Original Article

Association of Endothelial Nitric Oxide Synthase Gene Polymorphisms with Early-Onset Ischemic Stroke in South Indians

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***Aim*: The aim of this study was to investigate the association of T-786C, G894T and 4a/b polymorphisms in the endothelial nitric oxide synthase (*eNOS*) gene with early-onset ischemic stroke in South Indians.**
***Methods*: We enrolled 177 patients diagnosed with ischemic stroke aged between 15 to 45 years and 219 age- and gender-matched healthy controls. Genotypes of *eNOS* T-786C, G894T and 4a/b were identified by polymerase chain reaction and restriction fragment length polymorphism.**
***Results*: The allele and genotype frequencies of *eNOS* 4a/b, T-786C and G894T did not differ significantly in the patient group compared to controls. Logistic regression analysis indicated the 4a allele to be an independent predictor of ischemic stroke in females (dominant model: OR, 2.46; 95% CI, 1.11 to 5.43; $p=0.026$). Marked differences were found in the prevalence of the minor alleles of the three variants when comparing the South Indian population with the reported frequencies from Caucasians. There was also a contrast in the frequencies of 4ab and T-786C variants from other Asians. The genotypes of all three variants were found to be in Hardy-Weinberg equilibrium. There was a lack of significant linkage disequilibria among the variants, and none of the estimated haplotypes increased or decreased the risk of ischemic stroke.**
***Conclusion*: The *eNOS* intron 4a/b polymorphism can predict early-onset ischemic stroke in south Indian women.**

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Key words; *eNOS* 4a/b, T-786C, G894T, Stroke in the young

Introduction

Several lines of evidence suggest that ischemic stroke has a strong genetic component: the concordance rate of 17.7% in monozygotic twins as opposed to 3.6% in dizygotic twins[1)] being one of them. Consequently, ample genetic association studies have been performed but the results are inconsistent[2, 3)]. One of the potential explanations for the inconsistency might be the choice of age group of the study subjects. Most studies have targeted elderly populations whereas less attention has been given to early-onset cases. It has been suggested that less exposure to environmental confounders strengthens the influence of genetic determinants in young adults compared to older cases[4)]. The association between a family and personal history of stroke has been found to be stronger in younger stroke patients[5)]. Moreover, stroke in the young is not a rare event, particularly in countries of oriental origin, such as China, Korea and India, where it constitutes more than 5% of all strokes[6-8)]. A recent study has also shown that strokes occur in developing countries at a much earlier age than in developed countries[9)]. Hence, we targeted a young adult population for testing genetic associations with ischemic stroke.

Genetic variants of endothelial nitric oxide synthase (*eNOS*), the enzyme which generates nitric oxide in blood vessels and regulates vascular function, have been targets for case-control studies for various vascu-

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lar diseases. Several SNPs have been identified in the promoter, exons, and introns of the *eNOS* gene. G894T in exon 8 (also named rs1799983) leads to an amino acid change of glutamate (Glu) to aspartate (Asp) at site 298. The functional relevance of this polymorphism has been suggested to be due to the observed genotype-dependent alterations found in basal and shear-induced activation, altered caveolar localization and consequent impairment in the co-ordination of the regulatory cycle of the enzyme[10]. Mechanistically it is attributed to the altered protein-protein interaction of the mutant enzyme with caveolar protein-1. Another *eNOS* gene polymorphism, T-786C (also referred to as rs2070744) located in the promoter region of the *eNOS* gene, results from replacement of a thymidine by a cytosine at nucleotide −786. It has been reported that the T-786C reduces promoter activity of the *eNOS* gene, which results in the compromised production of endothelial nitric oxide[11]. The intronic 4ab variant, a 27bp, variable number of tandem repeat (VNTR) polymorphism, has also been widely studied[12-16]. Due to its location in the non-coding region, its chances of being associated with disease traits are minimal. In spite of this, the variant is shown to genetically contribute to basal levels of plasma nitric oxide[12], which is explained by its likely linkage to a functional locus.

