Full Length Research Paper

Evidence of association of a common variant of the endothelial nitric oxide synthase gene (Glu^{298} →Asp polymorphism) to coronary artery disease in South Indian population

Rabbani Syed[1,2] and Kaiser Jamil[1,2]*

[1]Genetics Department, Bhagwan Mahavir Medical Research Centre 10-1-1, Mahavir Marg, Hyderabad-500004 A.P., India.
[2]School of Biotechnology, Mahatma Gandhi National Institute of Research and Social Action, Hyderabad, A.P. India.

Genetic variants of endothelial nitric oxide synthase (eNOS) could influence individual susceptibility to coronary artery disease (CAD) with or without association with demographic factors. This study was designed to assess the Glu^{298}/Asp variant of the eNOS gene in 79 patients with CAD as compared to equal number (79) of controls. The genotype frequencies for eNOS gene polymorphism were determined by PCR and RFLP, and the results analyzed statistically. Demographic factors were recorded using structured questionnaire. The genotype frequencies for Glu^{298}/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 genotype frequencies differed significantly ($p < 0.05$) between the controls and cases. From our regression analysis we found that Glu/Asp variant in association with other factors such as hypertension, smoking, were independent risk factors of CAD, whereas other factors were not CAD independent risk factors. The individuals with eNOS Glu^{298}/Asp variant were at greater risk of CAD, and this might indicate genetic susceptibility to CAD and that eNOS gene (Glu^{298}/Asp) polymorphism could represent a useful genetic marker in identifying individuals at greater risk of developing atherosclerotic disease.

Key words: eNOS gene, polymorphisms, CAD, risk factors, genetic markers.

INTRODUCTION

Coronary atherosclerosis (CAD) is a common disease that causes ischemic heart diseases, such as angina pectoris and myocardial infarction (MI). Since as many as half of the patients have no symptoms, despite the presence of CAD, coronary deaths in India are expected to be the double over 20 years and reach two million by 2010 (Ghaffar et al., 2004). They may have silent ischemia or be unaware of the potentially dangerous abnormal heart rhythms (arrhythmias). The absence of chest pain or other common symptoms can also set the

stage for a heart attack that occurs without warning. Hence it is important to look at biomarkers for early detection of CAD. In addition, recently, the gene polymorphisms of angiotensin-converting enzyme (ACE) 1, apolipoprotein and eNOS gene have been reported as independent risk factors for myocardial infarction, although the genetic cause of this disorder has not been proved completely. Epidemiological studies also indicate that hyperlipidaemia, hypertension, cigarette smoking, diabetes, and obesity are risk factors for coronary artery disease (Bhopal 2000). Control of these environmental risk factors has, however, not been effective in completely predicting the development of the atherosclerotic process, suggesting that specific genetic predisposition

*Corresponding author. E-mail: rabbanisyd@gmail.com.

should be taken into account as well. Vascular endothelium modulates blood vessel wall 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 synthesized from L-arginine by the enzyme endothelial nitric oxide synthase (eNOS) (Furchogott et al., 1990). NO has diverse physiologic regulatory functions and is involved in smooth muscle relaxation, inhibition of platelet aggregation, immune regulation, neurotransmission and blood pressure regulation. Moreover, it has been shown that eNOS inhibition accelerates atherosclerosis in animal models, and that abnormalities of the endothelial NO pathway is present in humans with atherosclerosis. This evidence suggests that NO may inhibit several key steps in the atherosclerotic process and that an alteration of NO production within the vascular endothelium could contribute to the pathogenesis of atherosclerosis (Moncada and Higgs, 1993; Schmidt and Walter, 1994; Forte et al., 1997). Thus eNOS could be a candidate gene for atherosclerosis.

MATERIALS AND METHODS

Selection criteria

We included 79 patients with angiographically diagnosed CAD (52 males and 27 females; 50.79 ± 11.55 years) consecutively admitted to our Hospital with angiographically proven coronary artery disease (more than 50% stenosis affecting at least one vessel) and 79 healthy controls (43 males and 36 females; 49.2 ± 12.16 years old), in whom angiographic examination excluded the presence of coronary artery disease. 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.

