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Journal of Advanced Scientific Research

Available online through http://www.sciensage.info

ISSN
0976-9595
Research Article

ASSOCIATION OF *NOS3* GENE POLYMORPHISMS WITH ESSENTIAL HYPERTENSION IN SOUTH INDIAN POPULATION

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ABSTRACT

Essential hypertension involves both genetic and environmental factors. It is often described as silent killer, if left untreated it leads to heart failure, stroke and kidney failure. The present study aims to study the polymorphisms (894G>T in exon 7, 786T>C in the 5' flanking region, 4b/4a 27 bp VNTR Intron 4 and 10G>T in intron 23) of Endothelial Nitric Oxide Synthase (*NOS3*) gene implicated in hypertension. A PCR-PFLP analysis was carried for four NOS3 gene polymorphisms. The present study did not associate the T allele of G894G>T polymorphism as a risk factor for hypertension. Higher risk was obtained for individuals with CC genotypes for 786 T>C and for males who were homozygous for 4a allele of 27 bp VNTR intron 4 polymorphism at the *NOS3* locus.

Keywords: Essential Hypertension, Endothelial Nitric Oxide Synthase (*NOS3*), Gene polymorphism, PCR-RFLP

1. INTRODUCTION

Essential hypertension is a complex, multifactorial disorder that enhances the risk for heart failure, stroke, and kidney failure. Globally, it is estimated that 26% of the world's population (972 million people) has hypertension, and by 2025 the expected increase in prevalence is 29% [1]. In India, the prevalence is estimated to be 207 million persons including 112 million men and 95 million women [2]. The interplay of complex genetic and environmental factors that influence the phenotype makes elucidation of mechanisms underlying hypertension a difficult challenge. Case control studies and genome wide scans have identified several genes (around 300) and loci that regulate blood pressure either by constricting or dilating actions. The Endothelial Nitric Oxide Synthase (*NOS3*) gene synthesizes Nitric Oxide (NO), is a potent vasodilator and thus can play an important role in blood pressure regulation. The present study aims to study the polymorphisms (894G>T in exon 7, 786T>C in the 5' flanking region, 4b/4a 27 bp VNTR Intron 4 and 10G>T in intron 23) of Endothelial Nitric Oxide Synthase (*NOS3*) gene. The present study is expected to provide estimates of risk for individuals with particular genotype of *NOS3* gene for developing hypertension which also may add to our understanding of the mechanisms underlying hypertension.

2. MATERIAL AND METHODS

The study population included 492 individuals for NOS3 894 G>T, 460 individuals for 786T>C, 402 for 4a/4b polymorphism and intron 23 10G>T. Established patients who were on antihypertensive drugs were recruited from Gandhi Medical College and Hospital, Hyderabad, Telangana, India. Subjects diagnosed with secondary hypertension arising due to CAD, renal failure, thyroid disorder, pregnancy induced hypertension and other associated conditions were excluded from the study. Controls were recruited from various government and private organizations and personal contacts. 5ml of venous blood was collected in EDTA vacutainers from the subjects after an informed consent and DNA was isolated by non-enzymatic method [3]. A PCR-PFLP analysis was carried for four NOS3 gene polymorphisms, procedure details are summarized in Table 1, 2 & 3.The data generated was analysed by SPSS version 20.

Table 1: Primer sequences used for the *NOS3*polymorphisms selected for the present study

Intron 4 VNTR	F 5'-AGGCCCTATGGTAGTGCCTTT- 3' R 5'-TCTCTTAGTGCTGTGGTCAC- 3'
894 G>T	F 5'-CATGAGGCTCAGCCCCAGAAC-3' R 5'-AGTCAATCCCTTTGGTGCTCAC-3'
786 T>C	F 5'-ATGCTCCAAGGGCATCA-3' R 5'-GTCCTTGAGTCTGACATTAGGG-3'
10 G>T	F 5'-CCCCTGAGTCATCTAAGTATTC-3' R 5'-AGCTCTGGCACAGTCAAG-3'

Table 2: The restriction enzymes used, their recognition sites, incubation temperature and the effect of different mutations on the restriction sit

SNP	Restriction enzyme	Recognition site	Effect of Mutation	Incubation
894G>T	Mbo I	/GATC	Gain of site	37^0C, overnight
786T>C	NgoM IV	G/CCGGC	Gain of site	37^0C, overnight
10G>T	Hinc II	GTY/RAC	Gain of site	37^0C, overnight

