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Sequence variation in eNOS (Glu298Asp) gene and its impact on coronary artery disease: North Indian Study

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ABSTRACT

Endothelium-derived nitric oxide (NO) synthesized from L-arginine by endothelial nitric oxide synthase (eNOS) encoded by the NOS3 gene. Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis of NOS3 identifies G to T substitution at 984 position of exon 7 which changes Glu to Asp amino acid at codon 298. We have done case-control association study to investigate the relationship between Glu298Asp variant and coronary artery disease (CAD) of North Indian origin. The study consists of 199 unrelated patients with positive history of coronary artery disease and 153 unrelated healthy individuals without having history of CAD. Though we did not find any significant association of eNOS gene polymorphism with coronary artery disease (p=0.077), a stepwise increase in frequency of T allele of eNOS gene with the severity in terms of number of vessels involved was observed (T allele in one, two and three vessel; 10.2%, 14.5% and 16.4% respectively). Presence of diabetes, hypertension, smoking, elevated level of triglycerides and reduced level of HDL cholesterol were found as significant predictors of coronary artery disease on multivariable logistic regression analysis. A larger sample size study is needed to evaluate the association of eNOS polymorphism and CAD.

Key words Coronary artery disease, eNOS, Glu298Asp polymorphism

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INTRODUCTION

Nitic oxide (NO) a powerful short-lived vasoactive substance is constitutively produced from L-arginine by the enzyme eNOS (Moncada *et al.*, 1993). It plays a key role in the relaxation of vascular smooth muscle, inhibits adhesion of platelets and leukocytes to the endothelium and reduces vascular smooth muscle cells migration and proliferation. Moreover, it has been shown that eNOS inhibition accelerates atherosclerosis in animal models and that abnormalities in the endothelial NO pathways are associated with atherosclerosis in humans (Cayatte *et al.*, 1994; Ludmer *et al.*, 1986). Several polymorphisms have been identified in the eNOS gene. Among

them, a common variant located in exon 7 (G984T) of the eNOS gene that modifies its coding sequence (Glu298Asp) has been found to be linked by several groups to the risk for coronary spasm, coronary artery disease (CAD), and acute myocardial infarction. The aim of the study is to find out the association of eNOS gene polymorphism with coronary artery disease in CAD patients of north Indian origin.

MATERIAL AND METHODS

Study subjects

Patients, with evidence of more than 50% stenosis in coronary arteries, or a past history of prior angioplasty, or CAD by-pass grafting, were included in the present study. The patients were recruited from the outpatient and inpatient services of the Department of Cardiology, SGPGIMS.

The control group consisted of individuals without a CAD background and with negative results in treadmill stress tests. Both groups were age and sex matched. Clinical background entailing the presence of diabetes, hypertension, smoking habits, family history of CAD, body-mass index and complete lipid profile (serum triglycerides, total cholesterol, HDL, LDL and VLDL) were recorded as per the information provided by the family members of both the groups independently. The status of Diabetes and hypertension in patients and controls, were confirmed on prior medical records or standard clinical examination and tests, according to the standard definition described by Fauci *et al.*, 2006. Habitual smoking was defined as prevailing at least one year before CAD onset. A family history of CAD was defined based on its presence in first degree relatives. Body-mass index was calculated by dividing the weight in kilograms by the square of the height in meters (Fouci *et al.*, 2006). Exclusion criteria were cardiomyopathy, febrile condition, rheumatic heart disease, congenital heart disease and systemic disorders. A prior written informed consent was obtained from patients and controls. The study was approved by institutional ethics committee.

