An exonic G894T variant of endothelial nitric oxide synthase gene as a risk factor for ischemic stroke in North Indians

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INTRODUCTION

Stroke remains the third leading cause of death and the most familiar disease leading to functional impairments in the elderly, throughout the world (Roger et al. 2011). The incidence of stroke is rapidly rising in low and middle-income countries. WHO estimates that by 2050, nearly 80% of stroke cases in world would occur mainly in India and China (World Health Organization 2005).

Of all strokes, 87% are ischemic, 10% are intracerebral and 3% are subarachnoid hemorrhage (Roger et al. 2011). Along with some conventional risk factors such as hypertension, coronary heart disease, diabetes, dyslipidemia, smoking and obesity, common genetic polymorphisms with possible effects on function or protein expression within genes have been projected as possible genetic risk factors, contributing to the heterogeneous phenotypic profile of ischemic stroke. However, the genes concerned with pathogenesis of ischemic stroke are largely understudied (Hassan et al. 2004, Humpheries and Morgan 2004, Dichgans 2007).

eNOS (endothelial nitric oxide synthase) gene located on chromosome 7q35–36 contains 26 exons spanning approximately 21 kb of genomic DNA. It encodes eNOS enzyme playing a crucial role in the availability and exchange rate of nitric oxide (Wang and Wang 2000, Wattanapitayakul et al. 2001). Nitric oxide (NO), an endothelium-derived product of eNOS, is widely known to be a potent vasodilator, antithrombotic, anti-inflammatory, anti-proliferative and regulates blood
flow (Loscalzo 1995, Endres et al. 2004), all these actions being mediated by the activation of soluble guanylate cyclase and consequent increase in the concentration of cyclic GMP in target cells (Waldman and Murad 1988). As per the clinical and experimental data on the pathogenesis of ischemic stroke, endothelial dysfunction due to reduced bioavailability of NO plays a major role in vascular alteration (Yetik-Anacak and Catravas 2006). The clinical trials in eNOS knockout mice suggested that eNOS derived NO is needed for the vascular recruitment during ischemia and experimental inhibition of nitric oxide synthase accelerates the formation of early atherosclerotic lesions (Leeson et al. 2002). Similarly, decline in NO release predisposes humans to stroke, hypertension, thrombosis, vasospasm, and atherosclerosis (Petros et al. 1991, Calver et al. 1994, Cayatte et al. 1994, Markus et al. 2000), while restoration of NO activity induces the regression of preexisting intimal lesions (Cheng et al. 2008).

eNOS is widely studied candidate gene for stroke susceptibility and genetic variations in this could alter the expression and activity of eNOS enzyme, and therefore contribute to the development of ischemic stroke. Among investigated genetic variations in eNOS, G894T and intronic 4ab repeat polymorphisms have received the most attention. G894T variant in exon 7 of the eNOS leads to a change of glutamate to aspartate at site 298 and is said to have increased susceptibility to cleavage of eNOS enzyme (Joshi et al. 2007). The mutant T allele was reported to be associated with ischemic stroke (Berger et al. 2007). The intronic 4ab variant, a 27bp variable number of tandem repeats (VNTR) has been widely studied. A report by Chinese investigators indicated 4a allele of eNOS 4ab polymorphism to be associated with ischemic stroke (Hou et al. 2001). Due to its location in the non-coding region, its chances of being associated with disease traits are minimal. In spite, this intronic variant is shown to genetically contribute to basal levels of plasma NO (Markus et al. 1998). There are limited published reports on the association of G894T and intronic 4ab polymorphism with the risk of ischemic stroke from North India. Hence, our main aim was to investigate the association of eNOS genetic variability with ischemic stroke by assessing the comparative distribution of eNOS variants (G894T, 4ab) in ischemic stroke patients and healthy controls free of neurological diseases.

METHODOLOGY

Punjab with an area of 50,362 km² and population of 277.04 lakhs is situated in North India with population density of 550 per km². The cases for the present study were collected from Uppal Neuro hospital in Amritsar district of Punjab and controls from various areas around Amritsar. Stroke free status for the control population was determined using Questionnaire for Verifying Stroke Free Status (QVSFS) validated by Jones and others (2001). Informed consent from each participant was taken prior to sample collection. Institutional ethical committee of Guru Nanak Dev University (Number 94/HG) approved the study. The conventional risk factors (hypertension, diabetes mellitus, coronary artery disease, alcohol, smoking) were recorded as a part of patient’s clinical history. The examined study group represented mixed population, comprising of Sikhs and Hindus. The average ratio of consanguineous marriages in the studied population is noticed to be zero in detailed pedigree analysis of the subjects. Genomic DNA was extracted from whole blood samples using the standard phenol-chloroform method (Adeli and Ogbonna 1990) and stored at −20°C for further molecular analysis.

