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ENDOTHELIAL NITRIC OXIDE SYNTHASE GENES (INTRON 4A/B AND T786C) POLYMORPHISMS IN CORONARY ARTERY DISEASE IN NORTH INDIAN SUBJECTS

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ABSTRACT

Increased genetic propensity to develop Coronary artery disease (CAD) and increasing prevalence of cardiovascular risk factors are the reasons accounted for higher prevalence and greater severity of coronary artery diseases in Asian Indian. Intron 4a/b VNTR polymorphism and T786C in the promoter region of the endothelial nitric oxide synthase (eNOS) gene has been found to be associated with CAD in various ethnic groups. However, the results are conflicting. This prompted us to investigate the contribution of Intron 4a/b and T786C polymorphism of the eNOS gene in coronary artery disease patients in the northern Indian population. The DNA was isolated from the blood samples drawn from 200 controls and 227 CAD patients and their genotyping was carried out by polymerase chain reaction and restriction fragment length polymorphism techniques. In our study the observed genotypes were in accordance with the Hardy-Weinberg equilibrium in the study subjects in Intron 4a/b and T786C polymorphisms of the eNOS gene. Statistical significant difference was observed in alleles in Intron 4a/b gene between patients and controls (Chi-square = 9.2, p=0.002), whereas a borderline statistical significance was observed in the alleles of T786C of eNOS gene (Chi-square =3.55, p=0.058). Multivariate analysis (regressed for age and sex) revealed contribution of Intron 4a/b polymorphism of the eNOS gene suggesting that the individuals with the "a" allele were at 2 times higher odds to develop coronary artery disease. A borderline significant association was observed in T786C variant of eNOS gene with CAD.

Key words: Coronary artery diseases, eNOS, gene polymorphism, Intron 4a/b, T786C.

INTRODUCTION

The global burden of cardiovascular diseases (CVD) is rapidly increasing in developing countries. Increased genetic propensity to develop CVD and increasing prevalence of cardiovascular risk factors are the reasons accounted for higher prevalence of coronary artery diseases in Asian Indian [1-2]. Nitric oxide (NO) is a key molecule in endothelium-dependent vasodilatation and has been implicated in the development of hypertension, coronary artery diseases, and myocardial infarction [3-4]. Endothelial nitric oxide synthase (eNOS) modulates the synthesis of nitric oxide, encoded by eNOS gene is located on the chromosome 7q35-36 and contains 26 exons spanning 21 kilobases. Functionally important variants of the eNOS gene could influence individual susceptibility to atherosclerosis by altering the amount of NO generated by the endothelium [5-6]. Several polymorphisms have been reported in the eNOS gene, of these, a common variant located in exon 7 ($G^{984}\rightarrow T$) of the eNOS gene is the most studied in relation to cardiovascular diseases [3-11]. A number of variable Tandem Repeats (VNTR) of the Intron 4a/b VNTR polymorphism of eNOS gene has been found to be associated with coronary artery disease (CAD) in Japanese population [9]. Another variant T786C of the eNOS gene is the most important for the regulation of

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transcription rate is strongly associated with coronary spastic angina and myocardial infarction [10-11]. However, other studies have not revealed any association of these two polymorphisms (Intron 4a/b and T786C) with CAD [12-13]. The discrepancy in these studies on the association of Intron 4a/b VNTR and T786C of the eNOS gene polymorphism with CAD may be related to ethnic diversity. This led us to investigate the association between the CAD and two polymorphisms of eNOS gene, (Intron 4b/a, T786C) in Asian Indian patients with essential hypertension. This is the first case-control study demonstrating the contribution of Intron 4a/b and T786C polymorphism of the eNOS gene in coronary artery disease patients in the northern Indian population.

METHODOLOGY

Group 1. The 227 CAD patients [Age > 25 years<60 years] were selected from a series of consecutive outdoor patients attending coronary clinic and indoor patients of Department of Cardiology, AIIMS. Angiographically assessed patients who have 70% or more stenosis of at least one of the major coronary artery were selected for the study. Patients diagnosed for hypertension and diabetes mellitus were also included in the study. Patients with less than 70% stenosis, valvular disease or cardiomyopathy were excluded from the study. Prior informed consent from the patients and controls were taken.

