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ORIGINAL ARTICLE

Correlation and Identification of Variable number of Tandem repeats of eNOS Gene in Coronary artery disease (CAD)

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Abstract Endothelium-derived nitric oxide (NO) is synthesized from L-arginine by endothelial nitric oxide synthase (eNOS) encoded by the NOS3 gene on chromosome7. Since reduced NO synthesis has been implicated in the development of coronary atherosclerosis; polymorphisms of NOS gene might be associated with increased susceptibility to coronary artery disease (CAD). We therefore undertook this study to determine the association between the occurrence of CAD and eNOS4 b/a polymorphism in South Indian patients. We investigated the polymorphisms in the 27 base-pair tandem repeats in intron4 of the eNOS gene in 100 unrelated CAD patients with positive coronary angiograms and 100 age and sex matched control subjects without any history of symptomatic CAD. The eNOS gene intron4 b/a 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 63, 26 and 11 per cent in CAD subjects, and 72, 20 and 8 per cent in control subjects, respectively. The genotype frequencies did not differ significantly between the two groups. The frequency of the a allele was 0.24 per cent in CAD subjects and 0.18 per cent in control subjects and no significant association was found between patients and control group ($P = 0.57$, Odds ratio = 3.62). Plasma lipids, glucose and creatinine levels were significantly increased in CAD group. The genotypic frequencies and the allele frequency did not differ significantly between the CAD patients and controls indicating that this polymorphism was not an independent risk factor for the development of CAD in South Indian patients.

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1. Introduction

Millions of people under the age of 40 years across the world suffer asymptomatically from early coronary artery disease (CAD), which in many cases leads to heart attacks in later life. Hence it is important to look for biomarkers in early detection of CAD. Coronary risk factors, such as hypertension, hypercholesterolemia and diabetes mellitus are also known to cause

this disorder (Stehbens, 1990). Atherosclerosis is a systemic dysfunctional endothelial, chronic inflammatory, fibroprolifertive, angiogenic, prothrombotic, multifactorial disease of the arterial intima caused by the retention of modified low density lipoproteins, hemodynamic stress, and accelerated by redox stress (Hayden, 2001; Hayden and Tyagi, 1998, 2000, 2002). Researchers are now looking to pinpoint a gene or marker that can help predict the occurrence of CAD and identify individuals who are at an increased risk. Vascular endothelium modulates blood vessel wall and homeostasis through the production of factors regulating vessel tone, coagulation state, cell growth, cell death and leukocyte trafficking. One of the most important endothelial cell products is nitric oxide (NO), which is the most powerful endogenous vasodilator ever known. It can inhibit the adhesion, aggregation and recruitment of platelets; vascular smooth muscle cells migration and growth, also regulates some vessel-platelet interactions and limits the oxidation of atherogenic low density lipoproteins. Nitric oxide (NO) is synthesized from L-arginine and molecular oxygen by a family of three enzymes, the nitric oxide synthase (NOS) (Furchogott, 1990). 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. Several allelic variants of eNOS gene have been identified and their association with human diseases states has been studied. The evidence suggest that NO may inhibit several key steps in the atherosclerosis process and that an alteration in NO production within the vascular endothelium could contribute to pathogenesis of CAD. Emerging evidence suggests that coronary artery disease (CAD) is related to defects in the generation or action of NO. NO released from cells rapidly autooxidizes to yield NO_2, which interacts with hemoglobin to yield NO_3 (Ignarro et al., 1993). Because NO_2 + NO_3 (termed NO_x) is relatively stable in blood, the concentration of NO_x in blood may be an indicator of the endogenous formation of NO.

