Association of endothelial dysfunction with endothelin, nitric oxide and eNOS Glu298Asp gene polymorphism in coronary artery disease

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Abstract. The endothelial dysfunction has been implicated as a major event in the pathogenesis of atherosclerosis. Therefore, this study was planned to determine (a) role of endothelium-derived nitric oxide (NO) and endothelin as coronary artery disease (CAD) risk markers and (b) intergenotypic variation of endothelial nitric oxide synthase (eNOS) Glu298Asp polymorphism in CAD. The endothelin, NO and eNOS genotypes were determined in 60 patients with documented history of CAD. These were compared with 50 age- and sex- matched healthy controls. The genotype frequencies for eNOS gene polymorphism were determined by PCR and RFLP. The plasma endothelin in CAD patients was significantly higher ($p < 0.001$) whereas, the NO level in CAD group was significantly lower ($p < 0.001$) than the control group. The genotype frequencies for Glu298/Asp (Glu/Glu and Glu/Asp) genotypes were 75% and 25% in CAD subjects and 88% and 12% in control subjects, respectively. No Asp/Asp was found in any of the groups. The genotype frequencies differed significantly ($p < 0.05$) between the controls and cases. In conclusion, endothelin and NO may be used as markers of endothelial dysfunction in CAD. Asp allele might be a risk factor for CAD in the North Indian population.

Keywords: Nitric oxide, Coronary artery disease, Glu298Asp polymorphism, endothelin

1. Introduction

Coronary artery disease (CAD) is described as our modern “epidemic”. By 2015, this is expected to account for 34% of all male deaths and 32% of all female deaths in India [1]. Studies suggest that most CAD event rates are noted in individuals with one or more CAD risk factors [2]. However, at least 25 percent of coronary patients have sudden death or myocardial infarction without prior symptoms [3]. Hence, there is a need to focus attention on additional markers to predict coronary risk. It is well accepted that endothelial dysfunction occurs in response to cardiovascular risk factors and precedes the development of atherosclerosis [4]. Therefore, in this study, we have investigated

some of the factors which are considered to play a pivotal role in the maintaining endothelial homeostasis – nitric oxide (NO), endothelium derived bioactive peptide endothelin and G894T variant of endothelial nitric oxide synthase (eNOS) gene polymorphism.

Nitric oxide is one of the most important products synthesized by NO synthase (NOS) of endothelial cells, a signaling molecule that is able to exert profound functional and morphological effects on the vascular wall. Several experimental studies have demonstrated that NO inhibits many key steps of the atherosclerotic disease, and a defect of NO production could facilitate the progression of the atherosclerotic process [5]. NO generation is regulated through alterations in the expression of the eNOS enzyme. Due to the protective role of NO, the eNOS gene has gained special importance in the pathogenesis of CAD [6]. Further, the associations between eNOS genotypes and vascular disease could be population and disease specific. Functional DNA variants such as GT or TT alleles in the eNOS gene can

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lead to changes in the eNOS expression and enzyme activity, which may be modified by environmental factors. Understanding the nature of interaction will not only predict those at high risk for genotype-determined CAD risk, it will also build up interventional strategies.

eNOS is a key enzyme involved in maintenance of vascular homeostasis. A common variant located in exon 7 (G⁸⁹⁴T) of the eNOS gene that modifies its coding sequence (Glu²⁹⁸Asp) has been linked by several groups to the risk for coronary spasm, CAD, and acute myocardial infarction [7–9]. Some studies failed to find any relationship between the Asp variant and the risk of atherosclerosis [10,11] whereas other found that the risk for CAD is confined to individuals homozygous for the Asp allele of the Glu²⁹⁸Asp polymorphism, suggesting that homozygosity for aspartic acid in position 298 could produce a significant decrease in the amount of eNOS or its enzymatic activity [7,9].

