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Prevalence of endothelial nitric oxide synthase (*eNOS*) gene exon 7 Glu298Asp variant in North Eastern India

Priyanka Shankarishan, Prasanta Kumar Borah, Giasuddin Ahmed* & Jagadish Mahanta

*Regional Medical Research Centre, NE Region (ICMR), Dibrugarh & *Department of Biotechnology, Gauhati University, Guwahati, Assam, India*

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***Background & objectives*: Endothelial nitric oxide is a potent vasodilator and impairment of its generation brought about by gene polymorphism is considered a major predictor for several diseases. A single nucleotide polymorphism G894T within exon 7 of endothelial nitric oxide synthase (*eNOS-7*) gene, resulting in a replacement of glutamic acid by aspartic acid, has been studied as a putative candidate gene for cardiovascular diseases. The pattern of eNOS-7 Glu298Asp variant in the Indian population is poorly known. The present study was planned to determine the prevalence of the variant of this gene among tea garden community in Assam, North-East India with high prevalence of hypertension.**

***Methods*: Study participants of both sex aged ≥18 yr were recruited randomly from temporary field clinics established in tea gardens of Dibrugarh, Assam. Genomic DNA was extracted from 409 subjects by the conventional phenol-chloroform method. The prevalence of the eNOS exon 7 Glu298Asp variant was determined by polymerase chain reaction and restriction fragment length polymorphism analysis.**

***Results*: The study population was in Hardy-Weinberg Equilibrium. The frequency of the eNOS GG, GT and TT genotypes was found to be 75, 22 and 3 per cent respectively and did not show any significant difference in gender wise analysis.**

***Interpretation & conclusions*: Our results showed that the prevalence of the homozygous GG genotype was high (75%) and the rare mutant genotype (homozygous, TT) was 3 per cent in a population at risk with cardiovascular disease. Such population-based data on various polymorphisms can ultimately be exploited in pharmacogenomics.**

Key words Endothelial nitric oxide synthase (*eNOS*) gene - exon 7 Glu298Asp variant - north east India - tea garden community

Cardiovascular diseases are fast emerging as a major health hazard and it is estimated that by 2020, the burden of cardiovascular diseases in India would surpass any other country in the world[1]. Hypertension is the biggest and an almost entirely treatable cause of cardiovascular disease and even small ethnic differences in its optimum management have large implications for health resources. Genetic factors appear to be important in the pathogenesis of cardiovascular diseases but the molecular basis of this genetic predisposition remains largely unknown. The role of endothelial nitric oxide synthase (eNOS) in normal physiology suggests that it

could be a potential candidate gene for cardiovascular diseases. Yet no nationwide epidemiological studies to determine the prevalence of the candidate genes for cardiovascular diseases have been carried out. Sporadic studies from some parts provide data on the epidemiology of the candidate genes in India.

Nitric oxide, synthesized in vascular endothelial cells from the abundant amino acid L-arginine by the enzyme endothelial nitric oxide synthase (eNOS), is a potent vasodilator. At least three distinct NOS isoforms exist in mammalian cells: neuronal (nNOS, Type I), inducible (iNOS, Type II) and endothelial (eNOS Type III)[2]. In addition to its vasodilatory properties, NO exerts some anti-inflammatory effects in the blood vessel wall by inhibiting leukocyte adhesion to the endothelium. A single nucleotide polymorphism G894T within exon 7, resulting in a replacement of glutamic acid by aspartic acid at codon 298, a functional 27 bp variable tandem repeats (VNTR) in intron 4 (intron 4 a/b) of *eNOS* gene and a single nucleotide polymorphism T-786C at the promoter region has been extensively investigated with regard to its association with cardiovascular diseases. This replacement of glutamic acid by aspartic acid is said to reduce the activity of the enzyme, thereby resulting in a decrease in the production of nitric oxide. Impairment of NO bioavailability leads to endothelial dysfunction which is a pivotal event in the pathogenesis of many cardiovascular diseases such as hypertension, heart failure, and coronary artery disease[3,4]. Because of important evidences of *eNOS* gene exon 7 Glu298Asp variant involved in the blood pressure regulation, this has been studied as a putative candidate gene for cardiovascular diseases.

