

Original Research Article

Association of Intron4 VNTR Polymorphism with Essential Hypertension**Dr. G. Sasirekha¹, Dr. P. Bagavathiammal², Prof. Dr. R. Chitraa³, Dr. A. Leena Devi⁴, Dr. V. Anandhan⁵,
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Abstract: Endothelial nitric oxide synthase (eNOS) is the major enzyme responsible for nitric oxide production and its polymorphisms like exon7 (G to T) is linked to essential hypertension in many ethnic groups. The association of 27 bp variable number of tandem repeat (VNTR) polymorphism in intron 4 is more controversial and hence, it is proposed to explore its role in essential hypertension in our population. The aim of the study was to elucidate the allelic frequencies of eNOS intron4 VNTR in patients and controls, and to determine if there is any correlation between this polymorphism and essential hypertension. Genotype analysis was done for 150 essential hypertensives and 130 matched normotensive controls by Polymerase Chain Reaction. Homozygous 'aa' genotype was found to be more prevalent in hypertensives (4.6%), when compared to controls (3.1%). In contrast, both homozygous 'bb' and heterozygous 'ab' were more common in controls. But, the results were not statistically significant. eNOS intron4 VNTR polymorphism was not found to be an independent risk factor for essential hypertension, based on this study.**Keywords:** Essential hypertension, nitric oxide, endothelial nitric oxide synthase, variable number of tandem repeats (VNTR) polymorphism

INTRODUCTION

In India, hypertension is the predominant risk factor for coronary artery disease (CAD) in all ethnic groups. More than 90% of hypertensive individuals suffer from essential hypertension. It shows an earlier onset in men, than in women. In addition to conventional risk factors linked to essential hypertension such as age, obesity, smoking and stress, a strong genetic predisposition is also suggested [1]. It is estimated that, around 30% of variation in blood pressure is due to genetic factors [2]. Hypertension is more common in individuals who have one or two hypertensive parents and is closely correlated in monozygotic than di-zygotic twins [3]. Given the pivotal role of endothelial nitric oxide synthase (eNOS) in vascular homeostasis, the eNOS gene (NOS3) has emerged as a logical candidate gene in the investigation of hypertension genetics [4]. Variants of the NOS3 gene located in the 7q35-q36 region, with an entire length of 21kb have been investigated for association with hypertension and other cardiovascular disorders [5]. Of these, three polymorphisms have been widely examined for clinical relevance, based on their potential functional effects and their relatively high minor allele

frequency in various ethnic groups [6]. G894T substitution in exon7 resulting in a Glu to Asp substitution at codon 298, insertion-deletion in intron4 and T786C substitution in the promoter region. The association of these polymorphisms to hypertension has been controversial. The G to T polymorphism has been associated with hypertension [7] in many ethnic groups (Miyamoto et al., 1998), including the Indian population (Srivastava K, Narang R, Sreenivas V, Das S and Das N, 2008), and the 786 T variant has been associated with hypertension [8] and with coronary spasm [9]. But a 27 bp VNTR located in intron4 of eNOS gene, shows conflicting results.

In intron 4 of NOS3, there can be four 27 bp repeats (allele 'a') or five (allele 'b'). Presence of allele 'a', rather than the wild-type allele 'b', reduces its activity (Kato et al. 1999). Functional significance of this polymorphism was also identified in cases with endothelial dysfunction. Wang *et al.* [10] reported a significant association of this intron with CAD. Other studies on eNOS intron4 polymorphism showed positive association with renal disease [11] essential hypertension among Japanese [12] and stroke among

Chinese [13]. However, studies from Taiwan [14] did not reveal any association with premature CAD. The discrepancy in these studies on the association of *eNOS* intron 4b/a VNTR polymorphism with essential hypertension may be related to ethnic diversity. Hence, we investigated this polymorphism in the patients attending the hypertension clinic to find the extent of risk caused by this gene, in our ethnic group.