Endothelin, a marker of endothelial function and a potent vasoconstrictor peptide, induces smooth muscle cell proliferation and synthesis of extracellular matrix substances [12]. Increased plasma endothelin level may indicate early disturbances of endothelial function. Experimental studies have shown that endothelin may be produced by activated human macrophages and has also been implicated in cytokine and vascular cell adhesion molecule expression [13].

Here, we have investigated the role of emerging biomarkers of endothelial dysfunction that may increase the predictability of a coronary event in Indian population.

2. Materials and methods

This study was conducted in a tertiary care hospital of northern India and included 110 subjects (60 cases and 50 controls), after being approved by the ethical committee of the institution. Informed consent was taken from all the participants. Patients with documented history of coronary artery disease (on the basis of ECG and coronary angiography) were selected from the Out Patient Department of Medicine, Lady Hardinge Medical College and associated hospitals, New Delhi. Age and sex matched controls comprised of healthy volunteers with no clinical or ECG evidence of CAD and negative history of major CAD risk factors (past event of CAD or stroke, Diabetes Mellitus, hypertension, smoking, dyslipidemia and family history of CAD). Patients with recent history of acute coronary syndrome or cerebrovascular event (< 8 weeks),

chronic liver and kidney disease and cancer were excluded from the study. 5ml of overnight fasting blood sample was collected from all study participants, by venipuncture into evacuated tubes. Plasma obtained after centrifugation (10 min at 2500 x g), was divided into 2 aliquots- one for NO evaluation and second for endothelin. The remaining cell aggregate was kept for genotyping. Aliquots were stored at -40°C until batch analysed.

2.1. Estimation of NO

Determination of NO in plasma was performed indirectly by the measurement of stable decomposition product nitrite (NO₂), employing the Griess reaction (Mathew et al. (1996) [14]. Nitrite can be directly detected by observing the magenta colored azo dye that is formed from nitrite (NO₂) and the Griess reagent, the absorbance of which was determined at 543 nm using semiautoanalyser.

2.2. Estimation of endothelin

Plasma endothelin was estimated by ELISA using DRG's human Endothelin-1 Enzyme Immunometric Assay kit. The color generated was read at 450 nm. The measured optical density was directly proportional to the concentration of endothelin.

2.3. Genotyping

The DNA Extraction was done using Bioneer Genomic DNA Extraction Kit. This DNA was amplified by PCR using primers flanking polymorphic region of eNOS gene (Fig. 1). The resulting 457-bp amplification product was digested with Ban II restriction enzyme at 37°C for 20 hours. Ban II digested the amplified fragments into smaller fragments (137 bp and 320 bp) (Fig. 2). In case of a G to T substitution at position 894 of the exon 7 of eNOS gene, a Ban II restriction site was lost. The restricted fragments were resolved on 2% agarose gels and visualized by ethidium bromide staining.

3. Statistical analysis

Statistical analysis was done using SPSS version 14.0 software programme. The variables between patients and controls were compared using Student's t test and Chi square test. 'p' values below 0.05 were considered significant. All values are expressed in mean ± standard error of mean.

Fig. 1. Ethidium bromide-stained agarose gel used for genotyping PCR products (457 bp) with primers flanking Glu298 Asp polymorphic region of eNOS gene. (M-molecular wt. marker).

Fig. 2. eNOS gene digested with BanII restriction enzyme. Homozygotes (GG) showed 2 bands at 320 bp and 137 bp. Heterozygotes for this mutation (GT) showed 3 bands at 457 bp, 320 bp, and 137 bp.

4. Results

The study and the control group were age- and sex-matched. The demographic and biochemical characteristics of the study population are shown in Table 1 and 2 respectively. The mean age of the patients in the study group was 59.46 ± 11.334 years. The study population consisted of 58% females and 42% males. Hypertension and Diabetes mellitus were present in 70% and 36% of the study group, respectively, followed by hypertriglyceridemia (34%) and smoking (26%).

Plasma NO and endothelin levels in the study and the control group are shown in Table 3. The NO level was found out to be significantly lower in the CAD group than in the control group ($p < 0.001$) whereas endothelin was significantly higher in the CAD patients as compared to the healthy controls ($p < 0.001$).