The results of the genetic association of polymorphisms of *eNOS* with ischemic stroke have been inconsistent and controversial[3]. Further, there are few published reports on the association of candidate polymorphisms of the gene with the risk of early onset ischemic stroke. Howards and co-workers[17] have demonstrated the TT genotype of the *eNOS* T-786C variant as a potential risk factor for ischemic stroke in young women. Recently, the intron- 4a/b variant of *eNOS* has been reported to be an important risk factor for ischemic stroke in the young Chinese population[13]. Although the *eNOS* G894T variant has been reported to be possibly related to early atherogenesis[18], its relationship with ischemic stroke in the young is not clear and needs to be evaluated. Racial differences constitute an important component of the etiology of young strokes[19], and independent confirmation of genetic associations is very essential. To date, no reports are available for the association of *eNOS* variants with ischemic stroke in the Indian population. Hence, our main aim was to validate and extend the previous results by assessing the comparative distribution of *eNOS* variants in young Indian ischemic stroke patients and healthy controls.

Materials and Methods

Subjects

The study was approved by our Institutional Review Board and informed consent was obtained from all healthy and diseased volunteers. One hundred and seventy-seven patients aged between 15 to 45 years, who presented at the Neurological Services of the National Institute of Mental Health and Neuro Sciences (NIMHANS), a tertiary care centre for neuropsychiatric disorders located in Bangalore, India, were enrolled for the study. The diagnosis of ischemic stroke was confirmed by neuro-imaging studies (cranial CT scan/MRI). Patients diagnosed with haemorrhagic stroke, stroke secondary to neuroinfections, malignancy or other terminal illnesses were excluded from the study. The control group comprised of 219 age- and gender-matched healthy subjects who had no prior history of cerebrovascular disease. Detailed clinical and demographic data were recorded. Fasting venous blood samples were collected from all subjects. Genomic DNA was isolated from EDTA blood using Miller's protocol[20] and stored at −20℃ for molecular studies.

Genetic Analysis

eNOS 4ab Polymorphism

The *eNOS* 4a/b gene polymorphism was detected by the method of Wang *et al.*[14]. DNA was subjected to amplification by polymerase chain reaction (PCR) using the following primers: sense 5'-AGG CCC TAT GGT AGT GCC TTT-3' and antisense 5'-TCT CTT AGT GCT GTG GTC AC-3'. The amplified fragments were separated on 3% polyacrylamide gels with ethidium bromide staining. Three genotypes, containing 4, 5 repeats, namely 4/4- homozygous (4aa), 4/5-heterozygous (4ab), 5/5-homozygous (4bb), were identified. The allele containing 5 of 27bp repeats gave rise to PCR products of 420bp whereas that containing 4 repeats yielded a 393bp product.

eNOS G894T Polymorphism

DNA fragments (248bp) containing the G894T polymorphism were amplified with the following primers, 5'-AAG GCA GGA GAC AGT GGA TGG A-3' (sense) and 5'-CCC AGT CAA TCC CTT TGG TGC TCA-3' (antisense). PCR products were incubated at 37℃ for 4 hours using *Dpn II* (Bangalore Genei, India.). Digested products were separated by size on 2% gel with ethidium bromide staining. The DNA segment from TT homozygote was digested into 160 and 88bps. For the undigested GG wild homozygote, a single band of 248bp was observed.

eNOS T-786C Polymorphism

PCR was performed with the primers 5'-GTG TAC CCC ACC TGC ATT CT-3' (sense) and 5'-CCC AGC AAG GAT GTA GTG AC- 3' (antisense). The 306bp PCR product was checked for amplification and digested with the restriction enzyme *Nae I* (Bangalore Genei, India) at 37℃ for 4 hours. Digested products were separated by size on 2% gel with ethidium bromide staining. The DNA segment from TT homozygote was digested into 221 and 85bps.