AIMS AND OBJECTIVES

In this study, the aim is to elucidate the association between the intron4a/b polymorphism in *NOS3* gene and essential hypertension. The specific objective is to determine the genotype frequencies of the *eNOS* intron4 VNTR polymorphism in the extracted DNA of the patients with essential hypertension and controls, and to determine if there is any correlation between these polymorphisms and essential hypertension.

MATERIALS AND METHODS

STUDY POPULATION

It is a case control study, single centered, prospective and conducted in a tertiary health center over a period of 10 months. 150 unrelated essential hypertensive patients who were on treatment for 5-10 years were selected as cases, which included 131 males and 19 females, of mean age 50.59 ± 10.52 years. Those with secondary hypertension, diabetes mellitus, renal failure, fever, acute infections, chronic inflammatory states, chronic smokers and those on drugs like oral contraceptive pills, steroids were excluded from study group. 130 apparently healthy normotensives from the out-patient department, during their visit for master health checkup were selected as controls. Confounding factors like age, sex, smoking, alcoholism were matched.

METHODS:

Standard anthropometric data (height, weight) and resting blood pressure were recorded in each subject, after a thirty minutes rest on a couch. Blood samples were collected after overnight fasting in disodium EDTA tube. The tube was centrifuged at 2000 rpm for 20 minutes to get buffy coat for DNA extraction.

eNOS intron4 polymorphism screening:

DNA extraction was done from buffy coat by modified high salt method [15]. Extracted DNA was identified following an electrophoretic run using 0.8% agarose with a constant voltage of 7V/cm along with a comparison of known molecular weight 1kb DNA ladder and stored at -20°C for future use. (Figure: 1). PCR analysis of *eNOS* intron4 gene was carried out in a total reaction volume of 25 μL , which includes 12.5 μL of PCR master mix, 0.9 μL forward primer – 5'-AGGCCCTATGGTAGAGCCTT-3', 0.9 μL of reverse primer–5'-TCTCTTAGTGCTGTGGTCAC-3', 2.0 μL of extracted DNA and 8.7 μL of distilled water.

DNA was initially denatured at 94°C for 5min prior to amplification. PCR amplification was carried out in a thermal cycler for 30 cycles, which includes 1 min denaturation at 94°C , 1min annealing at 54°C and 1min extension at 72°C . Final extension was done at 72°C for 10 min. Amplified products are of two different sizes - 393 bp size for 'a' allele and 420 bp size product for 'b' allele. They are identified by 3% agarose gel electrophoresis by comparison with a known 100bp DNA ladder. Thus, each DNA sample revealed one of three possible patterns after electrophoresis viz., a 393 bp band (aa genotype), a 420 bp band (bb genotype) or both 393 and 420 bp bands (ab genotype) (Figure 2).

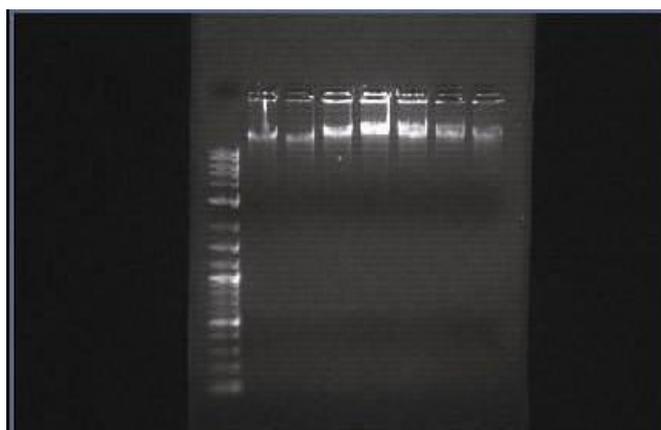


Fig-1: Extracted DNA (lane 2 to 8) was tested on 1% agarose gel using 1 kb ladder (lane 1). Ladder shows 1000, 800, 700, 600, 500, 400, 300, 200, 100bp fragments

