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Association of Variable Number of Tandem Repeats in Endothelial Nitric Oxide Synthase Gene with Coronary Artery Disease

*S Salimi [1], M Firoozrai [1], I Nourmohammadi[1], M Shabani [1], A Zavarehee [2], A Mohebbi [2]

[1]Dept. of Biochemistry, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
[2]Dept. of Cardiology, Shahid Rajaee Heart Hospital, Iran University of Medical Sciences, Tehran, Iran

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Abstract

Endo-derived nitric oxide (NO) is synthesized from L-arginine by endothelium nitric oxide synthase (eNOS). Since reduced NO synthesis has been implicated in the development of coronary atherosclerosis; we hypothesized that polymorphisms of NOS gene might be associated with increased susceptibility to this disorder and coronary artery disease (CAD). We studied the 27 base pair tandem repeat polymorphism in intron4 of the endothelial nitric oxide synthase (eNOS) gene in 141 unrelated CAD patients with positive coronary angiograms in Shahid Rajaee Heart Hospital and 159 age matched control subjects without a history of symptomatic CAD. The study protocol was approved by the Iran University of Medical Sciences Ethics Committee. The eNOS gene intron4a/b VNTR polymorphism was analyzed by polymerase chain reaction. The plasma lipids levels and other risk factors were also determined. The genotype frequencies for eNOS4b/b, eNOS4a/b and eNOS4a/a were 68.8, 29.1 and 2.1% in CAD subjects, and 81, 18.4 and 0.6 % in control subjects, respectively. The genotype frequencies differed significantly between the two groups (χ^2= 6.38 *P*= 0.041). The frequency of the allele was 16.7% in CAD subjects and 9.8% in control subjects and was significantly higher in the patients (χ^2= 6.18 *P*= 0.013, odds ratio=1.84). Plasma lipids, except HDL-C were also remarkablely increased in CAD group.

Keywords: *Coronary artery disease, Nitric oxide synthase gene, Polymorphism*

Introduction

Nitric Oxide is synthesized from L-arginine and molecular oxygen by a family of three enzymes, the nitric oxide synthase (NOS) (1, 2). NO is the most powerful endogenous vasodilator known. It can also inhibit the adhesion, aggregation and recruitment of platelets, vascular smooth muscle cells migration and growth, regulate some vessel-platelet interactions and limits the oxidation of atherogenic low density lipoproteins (1).

The inducible NOS is expressed in vessel walls and macrophages by certain cytokines and endotoxins lipopolysaccharides in pathological conditions (3). The constitutive neuronal NOS is expressed in the central and peripheral nervous systems as well as in macula densa of kidney. It plays important roles in physiological and pathophysiological conditions (4). The constitutive endothelial NO synthase (eNOS) is expressed in the endothelium, encoded by a 26 exon gene (NOS3) located on chromosome 7q35 to 36 with a total size of 21 kb and encodes an mRNA of 4052 nucleotides (5, 6).

The eNOS gene is expressionally and functionally regulated through multiple regulatory steps (7). Several allelic variants of eNOS gene have been identified and their association with human disease states studied. Moreover it has been shown that eNOS inhibition accelerates atherosclerosis in animal models and that abnormalities in the endothelial NO pathway is present in human with atherosclerosis (8).

The evidence suggest that NO may inhibit several key steps in the atherosclerosis process and

***Corresponding author:** Tel: +98 21 88059248, Fax: +98 21 88058719, E-mail: sasalimi@yahoo.com

that an alteration of NO production within the vascular endothelium could contribute to pathogenesis of atherosclerosis (9).