Statistical Analysis

Statistical analysis was performed using SPSS statistical package, version 15 and GraphPad InStat (GraphPad Software, San Diego, CA, USA). Data are expressed as the mean ± SD. The frequencies of the alleles and genotypes were compared between patient and control groups by the χ^2 test when appropriate. Genotypic distribution of gender, cigarette smoking, alcohol consumption, hypertension and diabetes between patients and controls was analysed by the generation of 3 × 2 contingency tables followed by χ^2 analyses. Fisher's exact test was used to compare the distribution of two genotype subgroups when the smallest of the four expected numbers was < 5. Logistic regression models were also developed to evaluate the independent roles of these genotypes in the stroke group. These logistic regression models included significant clinical risk factors. For all tests, a p value of < 0.05 was considered significant. Linkage disequilibrium was estimated amongst the three polymorphisms in our study subjects using Haploview software, (http://www.broad.mit.edu/mpg/haploview/contact.php). R-statistical language was used to determine the probabilities of the possible haplotypes based on the Expectation Maximization (EM) method[21].

Results

Demographic and clinical characteristics of patients and controls are reported in **Table 1**. There were 123 males and 54 females in the stroke group with a mean age of 29.71 ± 10.87 years. The control group consisted of 147 males and 72 females with a mean age of 28.32 ± 7.86 years. Among the vascular risk factors, cigarette smoking and alcohol consumption were found to be more prevalent in the patient group. Logistic regression analysis indicated that cigarette smoking and alcohol consumption were independent risk factors for the disease outcome. Other conventional risk factors, such as hypertension, diabetes mellitus and hyperlipidemia, were not significantly different between the two groups.

Table 1. Demographic and clinical characteristics of stroke patients and control subjects

Parameters	Patients $n = 177$	Controls $n = 219$	p value
Age (years) (Mean ± SD)	29.71 ± 10.82	28.42 ± 7.87	0.1940
Males, n (%)	123 (69.5)	147 (67.1)	0.6647
Smokers, n (%)	62 (35.03)	13 (5.9)	< 0.0001*
Drinkers, n (%)	52 (29.4)	20 (9.1)	< 0.0001*
Hypertension, n (%)	6 (3.4)	2 (0.91)	0.1469
Diabetes, n (%)	4 (2.3)	1 (0.47)	0.1774
Dyslipidemia, n (%)	2 (1.2)	0	0.1992

*Highly significant

Genotype and Allele Distribution of the *eNOS* Variants in Cases and Controls

All genotypes were found to be in Hardy-Weinberg equilibrium in both study groups. T894, C-786 and 4a were considered rare alleles and their relationship with study variables was analysed by dominant and recessive models.

For the *eNOS* G894T variant, the genotype distributions were as follows, GG 74.3%, GT 23.36% and TT 2.34%, with the frequency of the minor allele being 14.0% in the healthy control population. The variant was not associated with the risk for ischemic stroke (**Table 2**, **3**). Logistic regression analysis of the effect of genotype on the risk of ischemic stroke with covariates such as smoking, alcohol consumption, diabetes, and hypertension showed that the differences in genotype distributions remained insignificant (**Table 3**). Stratification of data for gender did not affect the distribution, as shown in **Table 2**.

Genotype distributions for the 4ab variant were: 4bb 61.2%, 4ab 34.6% and 4aa 4.2%, with the frequency of the minor allele being 21.5% in our controls (**Table 2**). On analyzing cases and controls, no difference was found in the distribution of allelic and genotypic frequencies ($p = 0.9811$) between cases and controls and the polymorphism was not associated with the risk for stroke (**Table 2**); however, gender stratification of samples showed the overall genotype distribution of the polymorphism to be differentially distributed between female patients and controls ($p = 0.0415$, $\chi^2 = 6.34$). The 4a allele was more prevalent in female cases than female controls, allele frequencies being 28.3% and 15.9% respectively. Logistic regression analysis of the effect of the variant indicated the 4a allele to be an independent predictor of ischemic stroke in females (dominant model: OR, 2.46; 95% CI, 1.11 to 5.43; $p = 0.026$); however, in males, the distribution of the genotypes was not sig-