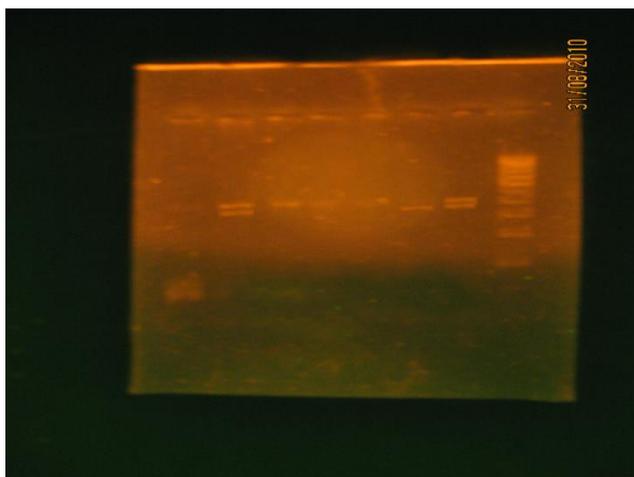


Fig-2: Electrophoretogram of intron4 eNOS gene product on 2% agarose gel, compared with 100bp DNA ladder. Each DNA sample revealed one of the three possible patterns viz., a 393 bp band (aa genotype), a 420 bp band (bb genotype) or both 393 and 420 bp bands (ab genotype).

Statistical Analysis

Allele frequencies were calculated by allele counting. Age, sex, smoking, alcoholism, BMI, plasma glucose were compared between control subjects and patients by students ‘t’ test and chi-square test (χ^2). Genotype frequency distribution between cases and controls were compared with a Pearson chi-square (χ^2) test for 2*2 contingency table. Logistic regression analysis was performed to evaluate the interaction between human eNOS intron4 gene and other variables in relation to essential hypertension. Independent variables included in this analysis were age, sex, smoking, alcoholism and plasma glucose. Relation between the number of essential hypertension and the gene was assessed by Spearman’s Rank Correlation analysis.

RESULTS

Table 1a and 1b shows age, sex, BMI and other conventional risk factors distribution in patients and control subjects with an insignificant p value. It shows cases and controls were perfectly matched regarding all

confounding factors. Tables 2 & 3 shows genotype distribution and allelic frequency of human eNOS intron 4 gene in patients with essential hypertension and controls. The allele frequencies were a/a=11, a/b=62, b/b=207. This was found to be in Hardy Weinberg equilibrium $\chi^2=7.61$ P=0.11 Homozygous ‘aa’ genotype and heterozygous ‘ab’ were found to be more prevalent in the hypertensives (4.6% and 25.2% respectively) when compared to controls (3.1% and 18.3%). In contrast, both homozygous ‘bb’ was more common in controls (78.6%) when compared with the hypertensives (70.2%). But these differences were not statistically significant

Pie diagrams 1 and 2 show the pattern of distribution of various genotype (aa, ab, bb) of intron 4 eNOS gene in cases and controls respectively. It shows bb genotype is more prevalent in our population. Bar diagrams 1 & 2 compares the genotype distribution and allele frequencies of human eNOS intron4 gene in patients with essential hypertension and controls.

Table-1a: Characteristics of Patients with Essential Hypertension and of control subjects.