Several of eNOS gene polymorphisms have been reported as 'susceptibility genes' in various cardiovascular and pulmonary diseases (10). Yashimora et al. discovered a GT substitution in exon7 in codon 298 of the human eNOS gene, which alters the amino acid at this residue from glutamate to aspartate (11). A T786 mutation in the 5′- flanking region of eNOS gene, which reduces eNOS promoter activity in vitro, was associated with coronary spam in Japanese population (12, 13). High numbers of CA repeat in intron 13 are also associated with an excess of risk of CAD (14). Among the reported polymorphisms of the eNOS gene, a significant association of the 4a/b polymorphism in intron 4 of the eNOS gene with coronary artery disease (CAD) and hypertension has been reported too. Wang et al. detected an association between homozygosity for the eNOS4a allele and an increased risk for CAD in current or ex-smokers (15). The variable number of tandem repeat (VNTR) polymorphism located in intron 4 of eNOS (eNOS4b/a polymorphism) was reported to be significantly associated with plasma NOx concentration (16). In repeats of a 27-bp consensus sequence, two alleles, a common large allele and a smaller allele, were explored. The larger allele (eNOS4b allele), designated 'b-insertion' has five tandem repeats, and the smaller allele (eNOS4a allele); 'a-deletion' has four repeats (6).

In the present study, we investigated the association between the occurrence of CAD and the intron4b/a polymorphism in Iranian patients.

Materials and Methods

Subjects Patients recruited in this study (n=141; 100 males and 41 females) were angiographically identified (>50% stenosis affected at least one coronary vessel) as CAD in Shahid Rajaee Heart Hospital. The control group consisted of 159 individuals (110 males and 49 females), within the same age range as patients who had no history of heart disease, chest pain, diabetes, hypertension and general illness. All participants were interviewed and data on smoking habitat, blood pressure, lipid profile and family history of CAD were recorded. Blood samples were obtained from all subjects after 12 h fasting and placed in EDTA tubes and stored at -80 ºC until the time of assay.

The study protocol was approved by Ethics Committee of Iran University of Medical Sciences.

Biochemical Analysis The serum concentrations of triglyceride (TG), total cholesterol, LDL cholesterol (LDL-C) and HDL cholesterol (HDL-C) were measured by standard methods in the clinical laboratory of the hospital.

DNA Analysis Genomic DNA was extracted from peripheral blood leukocytes by standard methods. Two oligonucleotide primers (sense) 5′-AGGCCCTATGGTAGTGCCTT-3′ and (antisense) 5′- TCTCTTAGTGCTGTGGTCAC-3′ based on the flanking sequences of the VNTR in the ecNOS gene were used to amplify the corresponding DNA fragment by the polymerase chain reaction. The intron4 VNTR polymorphism of the eNOS gene was detected by method of Wang et al. (15) with some modifications. The reaction was performed in a 25-µl final volume and contained 25 pmol of each primer, 0.2mmol of each deoxynucleoside triphosphate (Roche Germany), 1.5U *Taq* DNA polymerase (Fermentas Lithuania), 50mmol/L KCL, 2.5 mmol/ L MgCl2, 10mmol/ L Tris-Hcl (PH= 8.3) and 400ng of genomic DNA according to the following protocol: initial denaturation at 94 ºC for 5 min; 35 cycles of denaturation at 94 ºC for 30 s, annealing at 56 ºC for 1 min, and extension at 72 ºC for 1 min; and final extension at 72 ºC for 7 min. The PCR products were separated by electrophoresis on a 2% agarose gel and visualized by ethidum bromide staining.

Statistical Analysis All statistical analyses were performed with SPSS V. 11.5. Numeric data are presented as mean± SD. The differences between groups were examined by χ^2 test or an independent Student t-test when appropriate. Allele frequencies were estimated by the gene counting method. The frequencies of the alleles

and genotypes were compared between patient and control groups by the χ^2 test when appropriate. The odds ratio (OR) and 95% confidence interval (CI) were also estimated. We performed multivariate logistic regression analysis to adjust risk factors, in which CAD was a dependent variable and independent variables were BMI (Body Mass Index), smoking, family history, SBP (Systolic Blood Pressure), DBP (Diastolic Blood Pressure), TG, total cholesterol level, LDL-C, HDL-C, LDL-C/ HDL-C and eNOS genotype.

Results

The clinical characteristics of the study population are shown in Table 1. Control subjects were matched to the case patients for gender and age. Although BMI and HDL-C showed no significant differences between the patients and the control subjects, systolic and diastolic blood pressure, triglyceride, total cholesterol, LDL-C and LDL-C/HDL-C in patients were significantly higher. BMI and HDL-C did not differ between CAD patients and control subjects. Frequencies of smoking and family history in patients were higher than the controls.