Patient's characteristics

All the patients and controls were interviewed and epidemiological data / demographic data on smoking and alcohol habits, hypertension, diabetes, dyslipidemia, and family history of   were recorded. Informed consent was obtained from all patients and controls, as required by our ethics committee.

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 > 180 mg/dl, or who were receiving lipid lowering drugs, were considered dyslipidemic. Smoking history was recorded as either none or current smokers. Alcoholic are those who take 2 ounce /day (42% alcohol) are considered.

Each participating was interviewed to determine variables, such as smoking, alcohol use, family history ($\geq$1 first-degree relatives with MI or CAD), and to obtain consent for a blood sample for genetic analysis. A total of 79 CAD patients included in the current

study agreed to participate and to provide a blood sample for genetic analysis. The research protocol was approved by the institutional review boards of institution; all study participants provided written informed consent for clinical and genetic studies (EC-18 dt -26-4-09).

Biochemical analysis

Blood samples were collected from all subjects after 12 h fasting and placed in EDTA tubes and stored at -80°C until the time of assay for DNA extraction and for biochemical assays. The serum concentrations of triglyceride (TGL), total cholesterol (CHO), LDL cholesterol (LDL-C) and HDL cholesterol (HDL-C), urea, creatinine, fasting blood sugar (FBS) were measured by the standard methods (Auto-analyzer) used in the clinical laboratory of the hospital at the time of the patients diagnosis.

Analysis of the G→ T (Glu^{298}/Asp) polymorphism of exon 7 of eNOS gene

Genomic DNA was extracted from samples of whole blood by standard methods (salting out).The coding sequence variant was a G→ T substitution at position 894 in exon 7 which determines the Glu to Asp amino acid substitution (in codon 298) in the mature eNOS protein. According to previously described procedure, genotyping of all subjects was performed by polymerase chain reaction amplification (PCR) of exon 7 with the primers

5'-CATGAGGCTCAGCCCCAGAAC -3' (sense) and
5'-AGTCAATCCCTTTGGTGCTCAC -3' (antisense)

This was followed by *MboI* restriction enzyme digestion for 16 hours at 37°C. In the presence of a thymine (T) at nucleotide position 894 which corresponds to Asp298, the 139 base pair (bp) PCR product is cleaved into two fragments of 119 and 20 bp. The products of the digestion process were highlighted by electrophoresis on 3% agarose gel (Figure 1).

Statistical analysis

Genotype distributions in cases and controls were examined for significant deviation (p<.05) using Hardy-Weinberg equilibrium. The frequencies of risk-associated variants were compared in cases and controls by using the χ^2 test (SPSS 13.0 Exact Tests, SPSS Inc, Chicago, Ill). Allelic frequencies were calculated according to the number of different alleles observed and the total number of alleles examined. We performed multivariate logistic regression analysis to adjust risk factors, in which CAD was a dependent variable and independent variables were hypertension, smoking, alcohol intake habits, diabetes, TGL, total cholesterol level (CHO), LDL-C, HDL-C, CHO/HDL-C, LDL-C/HDL-C, FBS, urea, createnine and eNOS genotype. All the data were shown in mean ± SD.