Table 3: PCR product size and the number of fragments generated after digestion in homozygous normal, homozygous mutant and heterozygotes for different polymorphisms

Polymorphism	PCR product size	Analysed on	Homozygotes (Normal)	Heterozygotes	Homozygotes (Mutant)
Intron 4 VNTR	420 bp	8% PAGE	420 bp	420, 393 bp	393 bp
894G>T	206 bp	2.5% agarose	206 bp	206, 119, 87 bp	119, 87 bp
786T>C	236 bp	2.5% agarose	236 bp	236, 203, 33 bp	203 & 33 bp
10G>T	676 bp	2% agarose	577, 99 bp	577, 374, 203 & 99 bp	374, 203 & 99 bp

3. RESULTS AND DISCUSSION

3.1. NOS 3exon 7 (894G>T) (rs1799983)

Table 4 describes the percentage distribution of *NOS3* exon 7 (894G>T) genotypes in hypertensive and control groups. The overall genotypic frequencies of GG, GT and TT were 71.8 %, 19.3 % and 8.8 % in hypertensives, while they were 72.4 %, 19.3 % and 8.3 % in controls respectively. There was no significant difference observed in the distribution of these genotypic frequencies between hypertensives and control groups. The hypertensive and control data was divided into several cohorts (like gender, familial history, obesity, smoking and alcoholic consumption and dietary habit) and comparison was made between them. No significant deviation was observed in the cohorts. Several studies have shown positive association of 894G>Tpolymorphism of *NOS3* gene with hypertension [4, 8, 9], CAD [5, 6] and acute Myocardial infraction [7]. While, some studies did not show association of NOS3 gene with Hypertension [10], CAD [11, 12] and with MI in the South Indians [14]. Srivastava et al (2008) observed a higher prevalence of GT+TT genotypes in hypertensives when compared to controls and also association of T allele with hypertension [9]. Colomba et al (2008) reported a higher prevalence of GT+TT under dominant model in hypertensive patients who were prone to cardiovascular damage [8]. The present study did not associate the T allele as a risk factor for hypertension.

3.2. *NOS3*5' flanking region (786T>C) polymerphism (rs2070744)

The 786T>C polymorphism is present in the promoter region of *NOS3* gene. This variant results due to a substitution of thymidine by cytosine at 786 nucleotide and has been found to be associated with Coronary Spasm [14], Hypertension [15, 8], and CAD [6, 16]. The overall genotypic frequencies of TT, TC and CC were 63.1 %, 31.3 % and 5.6 % in hypertensives, while they were 63.0 %, 30.4 % and 6.6 % in controls respectively [Table 4]. There was no significant deviation observed between the distribution of the genotypic frequencies between hypertensives and control groups as well as in cohorts. However, within hypertensives, males had significantly higher proportion of TC (38.5%) and CC (6.8%) genotypes when compared to females (24.1%, 4.3% respectively; p=0.02).

Table 5 shows the risk estimation regarding different cohorts studied within the hypertensive group. Within the hypertensives, when the frequency of TT genotype was compared to TC genotype, significant risk was observed for gender (OR= 0.480; CI 0.271 to 0.849, p =0.006), this is because males had higher proportion of TC genotype than females. This shows that males with TT genotypes enjoy protection against the disease while those with TC genotype may be more prone to hypertension. Computation of risk for males with TC genotype revealed a 2-fold higher risk than males with TT genotype (OR=2.08; CI 1.175 to 3.609, p= 0.006). Similarly, when the frequency of TT was compared to CC genotypes, it is observed that individuals with TT genotype with a positive family history were protected against the disease when compared to non-familial patients(OR= 0.265; CI 0.07-1.004). Comparison of frequency of TC genotype with CC revealed that obese individuals with TC genotypes seem to enjoy protection against the disease (OR= 0.201; CI 0.048-0.0.85, p=0.14), where as individuals with CC genotype have 4.9 times higher risk for developing hypertension (OR= 4.963; CI 1.1171-21.028, p=0.14).

