Endothelial nitric oxide synthase (eNOS) Glu\textsuperscript{298}→Asp polymorphism (G894T) among south Indians

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Background & objectives: Nitric oxide (NO) synthesized by endothelial cells is known to be a potent vasodilator. It has been suggested that the single nucleotide polymorphism (SNP) in endothelial nitric oxide synthase (eNOS) (G894T) may affect the response of vascular endothelium to increased oxidative stress. The Glu298Asp (GT) polymorphism located in exon 7, may be associated with several diseases. The objective of the present study was to determine the presence of genotype frequencies of Glu298→Asp (G894T) single nucleotide polymorphism in the eNOS gene among south Indian male Tamil speaking population.

Methods: Polymerase chain reaction and restriction fragment analysis was done to detect the presence of Glu298→Asp (G894T) variant of the eNOS gene in 105 healthy male volunteers.

Results: The frequency of the eNOS GG, GT, and TT genotypes was found to be 74.3, 25.7, and 0 per cent respectively. The ‘T’ allele frequency among this population was found to be 0.13. Chi square analysis showed that the study population lies in accordance with Hardy-Weinberg equilibrium.

Interpretation & conclusion: The occurrence of the homozygous mutant (TT) was completely absent among the study population. Moreover, our result indicated that the presence of the homozygous mutant genotype for eNOS polymorphism was absent/less common among south Indian male Tamil population of Asian race.

Key words: Endothelium - genotype - Hardy-Weinberg equilibrium - nitric oxide - nucleotide polymorphism - single

Nitric oxide (NO) that is generated by endothelium, is a potent vasodilator and acts as an important key factor in the anti-atherosclerotic properties of the endothelium. It is a small molecule having a molecular weight of 30 Daltons, which is synthesized, via L-arginine oxidation, by a family of nitric oxide synthase (NOS) enzymes in which three isoforms have been identified: two constitutive, the neuronal NOS (nNOS, NOS-1) and endothelial (eNOS, NOS-3), and one inducible NOS (iNOS, NOS-2). Endothelial nitric oxide synthase (eNOS or NOS-3) is the main contributor of circulating NO. Apart from its role of vasodilation, nitric oxide has gained attention for its role as a neurotransmitter in the brain, and a cytotoxic agent that targets tumour cells. Reduction in the production of nitric oxide is the first step for the occurrence of many diseases.
showed that the south Indian population in general had higher prevalence of CVD when compared to north Indian population. “Ramachandran et al” reported about 4 per cent CHD prevalence in Chennai during 1994 while Mohan et al documented a higher prevalence (11%) during 1996-97.

It has been suggested that the SNP in eNOS (G894T) may affect the response of vascular endothelium to increased oxidative stress. The presence of eNOS polymorphism and its disease association have been reported in different populations including Japanese, Chinese, Koreans, Caucasians, Italians and north Indians. No studies have been done on the presence of the eNOS - G894T SNP among south Indian Tamil population. Hence an effort was made to explore the distribution of this SNP in eNOS among south Indian male Tamil speaking population. The main objective of the study was to determine the G894T SNP in the eNOS gene among south Indian male Tamil population.

Material & Methods

Study design: The Tamils represented in the present study are an ethnic group from the Indian subcontinent who belong to Dravidian origin. The Tamil identity is primarily linguistic. A total of 105 healthy male volunteers with no history of cardiovascular disease, diabetes, hypertension, renal failures, etc., who belonged to south Indian Tamil ethnicity were included for the study. The volunteers of Rajaji Government Hospital, Madurai were approached and the informed consent was obtained. The study protocol was approved by the local ethical committee. The males of age 30-45 (38 ± 6.4 yr) were selected and this study was conducted at PG & Research Department of Zoology and Biotechnology, Lady Doak College, Madurai, Tamil Nadu, India between January 2006 and May 2007. Blood was collected into EDTA coated tubes to assess the eNOS SNP.

DNA extraction: Genomic DNA was isolated from fresh blood by salting out procedure. For DNA extraction, 300 µl of the blood was used and the isolated DNA was stored at -20°C. The isolated DNA was confirmed by 0.7 per cent agarose gel electrophoresis and quantified by UV spectrophotometry (Hitachi, Japan).

PCR analysis of G894T SNP: PCR analysis was carried out using Eppendorf Master Cycler (Germany). Genomic DNA (~ 50 ng) was incubated in a total reaction volume of 50µl containing equal concentration of the forward primer 5’ TCC CTG AGG GCA TGA GGC T-3’ and reverse primers - 5’ TGA GGG TCA CAC AGG TTC CT-3’ (~70 picomoles) (GenScript Corp, USA), 200 µM deoxynucleotide triphosphate, 10X PCR buffer pH-8.3 containing MgCl, 15 mM and 1.5 units of Taq DNA polymerase (New England Biolabs, Beverly, MA).

DNA was initially denatured at 95°C for 5 min prior to amplification. PCR amplification was accomplished using 30 cycles consisting of 2 min denaturation at 95°C, 45 sec annealing at 62°C, and 1 min extension at 72°C. The final extension included a 1 min extension at 72°C.