Tea garden workers in Assam migrated from central and south India *viz*., Madhya Pradesh, Bihar, Orissa and Andhra Pradesh[5] in the pre-independence era as labourers in the tea industries, are now maintaining their own tradition and culture and living almost like a closed society, and are socio-economically lagging behind and are mostly illiterate. Dibrugarh in Assam has the largest land area for tea cultivation among all the districts of Assam. Earlier studies have shown that the prevalence of hypertension among them is very high[6]. Currently there are no studies investigating the prevalence of eNOS exon 7 Glu298Asp variant in the population of north-east India. Therefore, in the present study, an attempt was made to explore the prevalence of eNOS 7 Glu298Asp polymorphism in the said population.

Material & Methods

Study subjects: The sample size for the present study was determined by using standard statistical method. The preliminary estimate for prevalence of hypertension referred from a previous study[6] was found to be 60.8 per cent. With this prevalence and at confidence interval of 95 per cent with maximum allowable margin of error 5 per cent, the targeted sample size was found to be 369.

To achieve this sample size, a community based study was conducted through organization of temporary field clinics in 10 tea gardens selected randomly from a total of 129 tea gardens in Dibrugarh district, Assam during the year 2009. Health awareness campaigns was organized in the selected field sites and the people of either sex, aged 18 yr and above were invited to participate in this programme. Subjects participating voluntarily and giving informed consent for providing socio-demographic and clinical information and blood samples were eligible for the present study. By random selection, we recruited a total of 409 subjects from the selected tea gardens which included tea garden workers (378) and a few subjects from indigenous Assamese community (31). Ethical approval for the conduct of the study was obtained from the institutional ethical committee of Regional Medical Research Centre, NE Region (ICMR), Dibrugarh, Assam.

Collection of blood samples: Approximately 5 ml of peripheral blood samples were collected in a screw cap tube that contained EDTA. The specimen was transported to the laboratory at RMRC, NE Region (ICMR), Dibrugarh in ice box and stored at -20^{0}C till further analysis.

DNA extraction: DNA was extracted from whole blood samples containing EDTA as an anticoagulant by the standard phenol-chloroform method[7]. DNA purity and quantity were assessed by absorbance values in spectrophotometer and checked by 0.5 per cent agarose gel electrophoresis.

Determination of eNOS 7 polymorphism: To genotype the G894T polymorphism in the exon 7, polymerase chain reaction (PCR) amplification of 206 bp fragment encompassing the variant was performed, followed by restriction fragment length polymorphism (RFLP)[8] using the restriction endonuclease *Mbo1* (Promega, USA). Polymerase chain reaction consisted of 0.8 µl of each primer (Sigma, USA), 2.0 µl of 1X PCR buffer (Bangalore genei, Bangalore), 0.4 µl of dNTPs

(Bangalore genei), 0.18 µl of Taq. DNA polymerase (Bangalore genei) and 50-100 ng of genomic DNA. The PCR primers (Sigma) were 5'CAT GAG GCT CAG CCC CAG AAC 3' (sense) and 5' AGT CAA TCC CTT TGG TGC TCA C 3' (antisense). The PCR amplification conditions consisted of 94^{0}C for 5 min, followed by 40 cycles of 94^{0}C for 30 sec, 66^{0}C for 30 sec, 72^{0}C for 30 sec and then 72^{0}C for 8 min. The PCR amplicons were then incubated at 37^{0}C for 3 h. The 206 bp amplicon containing a thymine at nucleotide position 894 (corresponding to an aspartic acid at amino acid position 298) was cleaved into two fragments of 119 bp and 87 bp in length by *Mbo1* digestion but not for a guanine in this position. The restriction digest products were analyzed by electrophoresis on a 2.5 per cent agarose gel.