There are at least three isoenzymes of NOS: inducible NOS, constitutive neuronal NOS, and constitutive endothelial NOS (ecNOS), which constitute a ''gene family'', located on different chromosomes and expressed in different cell lines. Several eNOS gene polymorphisms have been reported as 'susceptibility genes' in various cardiovascular and pulmonary diseases Marsden et al. (1993). GT substitution in exon7 in codon 298 (Yashimura et al., 1998), T786 mutation in the 5 flanking region (Yashimura et al., 2000) and high numbers of CA, which have been repeated in intron 13 of eNOS gene are also known to be associated with excess of risk of CAD (Stangl et al., 2000). Among the reported polymorphisms of the eNOS gene, a significant association of the 4b/a polymorphism in intron 4 of the eNOS gene with coronary artery disease (CAD) and hypertension has also been reported. Wang et al. (1996) detected an association between homozygosity for the eNOS4a allele and an increased risk for CAD in current or ex-smokers. However, others did not detect a link between this gene variation and cardiovascular events (Ichihara et al., 1998; Park et al., 2000; Matyar et al., 2005). Reports say that the variable number of tandem repeat (VNTR) polymorphism located in intron 4 of eNOS (eNOS4b/a polymorphism) were significantly associated with plasma NO_x concentration Wang et al. (1997). 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. We investigated this association between the occurrence of CAD and the intron4 b/a polymorphism in South Indian Populations.

2. Materials and methods

2.1. Selection criteria

One hundred patients who were angiographically diagnosed as CAD patients (79males and 21females) consecutively admitted to the hospital with proven CAD (more than 50% stenosis affecting at least one vessel) were included in the study. The controls were those who came to the hospital with pain in the chest but did not have a history of angina pectoris or MI, and they showed a normal electrocardiogram (62 males and 38 females), in whom angiographic examination excluded the presence of CAD.

All the patients and controls were interviewed and epidemiological data/demographic data was recorded in a structured questionnaire, that included smoking habits, hypertension, diabetes, dyslipidemia, diet, occupation, treatment and any family history of CAD.

2.2. Data on risk factors

For CAD risk factors, the following definitions were used: subjects were defined as hypertensive if their blood pressure was $>140/90$ mm Hg or if they were receiving any antihypertensive treatment; those with a history of diabetes or who were receiving any anti diabetic drugs were considered to be diabetic; those with a total plasma cholesterol concentration of >200 mg/dl or a triglyceride concentration of >180 mg/dl, or who were receiving lipid lowering drugs, were considered dyslipidemic. Smoking history was recorded as either none or current smokers. A positive family history was the presence of a first degree relative with coronary artery disease at the age of 55years for men and <60years for women.

2.3. Protocol Approval

The institutional review boards of Bhagwan Mahavir Hospital and Research centre approved the research protocol. Informed consent was obtained from all patients and controls, as required by the hospital ethics committee, for clinical and genetic studies.

2.4. Angiographic study

All patients underwent coronary angiography. Coronary stenosis was considered significant in the presence of a luminal diameter narrowing of $>50\%$ of at least one pericardial coronary artery. The severity of coronary artery disease was expressed by the number of affected vessels (one, two, or three vessel) and also by means of the Duke scoring system—a prognostic index that includes the number of diseased major vessels, the presence of left main coronary artery disease, the percentage narrowing of the major vessels, and involvement of the left anterior descending coronary artery particularly when the proximal segment shows severe stenosis ($>95\%$).

The Duke score ranges from 0 to 100 (0 = no disease, 100 = the most severe disease).

2.5. Biochemical analysis

Blood samples (5 ml) were collected from all subjects after 12 h of fasting. Blood sample (2 ml) was placed in EDTA tubes for DNA isolation and 3 ml was used for serum collection.

The serum concentrations of triglycerides (TG), total cholesterol, LDL cholesterol (LDL-C) and HDL cholesterol (HDL-C), urea, creatinine, fasting blood sugar (FBS), were measured by the standard methods Auto-analyzer (Chemwell-USA) used in the clinical laboratory of the hospital at the time of diagnosis of the patients.

