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ENDOTHELIAL NITRIC OXIDE SYNTHASE GENE POLYMORPHISM (G894T) IN ESSENTIAL HYPERTENSION

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ABSTRACT

Background: Nitric oxide (NO) produced by endothelial nitric oxide synthase (eNOS) plays an important role in maintaining blood pressure homeostasis and vascular integrity. The single nucleotide polymorphism (G894T) in exon 7 of human *eNOS* gene has been found to be associated with the development of essential hypertension in different ethnic populations. **Aim and Objectives:** (a) The aim is to assess the G894T polymorphism of *eNOS* gene in patients with essential hypertension in a rural population of Salem, Tamil Nadu. (b) Also to determine the genotype and mutant allele ('T') frequency in them. **Materials and Methods:** Polymerase chain reaction and restriction fragment analysis was done to detect the presence of (G894T) variant of the *eNOS* gene in 41 hypertensive patients. **Results:** The frequency of the eNOS GG, GT, and TT genotypes was found to be 88%, 12%, and 0% respectively. The frequency of mutant (T) allele was found to be only 6.1%. **Conclusion:** The occurrence of homozygous wild G/G genotype is predominant and the homozygous mutant T/T genotype was not seen in the representative hypertensive subjects of our population in Salem. The presence of mutant (T) allele among the studied population was less common.

Key words: Essential hypertension, Nitric oxide, eNos gene polymorphism

INTRODUCTION

Essential or primary hypertension (HTN) is a major public health problem characterised by sustained elevation of blood pressure without any identifiable cause. Essential hypertension is one of the most prevalent chronic diseases in India and it is universally accepted that systemic hypertension is a distinct risk factor for various cardiovascular emergencies, particularly left ventricular failure, myocardial infarction, and stroke.[1] Both environmental and genetic factors may predispose individuals to essential hypertension. Since the underlying genetic pathways remain elusive, currently most studies focus on the genes coding for proteins that regulate blood pressure.

Clinical and experimental studies suggest that an alteration in nitric oxide (NO) metabolism may be a contributing factor in the pathogenesis of hypertension. NO is a potent vasodilator produced from L-arginine by endothelial nitric oxide synthase (eNOS) in vascular endothelium.[2] The impairment of NO generation causes elevation in blood pressure, and it is brought about mainly by *eNOS* gene polymorphism.[3] A gene and its selected polymorphism preferably should have the following features to make them a candidate target in the development of essential hypertension.[4]

- The gene product must be functionally relevant to hypertension
- Polymorphism within the gene must alter its function
- Hypertension needs to link to the chromosomal region harbouring the candidate gene.

Available studies demonstrate that the G894T polymorphism in *eNOS* gene fulfills the above mentioned criteria in the context of hypertension. At least three distinct NOS isoforms exist in mammalian cells: Neuronal (nNOS, Type I), inducible (iNOS, Type II) and endothelial (eNOS Type III). The gene encoding for eNOS is located on chromosome 7 (7q35-q36) and contains 26 exons with an entire length of 21 kb. The single nucleotide gene polymorphism i.e. the replacement of guanine (G) by thymine (T) at 894th position within the exon 7 of *eNOS-7* gene, results in a replacement of glutamic acid by aspartic acid in eNOS.[5] Thus the defective *eNOS* enzyme caused by G894T polymorphism in *eNOS* gene effects in elevated blood pressure due to impaired production of NO.

India, being a culturally and socially diverse nation, the genetic differences would be noted in the region-wise prevalence of hypertension. The genetic research regarding the nature of genetic contribution in accelerating the hypertension is inadequate in Salem district of Tamil Nadu. Therefore, the present study was planned to determine the frequency of mutant variant (T allele) of *eNOS* gene in our study population.

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MATERIALS AND METHODS

Study subjects

It is a cross-sectional study consisting of 41 hypertensive patients in different age groups (26-92 years) from both sexes [Table 1]. Study subjects were selected from patients attending Department of Medicine, Vinayaka Mission Kirupananda Variyar Medical College Hospital, Salem, Tamil Nadu. Institutional Ethical Committee clearance, and consent from the patients were obtained. With the use of a standardized questionnaire, a detailed medical history of patients was carefully recorded.

Inclusion criteria

Hypertensive patients with family history of hypertension were recruited for this study. All the patients recruited were undergoing treatment with one or more anti-hypertensive agents.

Exclusion criteria

Participants with heart disease, renal failure were excluded and the obese persons (body mass index >30), smokers and alcoholics also excluded.