Significant deviation for gender was observed when TT genotypes were compared to individuals with TC and CC genotypes put together, when tested under the dominant model (OR= 0.48; CI 0.279-0.827, p=0.004), indicating that individuals with TT genotype are protected against the disease. However, individuals with TC genotypes had significantly higher risk (1.9 times) when compared with TT and CC genotypes put together (OR= 1.964; CI 1.116-3.457,p=0.009). Comparison of frequency of individuals with CC genotypes with TT and TC genotypes put together revealed that individuals with CC genotype have 3.4 times risk for hypertension with positive family history (OR= 3.456; CI 0.926-12.902, p=0.032), and 3.1-fold higher risk if they happen to be obese (OR= 3.177; CI 0.915-11.030,p=0.034). Nakayama et al (1999) reported association of 786T>C polymorphism with Coronary spam [14]. They reported that the promoter activity decreased with CC genotype was associated under normoxic (52%) and hypoxic (62%) conditions compared with wild type promoter sequence. They suggested that presence of this 786T>C mutation reduces endothelial NO production in the coronary arteries and thereby predisposing patients carrying the mutant allele to coronary spasm. Miyamoto et al (2000) reported that the -786 C allele is associated with decreased*NOS3* mRNA levels and serum nitrite and nitrate levels [4]. Hyndman et al (2002) reported that patients with CC genotype had 2.16 times risk for developing hypertension [15]. Colombo et al (2003) reported that individuals with at least one C allele had higher mean CAD Duke score and were 1.6 times higher risk for CAD when compared to individuals with TT genotype[6]. Rossi et al (2003) confirmed the association of C allele with CAD in males, smokers, low HDL and obesity [16]. Colomba et al (2008) reported an increased prevalence of TC + CC genotypes with cardiovascular damage[8]. However, Kajiyama et al (2000) concluded that the 786 C allele is not associated with essential hypertension [17] and Arun Kumar et al 2013 did not find the association of this polymorphism with MI in South Indian population [13]. The present study revealed a positive association of 786T>C polymorphism with essential hypertension.

3.3. *NOS3* 4b/4a (27 bp VNTR) polymorphism

The 27 bp VNTR located in intron 4 of *NOS3* gene was reported to be associated with CAD [18]. They observed that individuals homozygous with 4a allele had higher risk for CAD among smokers. Other studies on *NOS3* intron 4 polymorphism showed positive association with renal disease [19, 20], essential hypertension among Japanese [21], CAD among Iranian [22] and stroke among the Chinese [23]. However, studies from Taiwan [24] failed to associate this polymorphism with premature CAD.

The overall genotypic frequencies of 4b/4b, 4b/4a and 4a/4a were 62.6 %, 29.9 % and 7.5 % in hypertensives, while they were 69.1 %, 27.1 % and 3.7 % in controls respectively. Comparison of male patients with male controls showed significant difference (p=0.023), that is because the proportion of 4b/4a and 4a/4a was higher in hypertensives (33.6% and 10.9%) than in controls (4b/4a-25.7 and 4a/4a- 2.7%). Significant deviation was also observed between the males and females within hypertensive group ((p=0.039), as the proportion of 4b/4a and 4a/4a was more in males (4b/4a-33.6 and 4a/4a- 10.9) when compared to females (4b/4a-26.0and 4a/4a- 3.8), (Table- 4).

When 4b/4b genotype were compared with 4b/4a genotype, protective effects were evident for 4b/4b genotype in males when compared to 4a/4a (OR= 0.188; CI 0.51 to 0.696, p=0.006, Table 5). Testing of

pooled genotypes under dominant model indicated the protective effect for males with 4b/4b genotype when compared with 4b/4a and 4a/4a genotypes put together (OR= 0.492; CI 0.282 to 0.857, p=0.006). When patients with 4a/4a genotype were compared with patients with 4b/4b and 4b/4a genotype, it was observed that homozygous recessive males have 4 fold higher risk for hypertension (OR= 4.48; CI 1.231 to 16.378.; Table 5). Uwabo et al (1998) have shown significant association of hypertension with a/a homozygosity [21]. They also suggested a linkage disequilibrium of this polymorphism with other genes related to essential hypertension. Other studies have shown a positive association of *NOS3* 4b/a polymorphism with CAD in Japanese [25] and Iranian populations [22]. Absence of such association was reported in German [26] and Taiwanese populations [24].

The present study revealed the association of intron 4 polymorphism in males with 4a/4a genotype with essential hypertension. These results suggest that the intron 4 polymorphism of *NOS3* can serve as a useful genetic marker for evaluation of susceptibility to essential hypertension.

3.4. *NOS3* intron 23 (10G>T) polymorphism

Some studies have failed to associate this polymorphism with essential hypertension [27, 28]. However, Yoon et al (2000), observed a significantly higher frequency of G- allele in CAD patients than in controls [11]. In the present study only GG genotypes were found both in patient and control groups indicting that either GG genotypes are fixed in our population or GT and TT genotypes are either absent or rare in our population.