RESULTS

Patients' characteristics and the details of the demographic and biochemical characteristics of the cases and controls are presented in Tables 1 and 2, respectively. In the present study we observed that age and alcoholism did not have significant role in CAD whereas other contributing factors such as diabetes, hypertension, hyperlipidemia, and smoking showed significant
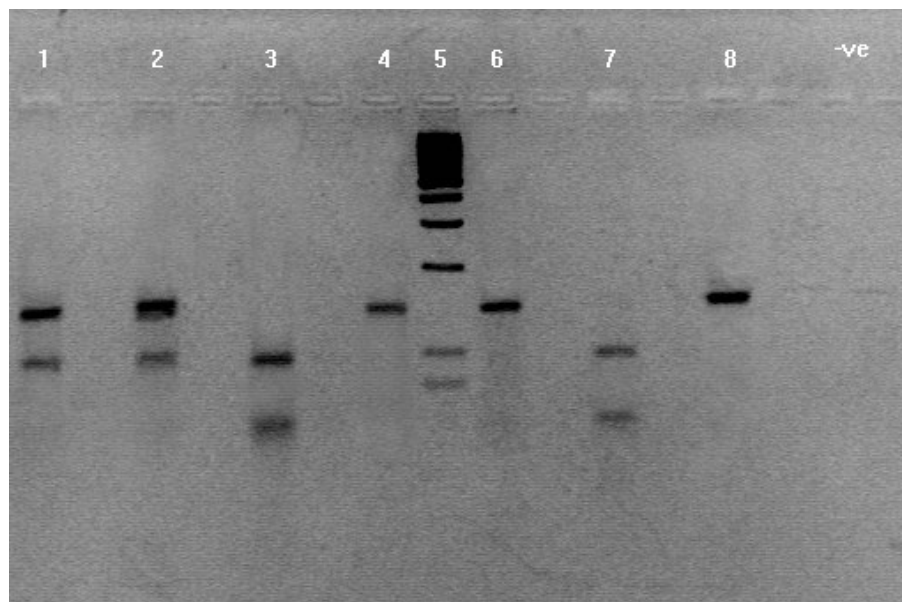
Figure 1. Bi-allelic polymorphism in exon 7 of the *NOS* gene detected by *Mbo*I restriction endonuclease digestion of the 139 bp PCR product: Lanes 1, 2, are restriction digested samples showing restriction patterns corresponding to heterozygocity for Asp/Glu. Lanes 3, 7 are restricted digested samples showing restriction patterns corresponding to homozygosity for Asp[298]. Lanes 4, 6, 8 are samples subjected to restriction digestion samples showing restriction patterns corresponding to homozygosity for Glu[298.]Lane 9 are negative control.

Table 1. Demographic characteristics of patients and controls.

Demographic factors	Cases n=79	Controls n = 79	p-value
Age males (23-76)*	50.92±11.92	50.79±11.55	0.56
Age females (18-62)*	52.69 10.93	49.2±12.16	0.65
Alcoholism %	22(27.8%)	12(11.39%)	0.11
Diabetics %	30(38.46%)	12(15.18%)	<0.001
Hypertension n %	43(55.12%)	11(13.92%)	<0.001
Smokers %	37(47.43%)	10(12.65%)	<0.001
Hyperlipidemia	27(34.17 %)	10(12.65%)	<0.001

*Values are represented as mean ± SD: $p < 0.05$ is considered significant.

Table 2. Biochemical characteristics of patients and controls.

Parameters	Cases n=79	Controls n=79	p-value
Total Cholesterol	203.49±45.87	206.91±15.71	0.56
Triglycerides (mgs/dl)	199 ±90.6	147.52±15.71	<0.001
HDL-C (mgs/dl)	42.16 ± 11.47	42.76±8.48	0.11
LDL-C (mgs/dl)	132.75±18.53	117.55 ±46.94	0.01
CHO/HDL-C (mgs/dl)	5.05 ±2.3	4.98±1.08	0.59
LDL-C/HDL-C(mgs/dl)	3.15 ±2.14	3.22±.88	0.21
FBS(mgs/dl)	213.15±118.21	124.72±6.80	<0.001
Urea (mgs/dl)	37.03 ±11.73	37.83±7.83	0.72
Creatinine (mgs/dl)	1.13 ±.6	1.18±.45	0.05

Values are represented as mean ± SD: $p < 0.05$ is considered significant.