Collection of blood samples

Approximately 2 ml of venous blood samples were collected in a screw cap ethylenediaminetetraacetic acid tube and the whole blood specimens were stored at −20°C (deep freezer) till further analysis.

Deoxyribonucleic acid extraction

Deoxyribonucleic acid (DNA) was extracted from whole blood samples using spin-column chromatography method, according to the protocol given by the manufacturer (Miniprep Kit, Helini Biomolecules, Chennai, Tamil Nadu, India). A silica based membrane technology used in the form of a convenient spin column by which the cellular components of the blood were lysed and the cellular DNA that bind to silica membrane are recovered after a series of "wash and spin" steps.

Table 1: Anthropometric parameters of the hypertensive patients

Hypertensive patients (*n*=41)	
Age	59.8±12.42
Sex: M/F	19/22
BMI cut-off value (<29.9)	24.9±2.49
Systolic blood pressure (mm/Hg)	134.5±13.44
Diastolic blood pressure (mm/Hg)	84.73±6.46

BMI: Body mass index

Gene amplification by polymerase chain reaction

The target DNA sequence of *eNOS* gene from the DNA samples was amplified by polymerase chain reaction (PCR) technique. The DNA was amplified in a final reaction volume of 15 µl containing 5 µl of master mix (GoTaq Green Master Mix, Promega, USA), 0.3 µl each of the primers (Eurofins Genomics Bangalore), 8.4 µl of nuclease free water and 1 µl of genomic DNA, and it was subjected to the suitable PCR conditions. The PCR primers used for the amplification of target DNA sequence were 5'CAT GAG GCT CAG CCC CAG AAC 3' (sense) and 5' AGT CAA TCC CTT TGG TGC TCA C 3' (anti-sense). PCR conditions were arrived after standardization. The PCR conditions consisted of 94°C for 10 min (initial denaturation), followed by 37 cycles with the conditions of 94°C for 45 s (denaturation), 56°C for 1 min (annealing), 72°C for 1 min 30 s (extension). The final extension was allowed for 10 min at 72°C and the PCR amplicons were stored at 4°C.

Restriction fragment length polymorphism analysis

To genotype the G894T polymorphism, restriction fragment length polymorphism analysis was done by agarose gel electrophoresis (AGE) after digesting the PCR amplicons using the restriction endonuclease *Ban II*. Restriction digestion was performed in a total volume of 10 µl consisting of 5 µl amplicon, 1 µl NE buffer and 8 units (4 µl) of *Ban II* enzyme (New England Bio Labs, New Delhi, India). Samples were then incubated at 37°C for 6 h and the digested products were subjected to AGE (2% gel stained with ethidium bromide). The DNA bands were visualized and documented using gel documentation system (Spectroline UV illuminator).

The allele containing a guanine nucleotide (the wild type) at position 894 of *eNOS* gene was cleaved into two fragments by *Ban II* digestion and is visualized as two separate bands with 125 bp and 82 bp in length. The thymine (the mutant type) containing amplicons (alleles) are not digested and it is visualized as a single band of intact 207 bp in length.

Statistical analysis

Allele and genotype frequencies were deduced using the gene-counting method and the Hardy–Weinberg (H-W) equilibrium checked by χ^2 test. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The present study investigated genotype distribution and allelic frequency of *eNOS* gene (G894T)

polymorphism in patients with essential hypertension in a rural population of Salem district, Tamil Nadu. The *eNOS* genotype distribution and the allele frequencies of study subjects are presented in Tables 2 and 3 respectively. On visualization, the agarose gel electrophoretic pattern of each DNA sample revealed one of the following genotypic patterns:

- Homozygous wild G/G genotype: In this genotype, both the two alleles with 'G' at 894th position are cleaved into two fragments (each 125 bp and 82 bp). Hence a total of four fragments were detected as two bands.
- Heterozygous G/T genotype: A total of three bands observed in this genotype. Of the two alleles, one of the allele which containing G formed two bands and another allele which contains 'T' formed a single band as it was not digested.
- Homozygous mutant T/T genotype (forming single band) was not detected in our studied subjects.

The genotype frequency of the homozygous wild G/G genotype and heterozygous G/T genotype among the studied hypertensive subjects was found to be 88%, (*n* = 36) and 12% (*n* = 5) respectively. The homozygous abnormal T/T (mutant type) genotype was not observed in our study population (*n* = 0). The respective frequencies of G and T allele among the studied hypertensive subjects were 93.9% and 6.1%.