The present study has some limitations. There are no measures of plasma nitric oxide levels that correlate with the investigated polymorphisms. The results can be further validated with large sample sizes and can be correlated with studies involving other genes that regulate blood pressure.

Table 4: Percentage distribution *NOS3* gene polymorphisms in hypertensive patients and control groups

Genotype	Total	hypertensives	Controls	p value
	n (%)	n (%)	n (%)	
894G>T	492	238	254	
GG	355 (72.15)	171 (71.8)	184 (72.4)	0.974
GT	95 (19.3)	46 (19.3)	49 (19.3)	
TT	42 (8.5)	21 (8.8)	21 (8.3)	
G	805 (0.82)	388 (0.82)	417 (0.82)	0.816
T	179 (0.18)	88 (0.18)	91 (0.18)	
786T>C	460	233	227	
TT	290 (63.0)	147(63.1)	143(63)	0.894
TC	142 (30.9)	73(31.3)	69 (30.4)	
CC	28 (6.1)	13(5.6)	15 (6.6)	
Allels				
T	722 (78.5)	367 (78.8)	355 (87.2)	0.835
C	198 (21.5)	99 (21.2)	99 (21.8)	
within Hypertensives		Males	Females	
TT		64 (54.7)	83 (71.6)	**0.02**
TC		45 (38.5)	28 (24.1)	
CC		8 (6.8)	5 (4.3)	
4a/4b	402	214	188	
4b/4b	264 (65.67)	134 (62.6)	130 (69.1)	0.184
4b/4a	115 (28.6)	64 (29.9)	51 (27.1)	
4a/4a	23 (5.72)	16 (7.5)	7 (3.7)	
Alleles				
4b	643 (80.0)	332 (77.6)	311 (82.7)	0.295
4a	161 (20.0)	96 (22.4)	65 (17.3)	
Between hypertensive and control Male				
4b/4b		61 (55.5)	81 (71.7)	**0.023**

4b/4a	37(33.6)	29 (25.7)	
4a/4a	12 (10.9)	3 (2.7)	
within Hypertensives	Males	Females	
4b/4b	61 (55.5)	73 (70.2)	**0.039**
4b/4a	37(33.6)	27 (26.0)	
4a/4a	12 (10.9)	4 (3.8)	

Table 5: Risk estimations for combinations of *NOS3*gene polymorphism genotypes for developing hypertension

Polymorphism	Cohort	Genotype	OR	CI	p	p_{fit}
786T>C						
	Gender	TT Vs TC	0.48	0.271-0.849	0.006	0.011
		TC Vs TT	2.08	1.175-3.698	0.006	0.011
		Family History				
		TT Vs CC	0.265	0.07-1.004	0.025	0.038
	Obese					
		TC Vs CC	0.201	0.048-0.854	0.014	0.19
		CC Vs TC	4.963	1.1171-21.028	0.014	0.19
		Pooled genotypic combinations				
	Gender	TT Vs (TC+CC)	0.48	0.279-0.827	0.004	0.007
		TC Vs (TT+CC)	1.964	1.116-3.457	0.009	0.018
	Family history	CC Vs (TT+TC)	3.456	0.926-12.902	0.032	0.051
	Obesity	CC Vs (TT+TC)	3.177	0.915-11.030	0.034	0.05
4a/4b						
	Males	4b/4b Vs 4a/4a	0.188	0.51-0.696	0.006	0.006
		Pooled genotypic combinations				
	Males	4b/4b Vs (4b/4a + 4a/4a)	0.492	0.282-0.857	0.006	0.012
		4a/4a Vs (4b/4b + 4b/4a)	4.48	1.231-16.378	0.011	0.012

4. CONCLUSION

The polymorphisms studied in *NOS3* gene seem to be playing an important role in the regulation of blood pressure by exerting their effect on the vascular tone. Higher risk was obtained for individuals with CC genotypes for 786 T>C and for males who were homozygous of 4a allele of 27 bp VNTR intron 4 polymorphisms at the *NOS3* locus. The results indicate the possible role of polymorphisms of NOS3 gene in Essential hypertension. Identifying these variants is necessary to ascertain their contribution to the pathophysiology of the disease. These results further help in formulating better prevention and management measures and thus will improve the quality of life afflicted. The role of NOS3 gene polymorphism has been studied worldwide and many studies have found a positive association while some failed to associate this gene with Essential Hypertension. The present study has observed certain genotypes in South Indians that are at higher risk for developing Essential Hypertension.

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