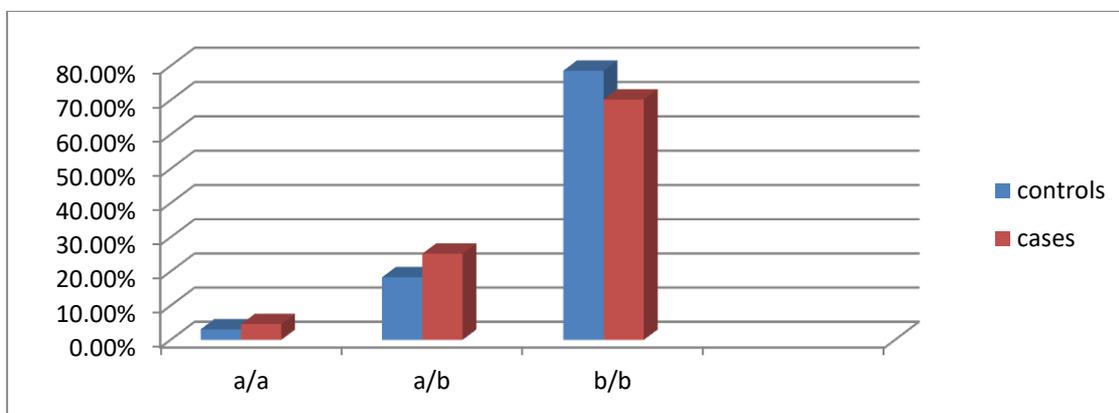
	Group				Student independent t-test
	Hypertensives		Control		
	Mean	SD	Mean	SD	
Age	50.59	10.52	51.83	11.60	t=0.94 P=0.36(NS)
Wt	66.23	9.78	64.42	10.39	t=1.51 P=0.13(NS)
Ht	163.78	6.88	162.15	7.75	t=1.86 P=0.06(NS)
BMI	25.13	3.90	24.47	3.41	t=1.47 P=0.14(NS)

Table-1b: Characteristics of Patients with Essential Hypertension and of control subjects.

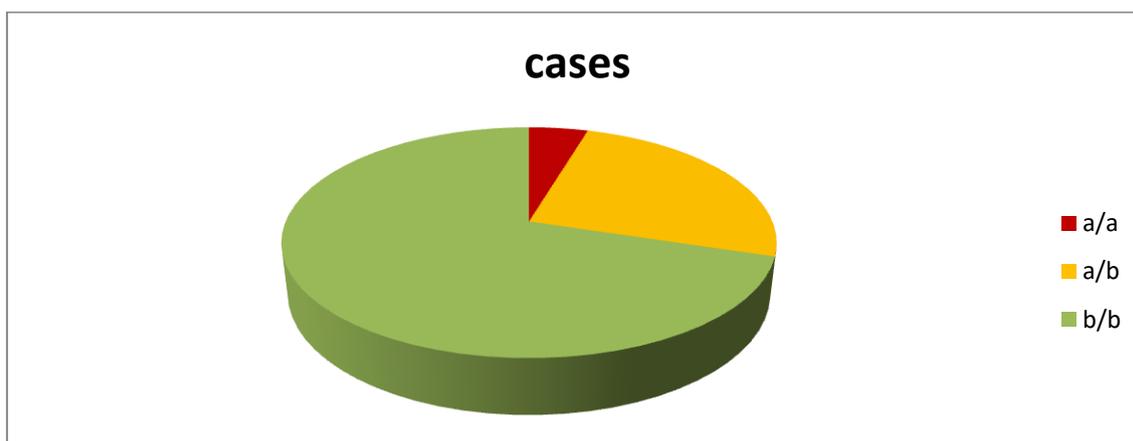
		Group				Pearson Chi-square test
		Hypertensives		Control		
		N	%	N	%	
Sex	Male	132	87.4%	118	90.1%	$\chi^2=0.49$ P=0.48(NS)
	Female	19	12.6%	13	9.9%	
Smoking	No	116	76.8%	96	73.3%	$\chi^2=0.47$ P=0.49(NS)
	Yes	35	23.2%	35	26.7%	
Alcoholism	No	122	81.5%	104	80.2%	$\chi^2=0.07$ P=0.88(NS)
	Yes	28	18.5%	26	19.8%	