association with CAD. The Glu[298] /Asp variant of the eNOS gene was detected by polymerase chain reaction/ restriction fragment length polymorphism analysis in 79 patients with CAD and 79 healthy controls. The geno- types Glu/Glu, Glu/Asp and Asp/Asp of the Glu[298]Asp polymorphism were present in 48 (60.75%), 25 (31.64%), and 6 (7.59%) of the 79 healthy control subjects and in 37 (46.83%), 24 (30.37%), and 18 (22.78%) of the 79 patients with CAD, respectively. The distribution of geno- types in the control group did differ significantly from that expected to be under Hardy-Weinberg equilibrium, and was a significant deviation from Hardy-Weinberg in the case group, with an excess of Asp homozygotes among the patients with CAD. The proportion of Asp homo- zygotes in the patients with CAD was 22.78% compared to 7.59% in controls (p = 0.05). In comparison, Glu homozygotes were present in 46.83%of CAD patients and in 60.75% of controls. The genotyping analysis and allele frequency are presented in Table 3. The proportion of Asp homozygotes among CAD cases was not significantly altered when subjects were subdivided by alcoholic, diabetics, hypertension, smoking status, or hyperlipidemia conditions. The odds ratio (see Table 4) for the Asp variant in this study is substantially greater than that reported previously for other CAD candidate gene polymorphisms [OR 3.59, CI 1.34-9.61, p < 0.05]. Regression analysis results given in Table 5 also reveals that Asp variant in association with contributing factors such as smoking (p = 0.02, Or ratio 2.8134 95% CI (1.1061 to 6.1561), hypertension (p = 0.01 OR ratio 3.3231 95% CI (1.3189 to 8.3728), levels were independent risk associated factors for the CAD.

DISCUSSION

Atherosclerosis is a multifactorial disease that involves complex interactions between genes and environmental factors (Tuomisto et al., 2005). Environmental and genetic factors influence a person's blood in terms of fat, or lipid levels, important risk factors for coronary artery disease (CAD). Epidemiologic, clinical, genetic, experimental, and pathological studies have all clearly established the major role of lipoproteins in athero- genesis (Havel et.al 1995). You should discuss about the other risk factors: smoking, hypertension, etc.

There was the expected clustering of CAD risk factors among cases. However, we detect an association between the Glu[298]/Asp polymorphism of the eNOS gene and other risk factors for CAD. Controversial results regarding this association have been reported. Previous studies from Japan and the United Kingdom have already suggested a role for the G894T polymorphism in the development of coronary atherosclerosis, with the risk being confined to TT894 homozygosity (Hiagorani et al., 1999; Hibi et al., 1998; Casa et al., 2004), as in our study. In one of the study a novel A→G polymorphism was

identified in intron 6 of eNOS in REPL patients, (Venkata et al., 2005) and also other study in which gender specific ACE gene polymorphism identified in essential hypertensive patients (Bhavani et al., 2004) from South India.

Some data suggested that the Asp[298] variant of eNOS could contribute to the generalized architecture of the vessels. This hypothesis is supported by in vivo evidence that eNOS mutant mice display a paradoxical increase in wall thickness accompanied by a hyperplasic response of the arterial wall after carotid artery ligation (Rudic et al., 1998). This suggests that a primary defect in the NOS/NO pathway may promote abnormal remodeling and pathological changes in vessel wall morphology associated with atherosclerosis. Thus, it is possible that in the process of atherosclerotic remodeling of adult human vessels, alterations in NO production resulting from the substitution of Glu[298] with Asp[298] could have a major impact on smooth muscle cell migration and proliferation (Stocker and Keaney, 2004). The mutant allele of the T-786C polymorphism in the promoter region of the eNOS gene has been associated with a reduced promoter activity and endothelial synthesis of NO, both of which predispose to coronary spasm in the Japanese population. Moreover, Yoshimura and colleagues found that the T-786C variant is in linkage disequilibrium with the eNOS gene intron 4b/a polymorphism, (Yoshimura et al., 2000), suggesting that the T-786C mutation underlies the functional characteristics of the intron 4a allele. Some results suggest that the eNOS gene locus is associated with diabetic nephropathy and the functional polymorphisms (-786T > C and 894G > T) might lead to a decreased expression of eNOS gene. eNOS intron 4 a/b polymorphism (presence of a allele) is a risk factor in addition to HT, DM, male gender, age and smoking for the development of CAD in Southern Turkey (Matyar et al., 2005). Patients with endothelial nitric oxide synthase (eNOS) intron 4 27 repeat homozygote's were more likely to develop severe coronary stenosis when they smoked (Xing and Jian, 2005). It is not known whether the associations that we reported in this study reflect a hypo functional enzyme or linkage disequilibrium between the Glu[298]/Asp polymorphism and another functional variant within the eNOS gene or another gene. Nishijima et al. (2007) reported that -786T/C polymorphism in the 5'- flanking region of the endothelial nitric oxide synthase (eNOS) gene is strongly associated with coronary spasm and is an independent risk factor for readmission due to recurrent attacks in these patients.