Table 2. Genotype and allele frequency of *eNOS* single nucleotide polymorphisms (SNPs) in ischemic stroke patients and controls

eNOS variant	Group	Genotype count (%)			*p*-value† (genotype)	Allele frequency	OR (95% CI), *p*-value§ (alleles)
		4bb	4a4b	4aa			
4a4b	All						
	Cases (*n* = 175)	106 (60.6)	61 (34.8)	8 (4.6)	0.9811	0.220	1.0 (0.73, 1.45)
	Controls (214)	131 (61.2)	74 (34.6)	9 (4.2)		0.215	*p* = 0.9304
	Females						
	Cases (*n* = 54)	28 (51.9)	22 (40.7)	4 (7.4)	0.0415*	0.278	2.13 (1.15, 3.96)
	Controls (*n* = 72)	53 (73.6)	16 (22.2)	3 (4.2)		0.153	*p* = 0.0183*
	Males						
	Cases (*n* = 121)	78 (64.5)	39 (32.2)	4 (3.3)	0.2922	0.194	0.74 (0.48, 1.12)
	Controls (*n* = 142)	78 (54.9)	58 (40.8)	6 (4.3)		0.246	*p* = 0.1717
		894GG	894GT	894TT			
G894T	All						
	Cases (*n* = 172)	124 (72.1)	43 (25.0)	5 (2.9)	0.8655	0.151	1.12 (0.75, 1.67)
	Controls (*n* = 214)	159 (74.3)	50 (23.36)	5 (2.34)		0.140	*p* = 0.6094
	Females						
	Cases (*n* = 53)	41 (77.36)	11 (20.75)	1 (1.89)	0.4258	0.123	0.87 (0.41, 1.86)
	Controls (*n* = 69)	51 (73.90)	18 (26.10)	0 (0.00)		0.130	*p* = 0.8488
	Males						
	Cases (*n* = 119)	83 (69.75)	32 (26.89)	4 (3.36)	0.6601	0.168	1.19 (0.74, 1.91)
	Controls (*n* = 145)	108 (74.48)	32 (22.1)	5 (3.45)		0.145	*p* = 0.4714
		-786TT	-786TC	-786CC			
T786C	All						
	Cases (*n* = 129)	70 (54.3)	50 (38.7)	9 (7.0)	0.4995	0.263	1.1 (0.72, 1.58)
	Controls (*n* = 129)	69 (53.5)	55 (42.6)	5 (3.9)		0.252	*p* = 0.8405
	Females						
	Cases (*n* = 32)	13 (40.6)	17 (53.1)	2 (6.3)	0.1550	0.328	1.5 (0.76, 2 98)
	Control (*n* = 53)	32 (60.4)	17 (32.1)	4 (7.5)		0.245	*p* = 0.2890
	Males						
	Cases (*n* = 97)	55 (56.7)	35 (36.1)	7 (7.2)	0.0584	0.252	0.98 (0.60, 1.59)
	Controls(*n* = 76)	37 (49)	38 (50)	1 (1.0)		0.256	*p* = 1.0000

*Statistically significant; †test of comparison of genotype distributions (Fisher's exact test); §test of comparison of allele distributions (Fisher's exact test); CI = confidence interval.

nificantly different ($p = 0.292$, $\chi^2 = 2.46$).

Genotype distributions for the T-786C variant were TT 53.5%, TC 42.6% and CC 3.9%, with the frequency of the minor allele being 25.2% in our healthy controls. The distribution of the C786 allele and the genotypic frequency of the polymorphism were not significantly different in stroke cases compared to controls (**Table 2**). Accepting a recessive model of inheritance, there was no significant association between the polymorphism of T-786C and the risk of ischemic stroke, although there was a trend (CC vs.TC + TT; OR = 1.86, 95% CI = 0.61 to 5.71. As shown in **Table 3**, logistic regression analysis after adjustment for covariates did not detect any association between the polymorphism and ischemic stroke (**Table 3**). Gender stratification also did not show any significant difference in the distribution pattern of the genotypes (Females: $p = 0.1550$, $\chi^2 = 3.72$; Males: $p = 0.0584$, $\chi^2 = 5.680$).

