Angiotensin Converting Enzyme Gene Insertion/Deletion and Endothelial Nitric Oxide Synthase Single Nucleotide Gene Polymorphisms among the patients of Essential Hypertension

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Abstract

Angiotensin converting Enzyme (ACE) and Nitric oxide (NO) produced by endothelial nitric oxide synthase (eNOS) plays an important role in maintaining blood pressure homeostasis and vascular integrity. The Insertion/Deletion polymorphism (I/D) in exon 7 of Angiotensin converting Enzyme (ACE) gene and the single nucleotide polymorphism (G894T) in exon 7 of human eNOS gene have been found to be associated with development of essential hypertension in different ethnic populations. The aim was to study the insertion/ deletion (I/D) polymorphism of ACE gene in patients with essential hypertension and the genotype distribution and to determine allelic frequency of ACE gene and also to assess the G894T polymorphism of eNOS gene in patients with essential hypertension and to determine the genotype and mutant allele (“T”) frequency in them. The target DNA sequence of ACE gene was amplified by Polymerase Chain Reaction (PCR) technique to detect the presence of ACE gene Insertion / Deletion (I/D) polymorphism in 41 patients with essential hypertension. Polymerase chain reaction and restriction fragment analysis was done to detect the presence of (G894T) variant of the eNOS gene in 41 hypertensive patients. The study results showed that the frequency of ACE I/I, D/D I/D genotypes and eNOS GG, GT, TT genotypes were found to be 44%, 27%, 29% and 88%, 12%, 0% respectively. The frequency of insertion (I) and deletion (D) alleles was found to be 58.54% and 41.46%. The study data indicates, the wild I/I genotype and homozygous wild G/G genotype are predominant and the homozygous mutant T/T genotype was not seen in the representative hypertensive subjects. The presence of mutant (T) allele among the studied population was less common only 6.1% and frequency of I allele is increasingly distributed in the studied hypertensive subjects of our study population.

Keywords: Essential hypertension, Nitric oxide, eNos gene polymorphism Angiotensin Converting Enzyme, ACE Gene Polymorphism.

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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 and an Insertion/Deletion polymorphism in the gene responsible for Angiotensin-I converting enzyme (ACE) may be a contributing factor in the pathogenesis of essential hypertension.

NO is a potent vasodilator produced from L- arginine by endothelial nitric oxide synthase (eNOS) in vascular endothelium [2]. The impairment of NO
The ACE-1 gene, encoding angiotensin converting enzyme (ACE-I) spans 21 kilobases, located on the long arm of chromosome 17 (17q23), and consists of 26 exons and 25 introns [3]. It contains a number of polymorphic variants that can be of potential use in genetic analysis of populations. The accumulated evidence points to the existence and the role of insertion/deletion polymorphism of ACE gene and its contribution in the development of essential hypertension. It has been shown that the ACE gene (ACE) contains a polymorphism based on the presence (insertion) or absence (deletion) of a 287 bp Alu repeat sequence (a nonsense DNA fragment) near the 3’ end of intron 16[4]. This insertion/deletion polymorphism in the ACE gene produces three genotypes in population: Deletion homozygotes (D/D); Insertion homozygotes (I/I); and Heterozygotes D/I. Since its identification, several studies have shown that the D/D (del/del) genotype in the ACE gene is associated with hypertension.

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 south Indian population.

Therefore, the present study was planned to determine the frequency of mutant variant (T allele) of eNOS gene and D allele of ACE gene in our study population.

**Aim & Objectives**

- The aim was to study the insertion/deletion (I/D) polymorphism of ACE gene and G894T polymorphism of eNOS gene in patients with essential hypertension.
- Also, to assess the genotype distribution and to determine allelic frequency of ACE gene and eNOS gene (G894T).

**Materials and Methods**

**Study subjects**

It is a cross-sectional study consisting of 41 hypertensive patients in different age groups (26-92 years) including both sexes [Table 1].

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 antihypertensive agents.

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

Collection of blood samples
About 2 ml of venous blood samples were collected in ethylene-diamine-tetra-acetic acid (EDTA) tube and the whole blood specimens were stored at -20°C (deep freezer) till further analysis.