Restriction analysis of G894T SNP: Restriction digestion was performed in a total volume of 10 µl consisting of 5 µl amplicon, 1 µl NE buffer (50 mM Potassium acetate, 20 mM Tris-acetate, 10 mM. Magnesium acetate, 1 mM Dithio thaeitol pH-7.9 at 25°C), and 8 units of BanII enzyme (New England Biolabs, Beverly, MA). Samples were then incubated for 5 h at 37°C and the digested PCR products were separated by 10 per cent polyacrylamide gel electrophoresis stained with ethidium bromide (Fig.) and documented using gel documentation system (Spectroline UV illuminator).

Sequencing of the eNOS gene: The PCR product obtained was subjected to DNA sequencing which was carried out by Sanger’s sequencing method in automated ABI 3100 Genetic Analyser (Bangalore GeNei sequencing services, India). The DNA sequencing was done to check and confirm whether the amplified product was eNOS gene sequence. The obtained sequence was then subjected to the BLAST N analysis to study the homology sequence of the amplified product.

Statistical analysis: Pearson chi-square (χ²) test was performed to find the statistical significance between the genotypes, and the gene frequency was calculated by allele counting.

Results & Discussion

When BLAST N analysis was performed with the DNA sequence of PCR product (457 bp), 98 per cent homology was found between the eNOS gene (accession number: NM 000603.3) and the submitted DNA sequence. The T and G allele frequency was found to be 0.13 and 0.87 respectively among Tamil male population. There were no individuals homozygous for
the mutation (T/T genotype) in the study population. The frequencies for the three genotypes in Tamil male population were as follows: homozygous wild-type G/G - 78 of 105 (74.3%), heterozygous genotype G/T – 27 of 105 (25.7%) and homozygous mutant T/T - none (0%). ($\chi^2$ test = 2.26, Degree of freedom=1, $P$>0.05 N.S). (Fig.). Hardy-Weinberg equilibrium analysis showed that the genotype distribution for the eNOS gene (G894T) in Tamil male population was in accordance with Hardy-Weinberg equilibrium.

Studies in Korean and Japanese populations reported the absence of homozygous mutant TT genotype. Similar results were obtained in our study where there was complete absence of mutant TT-(Asp/Asp) genotype among south Indian male Tamil population as reported in other Asian populations.

The 'T' allele frequency was found to be relatively higher (0.4) among Caucasians, and lower (0.1) among Asian populations: 0.04, 0.045 and 0.1 among Chinese, Koreans and Japanese, respectively. The 'T' allele variant was more common in Caucasians (34.5%) than in African-Americans (15.5%) or Asians (8.6%). The 'T' allele frequency of south Indian male Tamil population (0.13) observed in the present study was comparable to north Indian population (0.147) reported by Srivastava et al. The homozygous GG wild genotype was more (90.74%) among Chinese population and it was found to be less (37.6%) among Caucasians. Our study showed a frequency of 74.3 per cent, which was comparable to the frequency of north Indian population that showed 71.22 per cent. However, the prevalence of GG genotype was found to be decreased among South Indian male Tamil population when compared with other Asian populations.

The prevalence of heterozygous (GT) genotype for eNOS - G894T SNP was found to be highest among Italian population (51.8%) and the lowest heterozygous (GT) genotype was observed among Chinese population (8.3%). About 28.02 per cent of north Indian population had heterozygous GT genotype, which was comparable to that of the south Indian male Tamil population (25.7%) observed in the present study. The occurrence of homozygous mutant TT frequency was found to be very low (0.1%) among the Japanese population and a relatively higher frequency (15.3%) of homozygous TT genotype was observed among Caucasians. Homozygosity for the Asp298 or 'T' allele of the eNOS gene was rare in Asian population.

The genotype distribution for the eNOS gene (G894T) in Tamil population was in accordance with Hardy-Weinberg equilibrium.

It has been reported that Asian Indians are at higher risk for developing coronary artery diseases. Several studies have revealed the association of eNOS SNP and occurrence of T-allele with cardiovascular diseases and some reports suggest that eNOS is a candidate gene for several vascular diseases. Serrano et al studied the suggestive role of gene-disease association between the eNOS gene and systemic lupus erythematosus among Northwestern Colombian women where nitric oxide was found to be elevated.

The frequency of the eNOS gene SNPs varies among ethnic population where the genetic variations are found to be a common phenomenon that does not lend itself to clear boundaries among population groups. A maldistribution of eNOS among ethnic groups explains the inter-ethnic differences in nitric oxide mediated vasodilation. The occurrence of different genotypes in different populations may depend on race and ethnic background of the population. Apart from these two, environmental factors might also play an important role in disease...
pathogenesis. Gene–gene interactions are also likely to contribute the variation in different population and among people in same ethnic groups.

In conclusion, the findings of this study revealed that eNOS G894T mutation was less prevalent among male Tamil population. The TT genotype was completely absent among south Indian male Tamil population. However, the association between the eNOS gene and vascular diseases can be well understood by performing case-control studies.

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