DNA preparation and genotyping

Genomic DNA was extracted from EDTA peripheral blood leukocytes by the standard phenol-chloroform method (Poncz *et al.*, 1982). The quality of DNA was checked on 0.8% agarose (Sigma, USA) gel electrophoresis, and quantification was done on a UV spectrophotometer (Specgene Ltd, UK). The primers were synthesized from Genetix, France. Primers (Forward 5'-CATGAGGCTCAGCCCCAGAAC-3', Reverse 5'-AGTCAATCCCTTTGGTGCTCAC-3') for exon 7 of the eNOS gene. A modified procedure described by Hingorani *et al.*, 1999 was used for detecting the polymorphism. The PCR mixture was subjected for amplification in an automated thermocycler (BioRad PTC100, USA) with a protocol of initial denaturation at 94 °C for 4 min, followed by 30 cycles with 30 s at 94 °C, 45 s at 62 °C, 45 s at 72 °C and a 12 min final extension at 72 °C. The amplicon of 206 bp were digested with MboI (NEB UK) restriction enzyme as per manufacturer's instruction. The mutant T allele at nucleotide 894 creates the restriction enzyme site so the amplified 206-bp PCR product cleaved into two fragments of 119 bp and 87 bp. The digested product was separated on 3% agarose gel and documented on UVP Photo-doc-IT^{TM} UK, Imaging system.

Biochemical analyses

Fasting venous blood from patients and controls were collected in plain vials in order to analyze triglyceride (TG) levels. These levels were measured by lipoprotein lipaseperoxidase (Fossati *et al.*, 1982), those of total cholesterol (TC) by cholesterol oxidase (Allain *et al.*, 1974) and those of high density lipoprotein (HDL) by phosphotungstate magnesium chloride methodology (Lopes-Virella *et al.*, 1977). Very low density lipoprotein (VLDL) and low density lipoprotein (LDL)

levels were calculated indirectly by means of the Friedwald formula (Friedewald *et al.*, 1972) as (VLDL-C = TG/5; provided TG = 400 mg/dL) and LDL-C = TC – (HDL-C + VLDL-C).

Statistical analysis

Genotype and allele frequencies in CAD and control groups were compared by Chi square testing. The characteristics of patients and controls were evaluated by comparing biochemical findings using the Student t-test. Additionally, we performed multiple logistic regression model testing on the interaction of genotype and different classical CAD risk factors. All analyses were performed using SPSS v.11.5 (SPSS Inc., Chicago, USA) statistical analysis software. A two-tailed p value of $p < 0.05$ was considered statistically significant.

RESULTS

The study group included 199 angiographically proven CAD patients and 153 individuals as controls with no clinical history of CAD. The clinical and demographic data of patients and controls are presented in Table 1. Classical risk factors, viz., diabetes, hypertension, smoking and family history were significantly higher in the CAD group than in the control. The value of HDL- cholesterol was significantly lower in CAD patients than in controls, although serum triglyceride, total cholesterol, LDL-cholesterol and VLDL-cholesterol levels were found to be significantly higher in the former group.

Allele and genotype frequencies in both patient and control groups are described in Table 2. The distribution of genotypes within each group was in Hardy-Weinberg equilibrium. We found the prevalence of the GT genotype to be 18.1% in the patient and 24.2% in the control group, whereas mutant homozygous frequencies (5.0% and 1.3%) were observed in patients and controls, respectively. T allele frequency was found almost similar (14.0% in patients, 13.4% in controls) in both groups. An insignificant difference was observed between the two groups (p=0.79, OR 1.06 95% CI 0.67-1.67).

When we assessed the severity of the disease in terms of the number of vessels involved in CAD and eNOS gene polymorphism, we found variation in T allele prevalence in either one, two and three vessels [10.2%, 14.5% and 16.5% respectively] with stenosis in a CAD patient (Table 3).

On performing multiple logistic regression analysis; the influence of various risk factor like diabetes, hypertension, smoking, higher triglyceride and lower HDL-cholesterol on CAD precipitation (Table 4), was found significantly variable. Furthermore a concurrent increase in eNOS TT genotypes leads a greater CAD risk (6.45 times) than GG genotype.

DISCUSSION

The implication of eNOS gene in CAD pathogenesis has been very well studied in several ethnic groups (Jaramillo *et al.*, 2006; Wang *et al.*, 2001; Granath *et al.*, 2001; Mathew *et al.*, 2008). We found an insignificant association of T allele with CAD patients. The case control epidemiological studies conducted in past showed conflicting results. Our finding is consistent to Jaramillo *et al.*, 2006; Wang *et al.*, 2001; Granath *et al.*, 2001; Mathew *et al.*, 2008 where they have found an insignificant association of Glu298Asp gene polymorphism with coronary artery disease in Chilians, Taiwanese, Australian, Caucasian and Tamilians (South Indian) respectively. An insignificant difference of T allele frequency was found between presentations of CAD before 50 years and greater than 65 years in Canadians (Nassar *et al.*, 2001). Several authors reported significant association of the polymorphism with coronary artery disease in Tunisians

(Kerkeni *et al.,* 2006); Italians (Colombo *et al.,* 2003) and United kingdoms (Hingorani *et al.,* 1999).