Relationship between Vascular Risk Factors, Stroke and the Genotypes

Since cigarette smoking and alcohol consumption were found to be independent predictors of

Table 3. Odds ratios as estimates of relative risk for ischemic stroke in carriers of the -786C and 894T alleles

Polymorphisms	Model of inheritance	Crude OR	*p* value	Adjusted OR†	*p* value
eNOS 4ab	Dominant model	1.02 (0.68, 1.54)	0.917	1.14 (0.73, 1.76)	0.574
	Recessive model	1.10 (0.42, 2.93)	0.842	1.20 (0.43, 3.31)	0.730
	Homozygous for rare allele vs. homozygous for common allele	1.11 (0.41, 2.97)	0.837	1.25 (0.45, 3.5)	0.675
	Heterozygous vs. homozygous for common allele	1.012 (0.66, 1.55)	0.958	1.12 (0.71, 1.77)	0.626
eNOS T-786C	Dominant model	1.10 (0.67, 1.79)	0.708	1.03 (0.61, 1.74)	0.911
	Recessive model	1.86 (0.61, 5.71)	0.278	1.69 (0.50, 5.66)	0.396
	Homozygous for rare allele vs. homozygous for common allele	1.88 (0.60, 5.89)	0.279	1.67 (0.49, 5.72)	0.416
	Heterozygous vs. homozygous for common allele	1.02 (0.62, 1.70)	0.926	0.971 (0.56, 1.67)	0.916
eNOS G894T	Dominant model	1.15 (0.73, 1.81)	0.554	1.04 (0.64, 1.69)	0.886
	Recessive model	1.25 (0.36, 4.40)	0.726	1.34 (0.36, 4.98)	0.666
	Homozygous for rare allele vs. homozygous for common allele	1.29 (0.37, 4.56)	0.692	1.34 (0.36, 5.03)	0.665
	Heterozygous vs. homozygous for common allele	1.13 (0.71, 1.82)	0.606	1.01 (0.61, 1.67)	0.974

Odds ratios, ORs, with the effects of the minor alleles, -786C, 894T, and 4a assumed to be dominant (CC and CT combined vs TT, TT and GT combined vs. GG, and 4ab and 4aa combined vs. 4bb), and recessive (CC vs CT and TT combined, TT vs. GT and GG combined, and 4aa vs. 4ab and 4bb combined)
CI=Confidence interval; †Adjusted for smoking, alcohol consumption, hypertension and diabetes

stroke by logistic regression analysis, we investigated whether the combination of the *eNOS* variants and these atherogenic factors were associated with increased stroke risk. Heterozygous and homozygous allele carriers were combined because of the small numbers in the latter group after stratification for smoking and alcohol consumption. Smokers who were carriers of the *eNOS* -786C variant had decreased stroke risk (OR: 0.16, CI: 0.03 to 0.86, $p=0.0287$). A similar decreased risk of stroke was observed in alcohol consumers who had the *eNOS* 786C variant (OR: 0.26, CI: 0.036 to 0.71, $p=0.0171$). No interaction between the other vascular risk factors (hypertension, diabetes and dyslipidemia) and the mutant alleles was found.

Linkage Disequilibrium (LD) Analysis

Haploview was used to look for LD among the three studied polymorphisms but no significant LD was observed among the polymorphisms. Pairwise comparisons of the polymorphisms depicting the LD measures is presented in **Table 4**. Although not significant, the highest D' value of 0.436 was observed for the promoter and intron variant pair.

Table 4. Measures of linkage disequilibrium observed in a pair-wise comparison of the three polymorphisms of the *eNOS* gene

Variant 1	Variant 2	D'	LOD	r^2
T-786C	4ab	0.436	7.76	0.157
T-786C	G894T	0.101	0.04	0.0010
4ab	G894T	0.362	0.4	0.0070

Haplotype Analysis

None of the possible haplotypes was found to be associated with the risk of ischemic stroke, as presented in **Table 5**. The frequency distribution of the haplotypes was almost identical between cases and controls.