Deoxyribonucleic acid (DNA) extraction
Genomic DNA was extracted from the whole blood samples using spin-column chromatography method, according to the protocol given by the manufacturer (MiniPrep Kit, Helini Biomolecules, Chennai, Tamil Nadu, and 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. The purity and quantity of DNA were assessed by absorbance values in UV spectrophotometer and checked by 0.5 % agarose gel electrophoresis. Then the DNA samples were at -20°C till further analysis.

ACE gene polymorphism
Amplification of target DNA sequence by PCR
The DNA samples were amplified by polymerase chain reaction (PCR) technique. The target DNA fragment was amplified using two oligonucleotide primers flanking the ACE gene: the forward primer (sense): 5′-CTGGAGAGCCACTCCCATCCTTTCT-3′ and the reverse primer (anti-sense): 5′-GACGTCGCCATCACATTCGTCAGAT-3′. Each of the DNA samples was amplified in 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 1 μl of extracted DNA. 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.

Analysis of PCR products by Agarose Gel Electrophoresis (AGE)
Agarose gel plate prepared using 2g of agarose in 30 mL of 1X TBE (Tris-Borate-EDTA) buffer and the amplified PCR products were mixed with loading buffer and loaded into the separate sample wells created on the gel matrix. Molecular weight DNA marker (DNA ladder) was also run along with the test samples. Electrophoresis was performed at 80 V for 60 minutes at room temperature. Then the gel was stained in the presence of 50 ml of 1X TBE buffer containing ethidium bromide (0.5 μg /ml) for 30 min. The DNA bands were visualized on ultraviolet light transilluminator and photographed. On visualization, each DNA sample revealed one of three genotypic patterns:
- Homozygous I/I genotype: Insertion (I) alleles were detected as a single band of 490 bp fragment.
- Homozygous D/D genotype: Deletion (D) alleles were identified as a single band of 190 bp fragment.
- Heterozygous I/D genotype: Both 490 and 190 bp fragments (I and D) were detected as two separate bands.

eNOS gene polymorphism
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′CATGAG 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. The figure depicts a representative agarose gel electrophoretic pattern of PCR products of studied sample.

ACE Gene Polymorphism

![ACE Gene Polymorphism](image)

**STATISTICAL ANALYSIS**

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Hypertensive patients (n=41)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>59.8 ± 12.42</td>
</tr>
<tr>
<td>Sex: Male / Female</td>
<td>22/19</td>
</tr>
<tr>
<td>BMI (Body Mass Index)</td>
<td>24.9 ± 2.49</td>
</tr>
<tr>
<td>Cut-off value (≤ 29.9)</td>
<td>134.5 ± 13.44</td>
</tr>
<tr>
<td>Systolic Blood Pressure (mm/Hg)</td>
<td>84.73 ± 6.46</td>
</tr>
</tbody>
</table>

**RESULTS AND DISCUSSION**

The present study investigated genotype distribution and allelic frequency of \( eNOS \) gene.
(G894T) and ACE gene I/D polymorphism in patients with essential hypertension in a rural population of Salem district, Tamil Nadu. The eNOS genotype and ACE genotypes 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 \((H^2 - W)\) frequency) in our population \([Table 3; P = 0.67]\). This shows our study group lies in accordance with \(H^2 - W\) 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 \((8.6\%)\)\([9]\).

The occurrence of different genotypes in different populations may depend on race and ethnic background of the population \([10]\). This variance explains the interethnic differences in NO mediated vasodilation \([9]\). NO plays a pivotal role in the preservation of the endothelium homeostasis, regulation of vasmotor 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.

Among the three genotypes, the frequency of homozygous I/I genotype was higher and the least one in our population was homozygous D/D genotype. Heterozygous genotype showed an intermediate level of frequency. The genotype frequency of the homozygous I/I genotype, homozygous D/D genotype, and the heterozygous I/D genotype was found to be 44 %, \((n = 18)\), 27 % \((n=11)\), and 29 % \((n=12)\) percent respectively. The difference between the observed genotype and expected genotype frequencies in our population was found to be statistically significant \((p = 0.011)\). The respective frequencies of D and I allele among the study subjects were 41.46% and 58.54%. The frequency of I allele of ACE gene is found to be high when compared to D allele.