In the studied hypertensive subjects, the homozygous GG wild type was predominant (88%), and the frequency of T allele (mutant type) was only 6.1%. Chi-square analysis did not show any significant difference between the frequencies of observed genotype and expected genotype frequency (H-W frequency) in our population [Table 3; *P* = 0.67]. This shows our study group lies in accordance with H-W equilibrium.[6] The genotype frequencies and allele frequencies observed in our study are consistent with the previous studies conducted in the north Indian[1,7] and south Indian population.[8] Available studies demonstrated that, the 'T' allele variant was more common in Caucasians (34.5%) than in African-Americans (15.5%) or Asians (8.6%).[9]

Table 2: Genotype frequencies and the gender distribution in hypertensive patients

Genotypes	Number of patients			Genotype frequency (%)
	Male	Female	*n*	
G/G (homozygous wild type)	17	19	36	88
G/T (heterozygous)	2	3	5	12
T/T (homozygous mutant type)	0	0	0	0

Table 3: Genotype frequencies and the allele distribution in hypertensive patients

Genotypes	G/G	G/T	T/T	
Observed	36	5	0	Chi-square (χ^2) = 0.17
Expected H-W Freq.	36.15 (88.18%)	4.7 (11.45%)	0.15 (0.37%)	P-Value = 0.68
Allele Frequencies.	G = 77 (93.9%)		T = 5 (6.1%)	

H-W: Hardy-Weinberg frequency

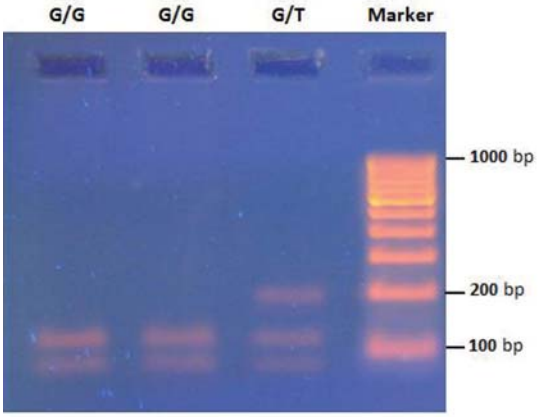
The occurrence of different genotypes in different populations may depend on race and ethnic background of the population.[10] This variance explains the inter-ethnic differences in NO mediated vasodilation.[9] NO plays a pivotal role in the preservation of the endothelium homeostasis, regulation of vasomotor tone and control of blood pressure. Many of the study reports demonstrate that whole-body NO production in patients with essential hypertension is diminished under basal conditions, as established by measurement of urinary and plasma nitrate.[11] The replacement of glutamic acid by aspartic acid at codon 298 caused by G894T missense variant, alters the structure of eNOS enzyme from helix to tight turn which results in the abnormalities in the activity of the eNOS and affects NO production.[12,13] In addition, there are multiple susceptibility genes with powerful environmental or gene-gene interactions influencing the development of hypertension.[10]

Since the contribution of genetic basis in essential hypertension varies with different race and ethnic background of the population, the results of most association studies regarding G894T polymorphism were inconsistent among different ethnicities and even among different populations from the same ethnicity. To our knowledge, this is the first study that has been designed to investigate the influence of G894T polymorphisms in hypertensive subjects of Salem population and we have not found any significant frequency of suspected mutant allele (T) in our study subjects. However, it is assumed that there could be the influence of other genetic factors in the development of essential hypertension in our target population. So the causative gene may be determined by knowing the genetic information about

the other clinically relevant polymorphisms in the *eNOS* gene such as T786C[14] and intron 4 (4b/a).[15] The other gene polymorphisms that have considerable clinical importance and influence the advent of this disease are ACE I/D,[4] AGT M268T,[16] and CYP11B2 C-344T.[17] Hence this attempt would help in identifying individuals at an increased risk of developing this disease and to initiate appropriate actions in them to avoid development or delay the onset of essential hypertension.

CONCLUSION

The present study data indicates the wild G/G genotype is predominant and the homozygous mutant T/T genotype is not seen in the hypertensive subjects of our population in Salem. The study revealed that G894T polymorphism of eNOS was less commonly found among the study subjects. Our findings may provide a data about genetic background of our population regarding G894T polymorphism, however a case-control study with larger sample size is needed to establish or refute the role of this polymorphism in the development of essential hypertension.



(**bp** - Base pairs)

The figure depicts a representative agarose gel electrophoretic pattern of PCR products of studied samples; showing the homozygous G/G genotype with two bands and heterozygous G/T genotype with three bands.

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