In this study we found 13.4% of T allele frequency in controls which is quite similar to the reported prevalence by Srivastava *et al.,* 2005 (T allele 14.7%). They estimated the prevalence of eNOS Glu298Asp polymorphism in healthy volunteers from Delhi and the surrounding areas. This might be due to the location of Delhi at north region of India and same ethinicity of the population studied.

In relation to severity of coronary artery disease for eNOS gene polymorphism, though we found an insignificant association; but the observation suggests a stepwise increase of T allele frequency in relation to disease severity. Our results are similar to Kerkeni *et al.,* 2006 and Colombo *et al.,* 2003.

In conclusion multivariable logistic regression analysis reveals the presence of diabetes; hypertension, smoking habit, higher serum triglyceride levels and lower HDL are the significant predictors for coronary artery disease (Table 4). While, presence of family history of CAD, serum totals cholesterol, LDL, VLDL and genotypes of eNOS are insignificant predictors for CAD.

Table 1: Clinical and biochemical characteristics of control and patients

Characteristics	Control	CAD Patient	P
N	153	199	
Age	56.82±9.13	56.83±9.54	0.99
Sex (M:F)	117:36	162:37	0.289
BMI	25.41±2.86	25.69±2.87	0.361
Diabetic: Non-diabetic	23:130	94:105	0.00
Hypertensive: Non-hypertensive	22:131	87:112	0.00
Smoker: Nonsmoker	6:147	47:152	0.00
Positive F/H : Negative F/H	1:152	10:189	0.027
Triglyceride (45-150 mg/dl)†	94.05±25.0	172.8±84.5	0.00
Total Cholesterol (125-250 mg/dl)†	158.75±45.72	175.35±49.68	0.001
HDL-cholesterol (23-60 mg/dl)†	38.32±7.95	36.57±6.85	0.028
LDL-cholesterol (92-148 mg/dl)†	101.86±36.14	109.02±43.03	0.09
VLDL-cholesterol (10-30 mg/dl)†	19.26±5.36	31.87±15.30	0.00

†*laboratory reference values, mean±SD*

Table 2: Distribution of eNOS genotypes in patients and controls

Genotype /Allele	Patients (N=199)	Controls (N=153)	p, OR (95% CI)
GG	153 (76.9%)	114 (74.5%)	0.077
GT	36 (18.1%)	37 (24.2%)	
TT	10 (5.0%)	2 (1.3%)	
G	342 (86.0%)	265 (86.6%)	0.79, 1.06 (0.67-1.67)
T	56 (14.0%)	41 (13.4%)	

Table 3: Genotype and allele frequencies of eNOS in relation to severity of CAD

	1 vessel (54)	2 vessel (69)	3 vessel (76)	p, OR (95% CI)
GG	45 (83.3%)	54 (78.3%)	54 (76.9%)	0.299
GT	7 (13.0%)	10 (14.5%)	19 (25.0%)	
TT	2 (3.7%)	5 (7.2%)	3 (3.9%)	
G allele	97 (89.8%)	118(85.5%)	127 (83.5%)	0.354
T allele	11(10.2%)	20 (14.5%)	25(16.5%)	

Table 4: Multivariable logistic regression

Risk factors	p-value	OR (95% CI)
Diabetes	0.00	5.97 (2.73-13.0)
Hypertension	0.00	8.49 (4.036-17.9)
Smoking	0.00	7.43 (2.41-22.7)
TG	0.00	1.045 (1.02-1.06)
TC	0.33	1.0 (1.0-1.03)
HDL	0.001	0.9 (0. .85-0.95)
LDL	0.105	1.023 (0.995-1.052)
VLDL	0.7	1.0 (0.89-1.085)
eNOS (GT)	0.884	1.064 (0.463-2.44)
eNOS (TT)	0.081	6.458 (0.796-52.376)

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