Group 2. The 200 controls were the age and sex matched volunteers [subject age >25<60 years] from the similar population group as patients, recruited from the staff of AIIMS and residents of Delhi and surrounding areas. Control subjects were with no history of endocrine, metabolic, ischaemic heart disease, renal and cerebrovascular disorders.

Sample collection and processing

The peripheral venous blood was drawn from the study subjects after 12 hours of fasting and packed cells were used for DNA isolation using the QIAamp® DNA Blood Mini Kit by QIAGEN®.

Genotyping of the eNOS gene

The eNOS 4a/b gene polymorphism was detected by the method of Wang *et al*., [9] with minor modifications. DNA samples were subjected to amplification by PCR using primer pairs that flank the region of the 27-bp direct repeat in Intron 4 of the eNOS gene. The PCR products were separated by electrophoresis on a 2.7% agarose gel and visualized by ethidum bromide staining. The wild type (4b/4b) contains five 27 bp tandem repeats, and homozygous mutant (4a/4a) has four 27-bp tandem repeats. The size of the PCR products were 393 bp and 420 bp for *a* and *b* alleles respectively. Genotyping has been followed by sequencing in 20% of the total samples in both the study subjects, to rules out any genotyping error in our study.

T786C polymorphism in the eNOS gene was detected by SNaPshot Multiplex Ready Reaction mix (ABI, Foster City, CA, USA). The mixture was analyzed by electrophoresis in a DNA analyzer (ABI Prism 3700). The software GeneScan analysis (ABI, Foster City, CA,USA) was used to analyze the results.

Biochemical determinations

Serum total cholesterol, triglycerides, Low density lipoprotein, high-density lipoprotein-cholesterol were determined using kits (Randox).

Results

Baseline characteristics of the study groups [200 controls and 227 CAD patients] are summarized in **Table 1**. Average age of the CAD patients was comparable to that of the control subjects The distribution of baseline parameters, examples, Total cholesterol, high density lipoprotein, low density lipoprotein and triglyceride levels were comparable in both the study groups.

Overall genotype analysis of eNOS Intron 4a/b and T786C gene polymorphisms in the study subjects

Results from our study found that the distribution of the observed genotype in both the study groups was in accordance with the Hardy-Weinberg equilibrium in both patients and controls, indicating that the screening method was appropriate. There was significant difference found in the alleles between patients and controls (Adjusted Yates corrected chi^2 value =9.2, P value = 0.002, odds ratio at 95% CI = 2.1 [1.30 - 4.11]) **Table 2.** A borderline significant difference in the genotype between patients and controls was observed (Chi-square =3.6, p=0.05), whereas a borderline statistical significance was observed with respect to alleles (Adjusted Yates corrected chi^2 value =3.55, P value = 0.058, odds ratio at 95% CI = 1.4 [0.85 – 2.22]), **Table 3**.

Statistical analysis

All computations were carried out with STATA program, version 8. Sample size was adequate for the present study which was determined using standard statistical method for case- control groups, at the significance level of 0.05 at power 80% as well as on the basis of prevalence of minor alleles referred from previous studies (4-11). Chi-square goodness of fit was used to verify the agreement of observed genotype frequencies with those expected (Hardy–Weinberg equilibrium). Difference between genotype groups were tested with analysis of variance (ANOVA). Odds ratios were determined by multivariate analysis of different genotypes/parameters after adjusting for age and sex. Student's t-test was used to compare various parameters detected in plasma between controls and patients. Statistical significance was defined as $p \leq 0.05$.

Table 1. Clinical parameters in the overall study subjects

Parameters	Controls [200] No./mean±s.d.	Patients [227]No./mean±s.d.
Age (years)	49.7±10.4	51.6+/-7.2
Sex (M/F)	147/53	196/31
Total cholesterol (mg/dl)	177.3±40.3	163.4±48.0
HDL (mg/dl)	48.5±8.3	38.6±7.6
LDL (mg/dl)	95.3±37.0	85.3±41.6
TG (mg/dl)	183.8±89.4	140±108
systolic blood pressure mm Hg,	120±3.47	145.5±14.05*
diastolic blood pressure mm Hg,	80.49±2.5	93.6±8.09*
Hypertension		8
Diabetes mellitus	0	9

*Patients groups were compared with controls with t-test of significance or by chi-square test; *p< 0.0001*

Table 2. Frequencies of eNOS Intron 4a/b genotypes and alleles in 200 controls and 227 CAD patients