Statistical analysis: Statistical analysis was done using Statistical package for social sciences for window (SPSS) version 13.0. Specific genotype frequencies of the population were compared by chi- square test. Allele frequencies were deduced from genotype frequencies. The Hardy-Weinberg Equilibrium was examined in Epi-info 2002. $P < 0.05$ was considered to be statistically significant.

Results

A total of 409 subjects of both the sexes (male = 45.5%), aged between 18-87 yr were randomly selected. The mean age of our study subjects was 39.8 ± 13.16 yr and had low BMI (18.85 ± 3.16 kg/m^2). Consumption of alcohol and smoking were predominantly observed in the participants. The mean systolic and diastolic blood pressures were 135.09 ± 23.35 and 83.63 ± 11.67 mm Hg respectively (Table I).

The Glu 298Asp polymorphism located in exon 7 of eNOS gene was detected in the two different ethnic groups. The 206 bp amplicon containing a thymine at nucleotide position 894 (corresponding to an aspartic acid at amino acid position 298) was cleaved into two fragments of 119 bp and 87 bp in length by *Mbo1* digestion but not for a guanine in this position. Thus, eNOS 7 Glu 298Asp polymorphism resulted in three different genotypes *viz*., GG (Glu/Glu homozygous; 206 bp), GT (Glu/Asp heterozygous; 206 bp, 119 bp and 87 bp) and TT (Asp/Asp homozygous; 119 bp and 87 bp).

The frequencies for the three genotypes in our study subjects (n=409) were homozygous wild-type G/G - 310 of 409 (75.79%), heterozygous genotype G/T - 88 of 409 (21.51%) and homozygous mutant T/T - 11 of 409 (2.69%) (Table II). The observed genotype and allele frequencies were in agreement with the frequencies predicted by the Hardy-Weinberg equilibrium (χ^2= 1.172, df =2, P>0.05). In the study population, the observed frequencies of the GG, GT and TT genotypes were 0.75, 0.22 and 0.03 and the allelic frequencies of G-allele and T-allele was 0.87 and 0.13. Gender wise distribution of genotypes in the study participants did not reveal any significant difference (Fig.).

In the study population, the frequencies of the GG, GT and TT genotypes among the Tea garden community were 77.0, 21.0 and 2.0 per cent and those among the Indigenous Assamese community were 65.0, 32.0 and 3.0 per cent respectively (Table II). The observed genotype frequency among the tea garden community and the indigenous Assamese community were in agreement with the frequencies predicted by

Table I. Demographic profile and clinical characteristics of the study subjects

Parameter	All subjects (N=409)	Tea garden community (n=378)	Indigenous Assamese community (n=31)
Age, yr (Mean ± SD)	39.83 ± 13.16	40.0 ± 13.16	37.68 ± 13.43
Gender, Male (%)	186 (45.5)	169 (44.7)	17 (54.8)
Alcohol consumption Yes (%)	211 (51.6)	198 (52.4)	13 (41.9)
Smoking habit Yes (%)	133 (32.5)	112 (29.6)	10 (32.3)
Body mass index (kg/m^2)	18.85 ± 3.16	18.67 ± 3.19	21.02 ± 1.45
Blood glucose (mg/dl)	93.40 ± 22.86	91.86 ± 20.11	112.06 ± 40.4
Blood urea (mg/dl)	28.74 ± 10.34	29.13 ± 10.54	23.97 ± 5.52
Serum creatinine (mg/dl)	0.96 ± 0.25	0.96 ± 0.26	0.98 ± 0.07
Systolic blood pressure (mmHg)	135.09 ± 23.35	135.35 ± 23.61	131.93 ± 20.0
Diastolic blood pressure (mmHg)	83.63 ± 11.67	83.74 ± 11.82	82.39 ± 9.7

Table II. *eNOS 7* genotype distribution among two different population groups of North Eastern India

