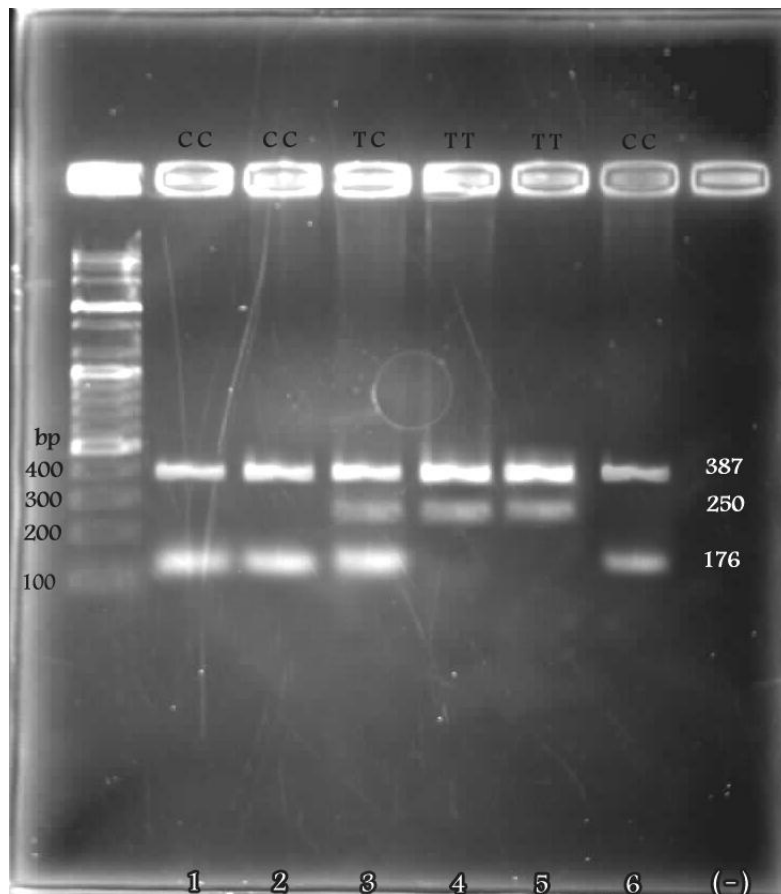


Figure 2. Products of PCR of T-786C polymorphism. Left, size marker 100 bp; right, negative control (no DNA). n = 1, 2, 6: CC genotype. n = 4, 5: TT genotype. n = 3: TC genotype.

Table 1. The demographic and clinical data of the patient (non- smoker) and control groups.

Characteristic	Patients (non-smoker) (n = 60) mean ± SD	Controls (n = 60) mean ± SD	P Value
Female/male (n)	30/30	30/30	NS
Age (years)	56 ± 6.81	54 ± 8.95	NS
Hypertension [n (%)]	28 (46.7%)	17(28.3%)	P<0.05
Family history [n (%)]	21 (35%)	16 (26.7%)	NS

NS, Non significant.

Table 2. The levels of VEF in patient (non-smoker) and control groups.

Parameter	Patients (non-smoker) (n = 60) mean ± SD	Controls (n = 60) mean ± SD	P Value
VEF (µM/L)	115.08 ± 23.15	140.38 ± 42.48	P<0.05

A p-value < 0.05 considered is significant.

Table 3. T-786C genotype frequencies in patients (non-smoker) and control groups.

Groups	Genotype (TT%)	Genotype (TC%)	Genotype (CC%)
Patient (non-smoker) N (%)	20 (33.3)	27 (45)	13 (21.7)
Control N (%)	39 (65)	16 (26.7)	5 (8.30)

of T/T, C/T and C/C genotypes was 33.3, 45 and 21.7%, respectively. In the control, the frequency of T/T, C/T and C/C genotypes was 65, 26.7 and 8.3%, respectively. The frequency of C allele was significantly higher in the patient group including non-smoker ($p<0.05$).

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) (Angeline et al., 2010; Ignarro et al., 1999); 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 decrease in production and bioavailability are associated with events that accelerate 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 (Jira et al., 2011; Elizalde et al., 2000). 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 (Jira et al., 2011; Elizalde et al., 2000).

In a study performed with 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 lower in patients. Scribner et al. (2003) reported that, NO levels are 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 (Scribner et al., 2003). In this study, serum levels of NO in patient groups were meaningfully higher than those of the control and our observations confirm data from previous studies. Recently, several studies revealed that there are various mutations 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 demonstrate 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 (Jira et al., 2011; Hyndman et al., 2002). 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 (Jira et al., 2011; Hyndman et al., 2002), the presence and severity of CAD (Rossi et al., 2003), and MI (Gluba et al., 2009; Nakayama et al., 2000). Rossi et al. (2003) reported that they found a significant association between the T-786C mutation and CAD. Colombo et al. (2003) showed a positive association between the polymorphisms and the extent of CAD in the Italian population (Colombo et al., 2003). T-786C polymorphism was also reported as being related to coronary spasm in the Japanese by Nakayama et al. (2000) and A-Kunnas et al. (2002). In this study, high frequency of C/C 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. (1999) and Narne et al. (2012), 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 (Jaramillo et al., 2008). The T-786C mutation in the promoter region of eNOS resulted in the reduction of eNOS promoter transcription rate (Tepliakov et al., 2010; Nakayama et al., 1999), leading to the reduced NO production in blood vessels and endothelial dysfunction (Kim et al., 2007).

Conclusions

It can be 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 vascular heart disease.

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