The distribution of genotypes of the Glu298Asp polymorphism is shown in Table 4. GG genotype was found in 45 subjects (75%) of study group and 44 subjects (88%) in the control group. GT genotype was found in 15 subjects (25%) of study group and 6 subjects (12%) of the control group. No TT was found in

Table 1
Demographic characteristics of CAD patients and controls

Demographic factors	Cases (n = 60)	Controls (n = 50)	p value
Age(years)	59.46 ± 1.603	58.32 ± 1.667	0.623
Diabetics %	36	–	<0.001
Hypertension %	70	–	<0.001
Smokers%	26	–	<0.001
Hypertriglyceridemia(> 150 mg/dl) %	34	–	<0.001
Hypercholesterolemia(> 200 mg/dl) %	22	–	<0.001
Family history of CAD %	18	–	<0.001
BMI (kg/m ²)	23.672 ± 0.643	22.836 ± 0.637	0.340

Values are represented as mean ± standard error of mean.

Table 2
Biochemical characteristics of CAD patients and controls

Parameters	Cases (n = 60)	Controls (n = 50)	p value
Total Cholesterol(mg/dl)	167.80 ± 5.997	169.30 ± 3.006	0.890
Triglycerides (mg/dl)	133.62 ± 8.701	129.12 ± 3.846	0.537
HDL-C (mg/dl)	29.90 ± 1.372	40.82 ± 0.882	0.001
LDL-C (mg/dl)	110.20 ± 5.356	103.00 ± 2.943	0.245
LDL-C/HDL-C	4.193 ± 0.311	2.597 ± 0.109	0.001
FBS(mg/dl)	104.20 ± 7.238	82.28 ± 1.188	0.001
Urea (mg/dl)	33.62 ± 1.720	30.10 ± 1.058	0.205
Creatinine(mg/dl)	0.968 ± 0.039	0.824 ± 0.027	0.003
Uric Acid (mg/dl)	4.594 ± 0.163	4.864 ± 0.192	0.271
Bilirubin (mg/dl)	0.754 ± 0.045	0.586 ± 0.027	0.008
ALT (IU/L)	30.74 ± 1.713	29.08 ± 1.938	0.307
AST (IU/L)	36.24 ± 2.925	29.70 ± 2.348	0.086
ALP(IU/L)	140.58 ± 5.56	128.62 ± 4.401	0.120
Total protein (g/dl)	6.88 ± 0.124	7.00 ± 0.097	0.612
Albumin(g/dl)	3.724 ± 0.076	3.810 ± 0.124	0.923

any group. The odd's ratio of the GT genotype in cases was 2.44 which was significantly high.

Table 5 shows the intergenotypic variation of the plasma nitric oxide and endothelin levels in the CAD and control group. NO level was found to be significantly lower in the GT genotype of both study and control group. Also, the NO level was significantly lower in the in the GG genotype of CAD group as compared to the controls.

5. Discussion

CAD is a major cause of mortality and morbidity around the world. The fact that traditional risk factors have failed to explain the excess risk of CAD has raised the possibility of a genetic susceptibility among Asian Indians [15]. We attempted to investigate the frequency of eNOS Glu298Asp polymorphism along with usefulness of some novel biomarkers in patients of CAD.

The mean age of the patients in the CAD group was 59.46 ± 11.33 years. It was found that 61–70 years age group had highest occurrence (34%) of CAD events

followed by 40–50 years age group (32%). This shows that though the occurrence of a CAD event is more common in elderly population, however the incidence is also increasing at an earlier age in Indian population. The study population consisted of 58% females and 42% males. 82% of the females were post-menopausal. As seen in many observational studies, menopausal women have higher risk of CAD [16]. The estrogen deprivation may be related to accelerating the risk atherosclerotic process after menopause [16]. The distribution of risk factors among the study group (Table 1) showed that hypertension(70%) and Diabetes mellitus(36%) were the most prevalent risk factors in CAD patients correlating with the earlier findings that hypertension and Diabetes are associated with marked increase in the risk of CAD [17–19]. There was no significant difference in the BMI between patients and controls (Refer Table 1). BMI of the entire study group (patients and controls) fell in the upper part of the normal range (18–25 kg/m²) indicating an increased susceptibility towards disease [20]. There was a significant difference($p < 0.001$) in the mean fasting blood sugar level of the study and control groups (Table 2). This may be due to the fact that 36% of the study pop-