Discussion

In India, the occurrence of stroke in the young is not a rare event; rather, it constitutes 13.5 to 32% of all strokes[8, 22], although the cause is not clear in many cases. In an earlier report, Lipska and co-workers[23] suggested that accelerated atherosclerosis might be the mechanism underlying the pathogenesis of early-onset stroke in the South Indian population. Endothelial

Table 5. Haplotype frequency distribution (Patients vs. Controls)

Haplotype	Patients	Controls	*p* (Fisher's exact test)	OR (95% CI)
T-4b-G	0.519	0.580	0.238	0.78 (0.53–1.15)
T-4b-T	0.121	0.099	0.533	1.26 (0.68–2.32)
T-4a-G	0.092	0.057	0.193	1.69 (0.80–3.59)
T-4a-T	0.015	0.019	1.000	0.77 (1.70–3.48)
C-4b-G	0.121	0.075	0.139	1.69 (0.80–3.27)
C-4b-T	0.029	0.028	1.000	1.03 (0.33–3.25)
C-4a-G	0.092	0.142	0.130	0.62 (0.33–1.13)
C-4a-T	0.010	0.000	0.242	5.20 (0.25–108.96)

Order of SNPs in *eNOS* haplotypes: T-786C, 4ab, G894T; OR = Odds ratio; CI = Confidence interval

dysfunction, an initial event of atherosclerosis, is an important feature associated with vascular diseases. Nitric oxide (NO) synthesized by endothelial nitric oxide synthase (*eNOS*), is a key mediator of endothelial function[24-26] playing a central role in the maintenance of vascular homeostasis, including regulation of cerebral circulation. It has been shown that a reduction in NO release predisposes humans to stroke[27]. Since endothelial NO exerts a variety of protective effects, the *eNOS* gene is a logical candidate gene for stroke susceptibility. Endothelial nitric oxide polymorphisms have been widely explored for their association with the occurrence of ischemic stroke across different populations, but there are limited data on their role in the etiology of stroke in the young.

The results of the association of the intronic 4ab variant with ischemic stroke are quite contradictory. Recently, the 4bb genotype of this polymorphism has been implicated as a predisposing genetic factor for early-onset ischemic stroke in Chinese population[13]; however, in 2001, Hou and coworkers[28] reported that the 4a allele was an independent predictor of the disease in elderly Chinese. In contrast, in an English population, the 4a allele was found to be protective, but the effect was confined to isolated symptomatic lacunar infarction[29]. In view of these contradictory observations, we tested the association of the variant with ischemic stroke in our population and found that the mutant 4a allele was an independent risk factor for ischemic stroke in young women. This gender-specific association of the gene with ischemic stroke could be explained by the mechanistic link between the female sex hormone, oestrogen, and the eNOS enzyme. The hormone is known to exert dual effects, both vasodilatory and vasoconstrictory actions, via eNOS. The enzyme is reported to switch its function from NO synthesis to superoxide production depending upon the microenvironment, such as availability of the substrate, arginine, or cofactors, such as BH4[30]. Balance between NO synthesis and superoxide generation has been suggested to regulate the dual actions of oestrogen. De-phosphorylation of certain important residues, such as T495, has also been demonstrated to perturb the balance between NO generation and oxygen reduction, which result from uncoupling of *eNOS*[31]. Due to its intronic location, any such direct functional impact of the 4ab variant on the activity of the enzyme is unlikely. Hence, we speculate that the contribution of the variant to the risk of stroke might be due to a nearby functional mutation.

In spite of functional relevance and reports on the association with endothelial function, most of the available data suggest a lack of association between the *eNOS* G894T variant and ischemic stroke. A study of a French population reported an association between the homozygosity of G allele and brain infarction[32]; however, comprehensive meta-analysis of studies on Caucasians has shown no significant increase in the risk of stroke in T allele (Asp 298) homozygotes compared to G allele (Glu298) carriers[2]. In our study we did not find any difference in the distribution of genotypic and allelic frequencies of the variant between patients and controls.