The above data suggests that the rate of the Asp geno- type alleles in the homozygous conditions is much higher in the CAD affected population compared to controls. This kind of a statistically significant difference in the frequency of and a specific allele/genotype between patients and controls may indicate a risk amounting to CAD. Regression analysis revealed that Asp/Asp genotypes together with hypertension and smoking were

Table 3. Genotype and Allele frequencies of $Glu^{298}\longrightarrow$ Asp polymorphism in CAD patients and controls.

Groups	Glu/Glu (%)	Glu/Asp (%)	Asp/Asp (%)	Total	Allele frequency		Total
					Glu (%)	Asp (%)	
CAD Cases	37(46.83)	24(30.37)	18(22.78)	79	98(60.20)	60(37.97)	158
Controls	48(60.75)	25(31.64)	6(7.59)	79	121(76.58)	37(23.41)	158

$\chi^2 = 7.44(2df)$, $p = 0.024$ for genotype; $\chi^2 = 7.87$ (1df), $p = 0.005$ for allele frequencies.

Table 4. OR of the $G\longrightarrow T$ (Glu^{298} /Asp) polymorphism in CAD Patients and Controls.

Genotypes	Patients n = 79 (%)	Controls n = 79 (%)	Odds ratio	95% CI
Glu/Glu	37(46.83)	48(60.75)	0.568	0.3-1.07
Glu/Asp	24(30.37)	25(31.64)	0.942	0.48-1.85
Asp/Asp	18(22.78)	6(7.59)	3.590	1.34-9.61

Table 5. Results for logistic regression.

Variables	Odds ratio (95% CI)	p-value
Diabetes	1.0097 (0.9999 to 1.0197)	0.0455
Hypertension	3.3231 (1.3189 to 8.3728)	0.0109
Smokers	2.8134 (1.1061 to 6.1561)	0.0269
Alcoholism	2.4231 (0.9721 to 6.0397)	0.0575
Urea	0.9889 (0.9610 to 1.0175)	0.4427
Creatinine	1.0529 (0.7302 to 1.5181)	0.7826
Total cholesterol	0.9904 (0.9691-1.0122)	0.3861
TGL	1.0088 (1.0013 to 1.0163)	0.0206
LDL–C	1.0094 (1.0019 to 1.0169)	0.0140
CHO/HDL-C	0.7941 (0.6242-0.1.0104)	0.0607
LDL-C/HDL-C	0.7459 (0.5269 to 1.0560)	0.0983

significant risk factors for CAD.