ulation were diabetic with or without medication. The difference between the mean HDL level and LDL/HDL ratio was also highly significant ($p < 0.001$). Lipoprotein profile is deranged in large proportion of CAD patients [21]. Low HDL and high LDL/HDL ratio has been associated with increased risk of CAD in many studies [22]. Low levels of HDL-C are reported to increase the risk of CAD even when total cholesterol is not elevated [23].

The mean plasma endothelin level in the CAD group was 9.78 ± 2.838 pg/ml and in the control group was 7.86 ± 2.204 pg/ml. The difference between the two was highly significant ($p < 0.001$). Increased plasma endothelin levels may indicate early disturbances of endothelial function. Endothelin is a potent vasoconstrictor, promotes smooth muscle cell proliferation and the synthesis of extracellular matrix substances. These factors play a major role in the pathogenesis of atherosclerotic process. Further to its vasoconstrictor and mitogenic properties, endothelin appears to be involved in the inflammatory process that underlies active atheromatous plaques [13]. According to Zouridakis et al. [24], plasma endothelin was raised in patients with coronary artery disease progression and may be a marker of risk of rapid stenosis progression. Significantly increased plasma concentrations were also found in patients with CAD, with the highest levels in a subgroup of 8 patients presenting with unstable angina [25]. In a recent study [26], the endothelin activity level was significantly elevated in subjects with significant obstructive CAD. Furthermore, high endothelin activity level was found to correlate with calcified plaque burden and to be an independent determinant of lesion of plaque severity.

Nitric oxide inhibits many key steps of the atherosclerotic disease. The fact that in a rabbit model, long term systemic inhibition of NO production with the NOS inhibitor L-NAME(N^G -nitro-L-arginine methyl ester) [27], enhances the formation of early atherosclerotic lesions further reinforces the protective role of NO in CAD. NO is a potent vasodilator. Besides, it also plays a major role in the inhibition of platelet adhesion and aggregation [28], of adhesion molecule and chemokine expression [29], of inflammatory cell infiltration [30] and, of smooth muscle cell proliferation [31]. A significant decrease in the NO level in atherosclerotic vessels may be due to oxidative stress in CAD. Increased superoxide production reacts rapidly with NO producing peroxynitrite which causes oxidative tissue damage by nitrosylation. Thus, endothelial function is impaired in CAD patients which may play a role in the pathogenesis

Table 3
Plasma NO and Endothelin level in the CAD and Control group

	CAD	Control
NO ($\mu\text{mol/L}$)	$11.56 \pm 1.151^*$	16.98 ± 1.248
Endothelin (pg/ml)	$*9.78 \pm 0.401^*$	7.86 ± 0.312

*($p < 0.001$).

of cardiovascular events [32]. In the present study, the mean plasma NO level was significantly lower in CAD group (11.56 ± 8.137 $\mu\text{mol/L}$) than in control group (16.98 ± 8.826 $\mu\text{mol/L}$). The difference between the two was highly significant ($p < 0.001$). Thus, our study interprets that NO has an antiatherogenic effect in the vasculature. Moreover, median plasma NO was found to be significantly higher in CAD patients than in controls in a study by Yoon et al. [33]. A recent study also showed significant low values of NO in non-smoking and non-diabetic patients of CAD as compared to controls [32].