Genotypes	Controls *N(%)*	Patients *N(%)*
bb	180 (90)	181 (79.7)
ab	19 (9.5)	41 (18.1)
1 (0.5)	5 (2.2)	aa
	x2= 0.41, p>0.05	x2=2.02, p>0.05
Unadjusted Yates corrected chi^2 value =7.805, P value = 0.0052, Unadjusted odds ratio at 95% CI = 2.29 [1.26-4.18] Adjusted Yates corrected chi^2 value =7.75, P value = 0.0058, Adjusted odds ratio at 95% CI = 2.3 [1.21 - 4.2]		
Alleles	**Controls**	**Patients**
a	0.05	0.11
b	0.95	0.89
Unadjusted Yates corrected chi^2 value = 9.1, p value = 0.0026, Unadjusted odds ratio at 95% CI =2.28 [1.3-4.0] Adjusted Yates corrected chi^2 value = 9.2, p value = 0.002, Adjusted Odds ratio 95% CI = 2.12[1.30- 4.11]		

p-value >0.05, non-significant

Table 3. Frequencies of eNOS Intron 4a/b genotypes and alleles in the study subjects

Genotypes	200 Controls *N(%)*	227 Patients *N(%)*
TT	135 (67.5)	132 (58.15)
TC	62 (31)	88 (38.77)
CC	3 (1.5)	7 (3.08)
	x^2=1.94, p>0.05	x2=2.89, p>0.05
Unadjusted Yates corrected chi^2 value =3.58, P value = 0.046, Unadjusted odds ratio at 95%CI =1.49 [0.98-2.27] Adjusted Yates corrected chi^2 value =3.60, P value = 0.05 Adjusted odds ratio at 95%CI = 1.48[0.98-2.26]		
Alleles	**Controls**	**Patients**
T	0.83	0.78
C	0.17	0.22
Unadjusted Yates corrected chi^2 value = 3.65, p-value = 0.056, Unadjusted Odds ratio 95% CI=1.42 [0.99-2.02] Adjusted Yates corrected chi^2 value = 3.55, p-value = 0.058 Adjusted Odds ratio at 95% CI = 1.4[0.85-2.22]		

p-value >0.05, non-significant

DISCUSSION AND CONCLUSION

The gene encoding Endothelial nitric oxide synthase (eNOS) has three clinically relevant polymorphisms: T786C in the promoter, the variable number of tandem repeats (VNTR) in Intron 4, and the Glu298Asp variant in exon 7 [3-8]. There are reports about the marked differences in the distribution of two clinically relevant eNOS polymorphisms (Intron 4a/b, T786C) among different ethnic groups. Nakayama *et al.,* [11] reported that the eNOS gene T786C polymorphisms have been linked to coronary artery spasm in Japanese population. Tanus-Santos et al [12] reported that the C786 variant was also more common in Caucasians (42.0%) than in African-Americans (17.5%) or Asians (13.8%). The finding of our study is important because Asian Indian population have a significant prevalence of hypertension [2]. On applying multivariate logistic regression analysis to our data, we have found borderline significant difference in the distribution of alleles between patients and controls. The need of the present study to be conducted in a larger sample size is warranted.

Another polymorphism of eNOS gene shows that homozygosity of a allele of Intron 4a/b has been found to be a risk factor for CAD among smokers [9]. In contrast, Singusch *et al.,* [13] and Hwang *et al.,* [14] did not find any association between this polymorphism and CAD in Germans and Taiwanese populations respectively.Tanus-Santos *et al*., [12] reported the 4a variant in Intron 4 was more common in African-Americans (26.5%) than in Caucasians (16.0%) or Asians (12.9%). In our study, there was significant difference in the genotypes and allele frequency between CAD patients and controls. Both univariate and multivariate (regressed for age and sex) analysis revealed that individuals with the "a" allele were at 2 times higher odds to develop coronary artery disease suggesting that Intron 4a/b gene polymorphism has been associated with the coronary artery disease. Systolic blood pressure, diastolic blood pressure, age, sex, family history and smoking have not emerged as independent risk factors of coronary artery diseases in our study. In conclusion, our results showed that the Intron 4a allele were significantly associated with coronary Artery Diseases. No association of T786C of eNOS gene with Coronary artery disease was found in patients with essential hypertension. This study also provides the baseline data on the normal distribution of Intron 4a/b and T786C polymorphisms of the eNOS gene in coronary artery disease in the northern Indian population.

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