Community	*eNOS 7* genotype			*P* value	eNOS 7 alleles	
	GG No. (%)	GT No. (%)	TT No. (%)		G	T
Tea garden community (n=378)	290 (77.0) (95% CI= 72.26-80.77)	78 (21.0) (95% CI=16.78-24.94)	10 (2.0) (95% CI= 1.35-4.66)	<0.05	0.87	0.13
Indigenous Assamese community (n=31)	20 (65.0) (95% CI=46.7-79.72)	10 (32.0) (95% CI=17.69-50.01)	1 (3.0) (95% CI=0.16-14.90)	<0.05	0.81	0.19

Numbers in bracket in brackets denote genotype frequency

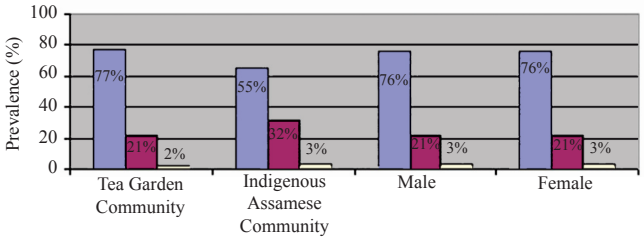

Fig. Distribution of eNOS 7 Glu298A sp polymorphism according to the communities and gender.

the Hardy-Weinberg equilibrium (χ^2= 0.832, df =2, *P*>0.05 and χ^2= 0.16, df =2, *P*>0.05 respectively). The allelic frequencies of G- and T-allele among the Tea garden community were 0.87 and 0.13 and that among the indigenous Assamese community were 0.81 and 0.19 respectively.

Discussion

There has been an increase in the prevalence of hypertension and coronary heart diseases throughout the world. Since its identification, several studies have shown the association between *eNOS* gene Glu298Asp variant and several cardiovascular diseases[8-10]. However, many other studies have failed to detect any such association. Genetic and environmental heterogeneity among different ethnic groups may account for this inconsistent result. Therefore, in order to develop public health policies and programmes it is necessary to know the prevalence and distribution of the candidate genes in the population.

The *eNOS* 7 homozygous GG wild genotype (75.8%) was predominant in our study population followed by *eNOS* 7 heterozygous GT genotype (21.5%) and *eNOS7* homozygous TT genotype (2.7%). Community-wise distribution of the *eNOS* gene Glu298Asp variant revealed that among the two communities, *i.e.*, the tea garden community and the indigenous Assamese population, the *eNOS 7* homozygous GG genotype was predominant. The higher frequency of the homozygous GG genotype in the present study is comparable to that of the north Indian[11] and south Indian population[12] with frequency of 71.22 and 74.3 per cent respectively. However, the prevalence of GG genotype was found to be low in our study population when compared with other Asian population[13].

The prevalence of heterozygous GT genotype was found to be 21.5 per cent, comparable to that of the north Indian[11] and south Indian population[12] that showed 28.0 and 25.7 per cent respectively. The prevalence of GT genotype for *eNOS* gene Glu298Asp variant is highest among the Italian population (51.8%)[8] and the lowest among the Chinese population (8.3%)[14].

A low (3%) frequency of the homozygous mutant TT genotype was observed among the two ethnic groups (2.6% in tea garden communities and 3.2% in Assamese communities). The study revealed a 'T' allele frequency of 0.13 and 0.19 among the tea garden community and the indigenous Assamese population respectively which is comparable to that observed among south Indian (0.13)[12] and north Indian population (0.147)[11]. The 'T' allele frequency is found to be relatively higher (0.4) among the Caucasians[15] and lower (0.1) among Asian populations[9,14,16].

The ethnic background and *eNOS* gene exon 7 Glu298Asp variant have been extensively studied across the globe. The analysis of distribution of *eNOS* Glu298Asp variant among different population will be useful in recognizing the mechanisms contributing to the development of common diseases like hypertension, myocardial infarction and coronary artery disease. Therefore, case control studies with large sample size and molecular based studies are necessary to understand the role of the *eNOS* gene exon 7 Glu298Asp variant in the development of cardiovascular diseases.

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Reprint requests: Dr Jagadish Mahanta, Director, Regional Medical Research Centre (ICMR), NE Region, Dibrugarh 786 001, Assam, India
e-mail: icmrrcdi@hub.nic.in