Since NO availability is regulated at the level of synthesis, the gene that encodes eNOS is a candidate for cardiovascular disease [34]. Several eNOS gene polymorphisms have been reported as 'susceptibility genes' in various cardiovascular and pulmonary diseases [35] of which we have studied Glu298Asp. This polymorphism is associated with CAD, end stage renal disease, and diabetic nephropathy in some [9,36–38] but not all studies [39–41]. The Asp allele from the Glu298Asp polymorphism was found to be an independent risk factor for premature ST elevation acute myocardial infarction in Mexican population [42]. However, in a study in Turkish population, neither the frequencies of the Glu298Asp genotypes nor the serum nitric oxide levels showed a significant difference between the patient and control groups [43].

In our study, GT genotype was more commonly present in patients of CAD(25%) as compared to the controls(12%). No TT was found in any group. The genotype frequencies differed significantly ($p < 0.05$) between the controls and cases showing that T allele may be the susceptibility allele for CAD in Indian population. Not much study has been done in India regarding eNOS Glu298Asp polymorphism genotype and allelic distribution in CAD patients. Kamna et al. [44] studied the prevalence of eNOS Glu298Asp polymorphism in 139 healthy volunteers from a region of Northern India. The distribution of GG, GT and TT genotypes was found to be 71.22%, 28.06% and 0.72% respectively and the allelic frequency of G and T allele were 0.853 and 0.148 respectively. In a recent study done in South Indian population, the genotype frequen-

Table 4
Genotype frequencies of Glu298Asp polymorphism in CAD patients and controls

Genotypes	Patients (n = 60)	Control (n = 50)	Odd's ratio
Glu/Glu	45 (75%)	44 (88%)	0.408
Glu/Asp	15 (25%)	6 (12%)	2.44
Asp/Asp	0	0	0

Table 5
Intergenotypic variation of the plasma NO and endothelin level in CAD and control group

Genotype	Nitric oxide ($\mu\text{mol/L}$)		Endothelin (pg/ml)	
	CAD	Control	CAD	Control
GG+GT	$11.56 \pm 1.15^{**}$	16.98 ± 1.25	$9.78 \pm 0.401^{**}$	7.86 ± 0.312
GG	$12.64 \pm 1.38^*$	17.68 ± 1.37	$9.79 \pm 0.46^{**}$	7.81 ± 0.32
GT	7.73 ± 1.34	11.83 ± 1.58	9.72 ± 0.81	8.17 ± 1.13

$^{**}p < 0.001$; $^*p < 0.01$.

cies for Glu298/Asp (Glu/Glu, Glu/Asp and Asp/Asp) genotypes were 46.83%, 30.37% and 22.78% in CAD subjects and 60.75%, 31.64% and 7.59% in control subjects, respectively, the distribution of which was significant ($p < 0.05$) between the controls and cases [45]. Some studies have recently shown that Asp allele is subjected to selective proteolytic cleavage in endothelial cells and vascular tissues [46,47] which may be responsible for the T allele being the risk factor for CAD.

In our study, it was found that in both CAD and control group, the mean NO level was significantly lower in GT genotype as compared to GG genotype. This shows that the T allele may be the susceptibility marker for endothelial dysfunction seen in CAD. We also demonstrated a significant decrease in plasma NO levels ($p < 0.01$) in the GG genotype of the CAD group ($12.641 \pm 8.643 \mu\text{mol/L}$) as compared to the GG genotype of the control group ($17.681 \pm 9.1 \mu\text{mol/L}$). Again in the GT genotype, the mean NO levels in the CAD group were lower ($7.727 \pm 4.452 \mu\text{mol/L}$) than in the control group ($11.833 \pm 3.868 \mu\text{mol/L}$). Moreover, plasma endothelin was found to be significantly higher in the GG genotype of the CAD patients as compared to the controls. This may interpret that there are certain factors other than the genetic predisposition also which are also responsible for the occurrence of CAD.

Thus, in conclusion, our study finds that endothelin and NO may be used for risk assessment especially in premature CAD and in individuals where traditional risk factors are not present. A significant association has been found between Glu298Asp polymorphism and CAD. Though the T allele is more frequently present in the CAD patients, further studies are also suggested for the conclusive evidence.

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