ACKNOWLEDGEMENTS

REFERENCES

Bhopal R (2000). What is the risk of coronary heart disease in South Asians? A review of UK research. J. Public Health Med., 22: 373-385.
Bhavani BA, Padma T, Sastry BKS, Krishna RN (2004). Gender specific Association o f Insertion / Deletion Polymorphism of Human Angiotensin converting enzyme gene with essential Hypertension. Int. Hum. Genet., 4(3): 207-213.
Casa JP, Bautista LE, Humphries SE, Hingorani AD (2004). Endothelial nitric oxide synthase genotype and ischemic heart disease: meta-analysis of 26 studies involving 23028 subjects. Circulation, 109: 1359-1365.
Furchogott RF (1990). Studies on endothelium-dependent vasodilatation and the endothelium-derived relaxing factor. Acta Physiol. Scand., 139: 257-270.
Forte P, Copland M, Smith LM, Milne E, Sutherland J, Benjamin N (1997). Basal nitric oxide synthesis in essential hypertension. Lancet, 349: 837-842.
Ghaffar A, Reddy KS, Singhi M (2004). Burden of non-communicable diseases in South Asia. BMJ. 328: 807-810.
Havel RJ, Rapaport E (1995). Management of primary hyperlipidemia. N. Engl. J. Med., 332: 1491-1497
Hiagorani AD, Liang CF, Fatibene J, Lyon A, Monteith S, Parsons A, Haydock S, Hopper RV, Stephens NG, O'Shaughnessy KM, Brown MJ (1999). A common variant of the endothelial nitric oxide synthase (Glu^{298} Asp) is a major risk for coronary artery disease in the UK. Oct 5. Circulation, 100: 1515-1520.
Hibi K, Lshigami T, Tamura K, Mizushima S, Nyui N, Fujita T, Ochiai H, Kosuge M, Watanabe Y, Yoshii Y, Kihara M, Kimura K, Ishii M, Umemura S (1998). Endothelial nitric oxide synthase gene polymorphism and acute myocardial infarction. Hypertension, 32: 521-526.
Moncada S, Higgs A (1993). The L-arginine-nitric oxide pathway. N. Engl. J. Med., 329: 2002-2012.
Matyar S, Attila G, Acarturk E, Akpinar O, Inal T (2005). eNOS gene intron4 a/bVNTR polymorphism is a risk factor for coronary artery disease in Southern Turkey. Clin Chim Acta, 354: 153-158.
Nishijima T, Nakayama M, Yoshimura M, Abe K, Yamamuro M, Suzuki S, Shono M, Sugiyama S, Saito Y, Miyamoto Y, Nakao K, Yasue H, Ogawa H (2007). The endothelial nitric oxide synthase gene -786T/C polymorphism is a predictive factor for reattacks of coronary spasm. Pharmacogenet. Genomics., 8: 581-587
Rudic RD, Shesely EG, Maeda N, Smithies O, Segal SS, Sessa WC (1998). Direct evidence for the importance of endothelium-derived nitric oxide in vascular remodeling. J. Clin. Invest., 101: 731-736.
Schmidt HH, Walter U (1994). NO at work. Cell, 78: 919-925.
Stocker R, Keaney Jr. JF (2004). Role of oxidative modifications in atherosclerosis. Physiol. Rev., 84: 1381-478.
Tuomisto TT, Binder BR, Yla-Herttuala S (2005). Genetics, genomics and proteomics in atherosclerosis research. Ann. Med., 37: 323-32.
Venkata S, Lakshmi R, Murthy K, Venkata P, Mamata D, Lalji S (2005). Recurrent early pregnancy loss and endothelial nitric oxide synthase gene polymorphisms. Springer Berlin, 274: 119-124.
Yoshimura M, Yasue H, Nakayama M, Shimasaki Y, Ogawa H,

Kugiyama K, Saito Y, Miyamoto Y, Ogawa Y, Kaneshige T, Hiramatsu H, Yoshioka T, Kamitani S, Teraoka H, Nakao K (2000). Genetic risk factors for coronary artery spasm: significance of endothelial nitric oxide synthase gene T-786→C and missense Glu298Asp variants. J. Invest. Med., 48: 367-374.

Xing LW, Jian W (2005). Smoking-gene interaction and disease development: Relevance to pancreatic cancer and atherosclerosis. Springer, 29(3): 344-353.