2.6. Analysis of VNTR eNOS gene polymorphisms

Extraction of DNA: genomic DNA was extracted from whole blood using standard phenol/chloroform methodologies with ethanol precipitation. The VNTR *b*/*a* genotype were identified by PCR amplification (21). The primer sequences used for amplification were:

5-AGGCCCTATGGTAGTGCCTT-3 (forward primer)

5-TCTCTTAGTGCTGTGGTCAC-3 (reverse primer)

The final concentration of the PCR mixture had 1.5 mM MgCl2, 50 mM KCl, 10 mM Tris–HCl (pH 8.8), 0.1% gelatin, 1% Triton X-100, 0.3 mM each of dNTPs, and 2U Taq DNA (Bioserve, Hyderabad) polymerase in each reaction tube. Amplification was carried out in a thermo cycler (TAKARA-China) under the following conditions with some changes in Salami et al. (2006): initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. The products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining.

2.7. Statistical analysis

All statistical analyses were performed with Sigma Stat. V 3.0. 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 Emery (1976). The χ^2 test was used for deviation of genotype distribution from Hardy–Weinberg equilibrium. The differences between groups were examined by χ^2 test or an independent student *t*-test whichever was 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 TG, total cholesterol level, LDL-C, HDL-C, LDL-C/HDL-C, urea, FBS, createnine and eNOS genotype.

3. Results

Base line demographics are present in Tables 1 and 2. The mean age at sampling of patients was 57.21 and 54.32years in control group. Two alleles of the VNTR in the human eNOS gene were observed one with four 27 bp 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. The genotype frequencies of 4b/b polymorphism in control subjects were 72%, 20% for b/a and 8% for a/a. On the other hand in CAD patients genotype frequencies were 63% for b/b, 26% for b/a and 11% for a/a (Table 3). There was no significant difference in the genotype of eNOS (χ^2 = 1.33, P = 0.24, odds ratio = 0.83) between the cases and controls. The frequencies of the a and b alleles were 24% and 76% for CAD patients and; 18 and 82% for the control subjects, respectively which was insignificant (χ^2 = 3.62, P = 0.057, odds ratio = 0.62).

As expected, both the control and patient group showed differences in the biochemical markers and other conventional risk factors for CAD. Dyslipidaemia, hypertension, diabetes mellitus, triglycerides and a previous record of CAD in the family are much higher in CAD patients than in controls. In average, LDL was also higher in the CAD group as well as

Table 1 Demographic characteristics of patients and controls.

	Cases n = 100	Controls n = 100	p-Value
Age males (21–82)	57.21 ± 14.05	54.32 ± 24	0.81
Age females (20–72)	51.69 ± 11.12	50.62 ± 10.72	0.67
Alcoholics (%)	26	10	< 0.01
Diabetics (%)	41	13	< 0.001
Hypertension (%)	34	10	< 0.01
Smokers (%)	37	8	< 0.001

Values are represented as mean ± SD: $p < 0.05$ in comparison to control group.

Table 2 Biochemical characteristics of patients and controls.

Parameters (mg/dl)	Cases n = 100	Control n = 100	p-Value
Total cholesterol	223.69 ± 62.32	203.34 ± 23.12	0.67
Triglycerides	203.34 ± 85.6	153.41 ± 17.81	< 0.0001
HDL-C	45.56 ± 13.95	41.16 ± 10.16	0.15
LDL-C	151.81 ± 63.08	130.12 ± 51.09	0.03
CHO/HDL-C	5.18 ± 2.29	5.34 ± 1.17	0.45
LDL-C/HDL-C	4.03 ± 2.14	3.18 ± 2.16	< 0.01
FBS	213.15 ± 118.21	124.72 ± 6.80	< 0.0001
Urea	39.19 ± 14.10	35.18 ± 7.83	0.72
Creatinine	1.16 ± 0.95	1.12 ± 0.98	0.75

Values are represented as mean ± SD: $p < 0.05$ in comparison to control group.

Table 3 Distribution of VNTR of eNOS genotypes and allelic frequency of the study population.

Study group	VNTR genotype				Allelic frequency		
N = 100	4b/b	4b/a	4a/a	Total	b	a	Total
Patients	63	26	11	100	152 (0.76)	48 (0.24)	200
Control	72	20	8	100	164 (0.82)	36 (0.18)	200

χ^2 = 1.33(2df), p = 0.24 for genotype; χ^2 = 3.26 (1df), p = 0.057 for allelic frequency.