Table-2: Genotype distribution of human eNOS intron 4 gene

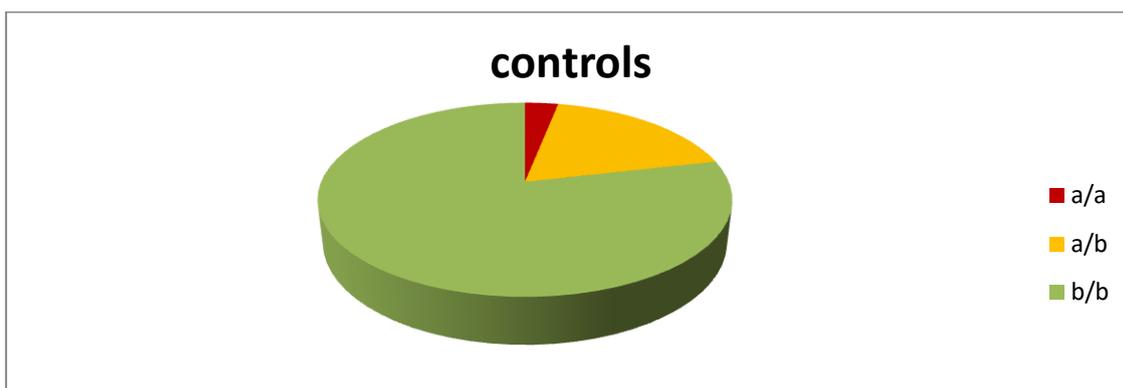
Genotype		Group		Pearson Chi-square test
		Hypertensives	Control	
a/a	7(4.6%)	4(3.1%)	$\chi^2=7.61$ P=0.11	
a/b	38(25.2%)	24(18.3%)		
b/b	105(70.2%)	102(78.6%)		
Total	150	130		



Bar diagram 1: Comparison of eNOS intron4 gene distribution in cases and controls



PIE DIAGRAM 1: Genotype distribution in hypertensives

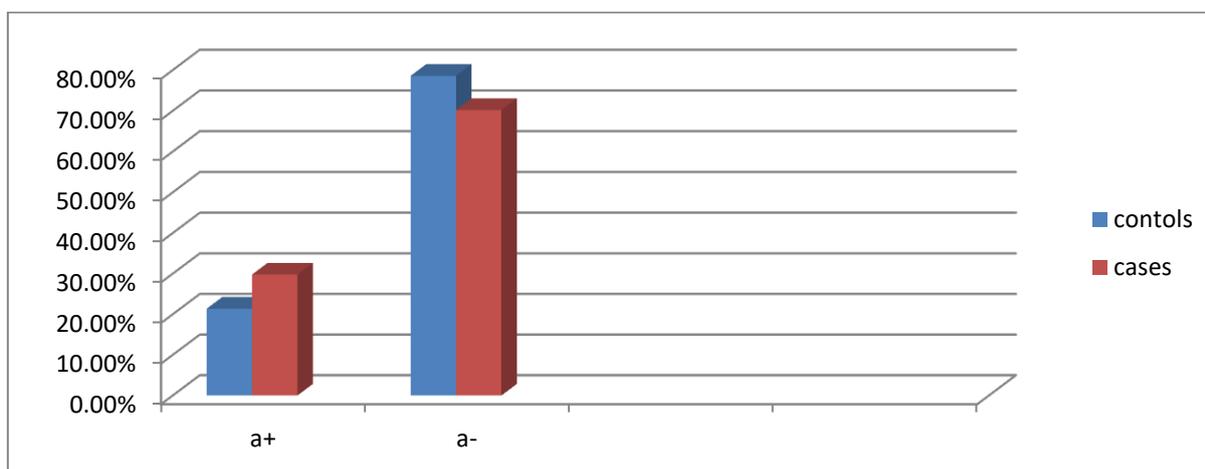


PIE DIAGRAM 2: Genotype distribution in controls

Table-3: Allele frequencies of human eNOS gene INTRON4 gene

Genotype	Hypertensives	Control	P value
a+*	45 (29.8%)	28 (21.4%)	Chi sq = 0.45 p = 0.5
a-*	105 (70.2%)	102 (78.6%)	

a+* = a/a + a/b ; a-* = b/b



Bar diagram 2: Comparison of eNOS intron4 allelic distribution between cases and controls