These observations are in line with many of the previous studies conducted in other parts of India including the studies conducted by Randhawa et al. and Pasha et al.\([5,6]\). These studies have reported that the homozygous I/I genotype is increasingly distributed...
than the D/D genotype and there was no significant difference noted between the genotype frequencies of hypertensives and control. In our study, the difference between the observed genotype and the expected genotype frequencies in the studied subjects was found to be statistically significant. This shows that the observed genotype frequency is not in accordance with Hardy-Weinberg equilibrium. Our results showed that, the frequency of I allele was higher than the D allele in the studied population. As in our study, many of the Indian studies showed an increased distribution of I allele in hypertensive population. A significant association of I allele with hypertension has been demonstrated in the study conducted by Srivastava et al. [7].

In ACE gene polymorphism, the plasma ACE level of D/D genotype is reported to be about double that of I/I genotype; and intermediate level in I/D type [8]. The molecular mechanism underlying this ACE insertion/deletion polymorphism is not clear. It is suggested that the interaction with regulatory elements may be altered due to polymorphism, thus causing increased production of ACE [9]. A significant association of high ACE producing D allele with hypertension has been demonstrated in the African-American, Chinese and Japanese populations [10-13]. A few of the Indian studies also have reported the increased distribution of D allele in hypertensive patients. Interestingly, studies conducted in many other countries were inconsistent with Indian studies including the present study [14, 15]. The inconsistent results in different populations may be due to varied ethnicity or the various other genetic and environmental factors implicated in the regulation of blood pressure. In addition to these factors, the result of present study may also be influenced by the study design and the composition of the sample population.

Since hypertension is a complex genetic disorder, it is important to know the genetic information about the other polymorphisms that interact and influence the advent of this disease. Of the various polymorphisms suggested, the AT1R A1166C, AGT M268T, CYP11B2 C-344T, eNOS G894T and eNOS 4a/b polymorphisms have attracted considerable clinical importance [16]. Hence this attempt would help in identifying individuals at an increased risk of developing this disease and to initiate appropriate actions in the m to avoid development or delay the onset of essential hypertension.

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

<table>
<thead>
<tr>
<th>ACE Gene Polymorphism</th>
<th>Genotypes</th>
<th>Genotype frequency (%)</th>
<th>Male</th>
<th>Female</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I/I</td>
<td></td>
<td>9</td>
<td>9</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>D/D</td>
<td></td>
<td>4</td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>I/D</td>
<td></td>
<td>6</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>19</td>
<td>22</td>
<td>41</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>eNOS Gene Polymorphism</th>
<th>Genotypes</th>
<th>Genotype frequency (%)</th>
<th>Male</th>
<th>Female</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G/G (homozygous wild type)</td>
<td></td>
<td>17</td>
<td>19</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>G/T (heterozygous)</td>
<td></td>
<td>2</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>T/T (homozygous mutant type)</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>19</td>
<td>22</td>
<td>41</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>ACE Gene Polymorphism</th>
<th>Genotypes</th>
<th>Chi square ($\chi^2$)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I/I</td>
<td>18</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>D/D</td>
<td>14.05 (34.27%)</td>
<td>19.9</td>
</tr>
<tr>
<td></td>
<td>I/D</td>
<td>7.05 (17.19%)</td>
<td>11</td>
</tr>
<tr>
<td>Allele frequency</td>
<td>I= 48 (58.54%)</td>
<td></td>
<td>D=34 (41.46%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>G/G</th>
<th>G/T</th>
<th>T/T</th>
<th>Chi square ($\chi^2$)=0.17</th>
<th>P value = 0.68</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed</td>
<td>36</td>
<td>5</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expected</td>
<td>36.15 (88.18%)</td>
<td>4.7</td>
<td>0.15 (11.45)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H.W Freq.</td>
<td>36.15</td>
<td>4.7</td>
<td>0.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allele frequency</td>
<td>I= 48 (58.54%)</td>
<td>D=34 (41.46%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

H.W: Hardy Weinberg Frequency
CONCLUSION

The present study data indicates, the wild I/I genotype is predominant and the frequency of I allele is increasingly distributed and the wild G/G genotype is predominant and the homozygous mutant T/T genotype is not seen in the studied hypertensive subjects of our population. 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 ACE I/D polymorphism and 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.

REFERENCES