Odds ratio for b/a genotype, a/a genotype and a allele are 0.83 (95% CI; 0.6–1.13), 0.81 (95% CI; 0.53–1.23), and 0.62 (95% CI; 0.62–0.99) respectively.

total cholesterol. We found that the biochemical parameters like triglycerides, LDL, glycemia, CAD history, smoking habit, dyslipidaemia to be independently related to CAD.

4. Discussion

Prevention of CAD and reduction of its mortality and morbidity remains one of the greatest public health challenges throughout the world. Over the last two decades, function of the vascular endothelium has emerged as a strong marker for monitoring cardiovascular (CV) health on the basis that impairment of endothelial function is the earliest event in the process of developing CAD. Endothelial dysfunction has been frequently associated with all common CV risk factors predisposing to atherosclerosis (Bergholm et al., 2003), such as age (Celermajer et al., 1994), obesity (Steinberg et al., 1996), hypertension (Linder et al., 1990), hypercholesterolemia (Heitzer et al., 1996), diabetes (Meigs et al., 2006), smoking (Celermajer et al., 1996) and physical inactivity (Franzoni et al., 2005).

In the presence of endothelial dysfunction, improvement in the endothelial function has been observed with various pharmacological treatments and also was evident with behavioral modifications including increased physical activity. Over the last decade, a remarkable burst of evidence has accumulated; offering the new perspective that NO plays a pivotal role in CAD. Even though nitric oxide may not be the only answer, but it was declared THE MOLECULE OF THE YEAR in 1998 and the discoverers were awarded Noble prize for its role in cardiovascular diseases. Nitric oxide is a highly diffusible and versatile molecule that affects almost every biological system. Increase in cGMP activates the protein kinase G family, thus resulting in a cascade of responses at the transcriptional and translational levels. Increased cGMP causes relaxation of vascular smooth muscle, mediates shear stress-induced endothelial dependent vasodilation, inhibits platelet aggregation, monocyte adhesion to the endothelium, inhibits vascular smooth muscle cell (VSMC) growth and migration, and finally, reduces the oxidation of LDL cholesterol. Recent studies have found much evidence for the role of oxidative stress in the degradation of NO. This reduction in vascular NO bioavailability has been shown to contribute to altered vasomotor tone, hypertension, endothelial dysfunction and development of atherosclerosis (Patel et al., 2000; Bloodsworth et al., 2000). It has been reported that different VNTR alleles of the eNOS gene may be associated with CAD and hyperlipidemia (Moreel et al., 1992; Cavalli et al., 2000). Some investigators identified an association of the 27-bp tandem repeat polymorphism with the risk of MI, CAD and EH (essential hypertension) (Shoji et al., 2000; Gardemann et al., 2002), whereas others did not detect any link between CAD, MI and EH (Hibi et al., 1998). eNOS intron4 a/b polymorphism was found to be a risk factor in addition to HT, DM, male gender, age and smoking for the development of CAD in Southern Turkey. Patients with endothelial nitric oxide synthase (eNOS) intron4 27-bp repeat homozygotes were more likely to develop severe coronary stenosis when they smoked Wang and Wang (2000).

Studies in Indians population show lack of association of intron4 VNTR of eNOS gene with DR (dilated retinopathy) in Southern India (Uthra et al., 2007). Venkata et al. (2005) found strong association of eNOS with RFLP and also studies from Delhi also confers association of eNOS gene with COPD. Interestingly, the association of ApoB100 VNTR and RFLPs with plasma lipid concentration or coronary artery disease varies in different ethnic groups and has not always been found to be associated with CAD.

There was no statistically significant difference in eNOS b/a allele percentages between cases and controls. In fact, discrepancies in association studies may also result from consideration limited to only one polymorphism rather than combinations of polymorphisms. However, it does emphasize the importance of such studies, which may help in future to delineate the high-risk group for CAD, and may be of use in the genetic screening of patients with CAD belonging to different populations.

5. Conflict of interest

The authors declare that they have no conflict of interest.

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