DISCUSSION

Genetic factors in combination with a number of environmental risk factors are involved in essential hypertension. Variation in the expression and activity of eNOS is being linked to essential hypertension in various animal models. NOS3 gene is located in the chromosome 7, corresponding to the 7q35-q36 region, contains 26 exons with an entire length of 21kb. In normotensive animals, administration of methylated arginines, which are competitive eNOS inhibitors, caused marked and dose-dependent elevations in blood pressure level [16]. When normotensive mice were engineered to express the constitutively active eNOS in excess, their mean blood pressure levels were 18 mm Hg lower than normal [17]. In spontaneously hypertensive rats, a single intravenous injection of human complementary DNA encoding eNOS almost completely normalized blood pressure for 5 to 6 weeks [18]. The eNOS^{-/-} knockout mouse develops mild-to-

moderate hypertension, with basal blood pressure levels typically 20 mm Hg higher than in control littermates [19]. It was found that these mice have decrease in eNOS protein expression [20] and positively associated with systemic and pulmonary hypertension [21], altered vascular remodeling [22] and impaired angiogenesis [23]. This down-regulation of eNOS mRNA, undoubtedly contributes to the reduced endothelial NO production and defective endothelium-dependent vasorelaxation [24] in diseased atherosclerotic vessel. Decreased eNOS expression due to its various gene polymorphisms is suggested as cause of essential hypertension in humans, by influencing serum NO level [25]. A 27bp repeat polymorphism in intron 4 of NOS3 gene is the one which is of conflicting interest and its role in essential hypertension is being explored. Hence, it is proposed to study the association of serum NO level and eNOS intron4 polymorphism in essential hypertension.

In this study, the hypertensives and controls were perfectly matched with respect to the confounding variables like age, sex, BMI, smoking and alcoholism. The main area of study was focused on eNOS intron4 polymorphism screening (genotypes aa, ab, bb). In the present study the frequency of 'a' allele was found to be approximately 0.26, which is little higher than that found in other populations viz., Iranian (0.1), Japanese (0.1 to 0.13), Turkish (0.14), Australian (0.17) and little lower than that of African Americans (0.28). The differences in the ethnic origin and sample sizes might have an influence in the results obtained regarding the distribution of the eNOS intron4 polymorphism studied in these populations. This study did not find any correlation between the presence of the eNOS 4a/b variant and essential hypertension. This may be due to the small sample size. According to the literature survey, only two studies, one on Caucasians in general [26], and another on the Ukrainian population, have shown a correlation between this polymorphism and essential hypertension. No such correlation have been found in other studies.

Though intron 4a/b polymorphism of NOS3 had no association with essential hypertension, a trend towards a higher frequency of the 'a' allele was apparent in the hypertensives. In other studies conducted in India, the same trend was observed. This study needs to be expanded by conducting a large cohort study, to elucidate in depth, a link of any association of essential hypertension with intron4 gene polymorphism.

CONCLUSION

eNOS intron4 polymorphism was not found to be a risk factor for essential hypertension in this study. The presence of 'a' allele makes a person more susceptible to essential hypertension probably because of its nitric oxide lowering effect. 'b' allele was found to be protective. As the study population was confined to the hypertensives attending a single govt. general hospital, the expected role of eNOS intron4 polymorphism as a risk factor could not be framed. This study needs to be expanded to cover a larger population, to define the actual role of eNOS intron4 polymorphism in essential hypertension. Various other eNOS genes have to be explored and their association with eNOS activity and essential hypertension to be studied. GENE THERAPY may be ventured if there is an association of the gene polymorphism with essential hypertension.

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