The CC genotype of T-786C promoter polymorphism has been reported to predispose the Chinese population to ischemic stroke[33]. On the other hand, the TT genotype has been found to be more prevalent in young African women with ischemic stroke than controls[17]. We observed a higher prevalence of the CC genotype in stroke patients compared to controls, although the distribution was not significant. Gender stratification of our data did not indicate any risk associated with the polymorphism in young Indian women. As our study was limited by the small sample size, tests of association on a larger population might give better insight into the role of this polymorphism in the etiology of ischemic stroke in the Indian population.

Table 6. Prevalence of minor alleles of *eNOS* variants in controls by ethnic differences

Allele	Japanese (%)	Chinese (%)	Koreans (%)	Caucasians (%)	African-Americans (%)	South Indians (present study) (%)
894T	5–10.2[a, b, c]	11.49[d]	9[e]	30–37[f, g, h]	15–15.5[g, i]	14.0
4a	10.9, 11[a, b]	7.7,9.1[j, k]	15[e]	16[g]	27,29.2[g, i]	21.5
786C	3.4[l]	10.03[d]	8.6[m]	42[g]	17.5, 16.7[g, i]	25.2

[a]Miyamoto et al.[39], [b]Hibi et al.[40], [c]Shimasaki et al.[41], [d]Cheng et al.[33], [e]Park et al.[45], [f]Hingorani et al.[42], [g]Tanus-Santos et al.[38], [h]Markus et al.[43], [i]Li et al.[44], [l]Nakayama et al.[11], [j]Congning et al.[13], [k]Li et al.[46], [m]Song et al. [47]

Interactions between *eNOS* polymorphisms and environmental factors have been well documented. Smoking has been the main focus of attention, particularly in studies of T-786C polymorphisms in Asian populations. Among smokers, homozygosity for the -786C allele has been shown to be associated with lower cerebral blood flow as well as higher cerebral vascular resistance in comparison with -786T allele carriers[34]. In contrast, the results of our study indicate that the wild genotype of T-786C increases the risk of stroke in smokers and alcohol consumers. Again, these results should be viewed with caution due to the small sample size of the study.

There are reports of linkage disequilibrium amongst variants of the gene[15, 16, 35, 36]. Haplotype-specific co-ordination between promoter (T-786C) and intronic (4ab) variants of the gene has been shown to regulate its expression pattern *in vitro*[37]. In view of these earlier reports, we performed the test for LD amongst the three variants and did not find any such effect in our study subjects. In addition, haplotype-based analysis for the possible haplotype combinations also did not indicate any association of the gene with the disease phenotype.

In line with earlier observations of a significant disparity in the distribution of *eNOS* variants among various populations[38], the influence of ethnicity on the prevalence of gene polymorphisms was also established by our study. We carried out comparative analysis of the allele frequencies in our control population with the reported frequencies. Marked differences were found in the prevalence of the minor alleles of the three variants when comparing the South Indian population with the documented frequencies in Caucasians, as shown in **Table 6**. There was also a prominent contrast in the frequencies of 4ab and T-786C variants even from other Asians (Japanese, Chinese and Koreans). The higher prevalence of 4ab and T-786C in our population could possibly be associated with the risk of ischemic stroke. This is justified by the observed increased risk for ischemic stroke in women who were carriers of the 4a allele in our study. Although we did not find any association of the T-786C variant with ischemic stroke, a study in a larger sample size would be worthwhile.

In conclusion, we observed a positive association between carriers of the *eNOS* 4a allele and the risk of ischemic stroke in young Indian women. Our study could possibly explain the genetic basis underlying gender differences in ischemic stroke. To the best of our knowledge, this is the first case-control study that has evaluated the risk associated with *eNOS* gene polymorphisms with early-onset ischemic stroke in an Indian population. Larger studies in other ethnic populations are warranted to determine the role of this polymorphism in the etiology of stroke, especially stroke in the young.

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