Two alleles of the VNTR in the human eNOS gene were detected in Iranian subjects (Fig. 1). One allele contained four of 27bp repeats (eNOS4a allele) giving rise to a PCR product of 393 bp, whereas the other contained five of such repeats (eNOS4b allele), yielding a PCR product of 420 bp. The genotype frequencies of 4b/a polymorphism in control subjects were 81%. For b/b, 18.4% for b/a and 0.6% for a/a.

On the other hand in CAD patients genotype frequencies were 68.79% for b/b, 29.08% for b/a and 2.13% for a/a (Table 2). ecNOS genotype was significantly associated with CAD (χ^2= 6.38 *P*= 0.041).

The frequencies of the a and b alleles were 16.7 and 83.3% for CAD patients and; 9.8 and 90.2% for the control subjects, respectively, and differed significantly between two groups (χ^2= 6.18, *P*= 0.013, odds ratio= 1.84).

The logistic regression analysis revealed that SBP, DBP, smoking and family history were independent risk factors of CAD (*P*= 0.003, *P*= 0.053, *P*= 0.002 and *P*= 0.012, respectively), whereas 4b/a genotype, total cholesterol, LDL-C and TG were not CAD independent risk factors.

Table 1: Clinical characteristics of the study population

	CAD Patients	**Controls**	***P***
No. of subjects	141	158	
Age (yr)	53.1± 9.7	51.9±8.8	NS
BMI(kg/m²)	27.4± 6.5	27.2±18.2	NS
Smoking (%)	36.88	25.3	χ^2=4.67 *P*=0.031
Family history (%)	39.7	21.5	χ^2=11.7 *P*=0.001
SBP (mmHg)	84.8± 13.3	76.1± 6.7	*P*= 0.000
DBP (mmHg)	130.1± 19.2	117± 14.9	*P*= 0.000
Cholesterol (mg/dl)	204.99± 45.5	189.8± 32.8	*P*= 0.001
TG (mg/dl)	200.6± 107.8	168.7± 86.8	*P*= 0.005
LDL-C (mg/dl)	118.5± 37.2	107.6± 33.7	*P*= 0.009
HDL-C (mg/dl)	46.43± 10.8	48.42± 11.8	NS
LDL-C/HDL-C	2.63± 0.93	2.34± 0.88	*P*= 0.006

NS= Non Significant

Table 2: Genotype and allele frequencies of 4b/a polymorphism of the ecNOS gene in CAD patients and controls

	CAD patients	Controls	χ^2	*P*v	odds ratio(95% CI)
4b/a polymorphism			6.38	0.041	
b/b, n (%)	97(68.8)	128(81)			
b/a, n (%)	41(29.1)	29(18.4)			
a/a, n (%)	3(2.1)	1(0.6)			
total	141	158			
			5.97	0.015	1.94
b/b, n (%)	97(68.8)	128(81)			
b/a + a/a, n (%)	44(31.2)	30(19)			
total	141	158			
allele					
b (%)	83.3	90.2	6.18	0.013	1.84
a	16.7	9.8			
total	100	100			

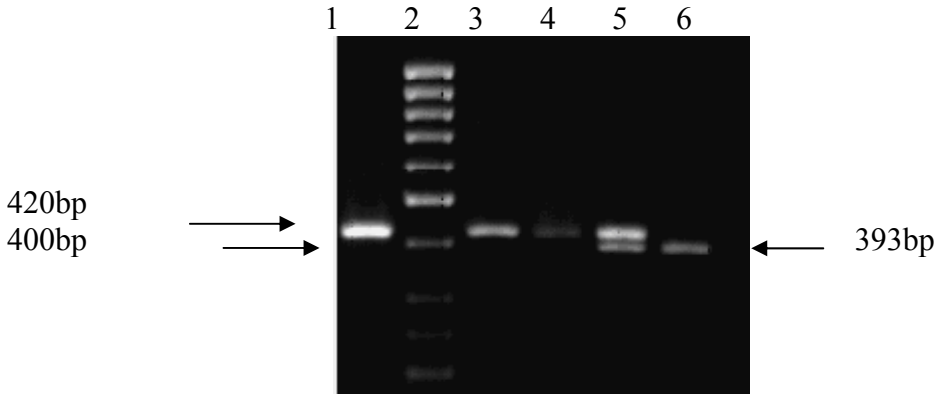

Fig. 1: Genotyping of the VNTR in intron4 of eNOS gene. The direct PCR product was separated by electrophoresis in 2% agarose gel and visualized by ethidum bromide staining. Lane 1; four-repeats homozygous, lane 2; four- and five – repeats heterozygous, lanes 3, 4 and 6; five- repeats homozygous. Lane 5 is the DNA marker and the band of 420bp indicates five repeats and the band of 393bp indicates four repeats of the 27bp.

Discussion

In addition to established risk factors, genetic risk factors may have important roles in the pathogenesis of coronary atherosclerosis and acute myocardial infraction. Using the approach of epidemiological studies it is possible to identify weak susceptibility genes in polygenic diseases like coronary heart disease (17). In the last decay, the potential link between an increasing number of gene variants and coronary heart disease has been analyzed by several investigators. Due to the protective roles of nitric oxide against important events during atherogenesis, the endothelial nitric oxide synthase gene has been identified as a further susceptibility of coronary heart disease (18, 19). Yashimura et al. described a point mutation of guanine to thymine at nucleotide 1917 in exon7 of the eNOS gene that resulted in replacement of glutamic acid by aspartic acid at codon 296. In intron 4 of the eNOS gene a repeat polymorphism was identified; the larger allele, eNOS4b, contains five tandem 27-bp repeats, the smaller allele, eNOS4a, only four repeats (11). Later investigators describe other polymorphisms of eNOS gene in the 5' flanking and some introns. In the original study of Wang et al. there was in current smokers, but not in non smokers, an excess of homozygotes for the rare eNOS 4a allele in patients with severely stenosed arter-

ies, compared with those with no or mild stenosis (15). In contrary, among Japanese population, the eNOS 4a allele was an independent risk factor for myocardial infraction (MI) and with no differences between smokers and non-smokers (20).

Some other studies also identified an association of the 27-bp repeat polymorphism with the risk of MI, CAD, EH (Essential Hypertension) (21, 22). Whereas other investigators did not detect a link to CAD, MI and EH (23-25).

The reason for this discrepancy remains unclear. The discrepancy between these different studies may be attributable to various factors such as diagnostic criteria or race, for example the frequency of the four repeat allele was approximately 0.1 in our subjects is similar to previously reported in Japanese (0.1-0.13) (20, 26), Spanish (0.13) (27) and Turkish (0.14) (28), but lower than the observed among the Caucasian of Australia (0.17) (29) and African-Americans (0.26-0.3) (30, 31).

We showed that eNOS 4a/b polymorphism was associated with CAD in the Iranian population. Similarly, Matyar et al. found that the 4a/b VNTR polymorphism is associated with CAD in Southern Turkey (21) and also Kunnas et al. found that this polymorphism is associated with risk of CAD and MI in middle age men (30). In addition, Rao and Park found that eNOS 4a/b polymorphism is associated with CAD and acute coronary syndrome in African-American and Koreans respectively (31, 32). In contrast Gardemann did not detect a link in this polymorphism to CAD in Germans (23).

By the multiple regression analysis, it became clear that SBP, DBP, family history and smoking but not eNOS gene 4a/b polymorphism were independent risk factors of CAD. It was probably due to low number of samples.

Our study had several limitations. The first was the lack of functional studies. Second, the 4a allele of the eNOS4a/b polymorphism had an estimated frequency of only 0.1, and the association between this polymorphism was based on only 4 individuals who were homozygous for this polymorphism. Thus, a larger sample should be examined to confirm the relation between this polymorphism and CAD.

In conclusion in the present study we demonstrated that eNOS4a/b polymorphism was associated with CAD. But despite of other risk factors such as SBP, DBP, family history and smoking, this polymorphism was not an independent risk factor however, larger sample sizes are necessary for confirming the relationship between CAD and this variant.

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