GENOTYPIC ANALYSIS

eNOS G894T polymorphism

G894T genetic polymorphism was determined using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique using primer pairs: 5'-AAGGCGAGGAGGATGAGGATGGA-3' (sense) and 5'-CCCAGTCAATCCCTTGGTCTA-3' (anti-
eNOS variants in ischemic stroke

**eNOS variants in ischemic stroke**

For G894T genotypic analysis, the amplified PCR products were analysed on 2.2% ethidium bromide stained agarose gel, followed by digestion of PCR products with restriction enzyme Dpn II according to the manufacturer instructions (New England Biolabs, Beverly, MA). The restriction digestion products were analysed on 2.8% ethidium bromide stained agarose gel. The DNA fragment from TT homozygote was digested into 160 and 88bp fragments, while for undigested GG homozygote, a single 248bp fragment was observed on the gel.

**eNOS 4ab Polymorphism**

eNOS 4ab genetic polymorphism was determined using direct PCR technique with following primer sequences: AGGCCCTATGGTAGTGCCCTTT-3' (sense) and 5'-TCTCTTAGTGCTGTGGTCAC-3' (antisense) with slight modifications for PCR conditions (Wang et al. 1996). The amplified fragments were separated on 2.5% ethidium bromide stained agarose gel. The genotypes to the subjects were assigned on the basis of expected fragments obtained after PCR on 2.3% ethidium bromide stained agarose gel. Three genotypes namely homozygous 4aa (393bp) and 4bb (420bp) and heterozygous 4ab (420bp, 393bp) were observed on gel.

A negative control without DNA template was included in each reaction. The genotyping was done blindly without the knowledge of the clinical status of the subjects. Ten percent of DNA samples were reanalysed and results of both sets were 100% concordant.

**Statistical Analysis**

Continuous variables were expressed as mean and standard deviation. Categorical variables were expressed as percentages. Hardy Weinberg Equilibrium (HWE) was tested by comparing the observed to expected genotype frequencies in cases and controls using Chi-square ($\chi^2$) test. The difference in allelic and genotypic frequency distribution between cases and controls was evaluated through Chi-square ($\chi^2$) test. The relative risk of genotypes and alleles was determined by odds ratio (OR) and 95% confidence intervals (95% CI). The $p$ values were two tailed, and statistical significance was accepted as $p \leq 0.05$. All the statistical values were calculated using SPSS (Version 16, SPSS Inc, Chicago, IL).

**RESULTS**

This case-control study consisted of 120 ischemic stroke patients (61 females and 59 males) and 101 age-matched unrelated healthy control individuals (49 females and 52 males). The mean age was 62.1±11.74 years (range: 35–90 years) for the cases and 58.03±10.75 years (35–85 years) for the controls.

**Association between eNOS polymorphisms and ischemic stroke**

Genotypic analysis of the two polymorphisms (G894T and 4ab) of eNOS is presented in Table I and II respectively. The observed genotype frequencies of G894T and 4ab variants were found to be in Hardy Weinberg equilibrium ($p>0.05$). The alleles 894T and 4a were considered as rare and their relationship with ischemic stroke was evaluated with various genetic models (dominant, co dominant and recessive models).

Table I summarizes the genotypic and allelic distributions of eNOS G894T polymorphism in ischemic stroke patients and controls. Both cases and controls were found to be in Hardy Weinberg equilibrium (cases: $p=0.142$; controls: $p=0.901$). In the present study, 70% of cases with ischemic stroke have homozygous wild type genotype (GG), while 25% and 5% cases were having heterozygous (GT) and homozygous variant (TT) genotype respectively. Among control group, 69.2%, 14.2% and 0.8% showed GG, GT and TT genotypes respectively, with the minor allele frequency being 9.4%. The genotypic distribution of eNOS G894T polymorphism showed significant difference between cases and controls ($\chi^2=5.976$, $p=0.05$) and similarly the difference of allelic distribution between cases and controls was also observed to be significant ($\chi^2=6.04$, $p=0.014$). There was a suggestive evidence of an association of G894T variant in dominant model (GT/TT vs. GG: OR=1.98, 95% CI 1.04–3.76, $p=0.034$) as well as in co dominant model (GT/ GG vs. TT: OR=1.93, 95% CI 1.10–3.39, $p=0.017$) with the occurrence of ischemic stroke among North Indians. The recessive model (GG/GT vs. TT) also indicated weak association of eNOS G894T variant with ischemic stroke (OR=5.26, 95% CI 0.62–44.46, $p=0.072$).
Table II represents the genotypic and allelic distribution of eNOS 4ab polymorphism in ischemic stroke patients and controls. Nearly, 82.5% ischemic stroke patients exhibited 4bb genotype, whereas 23.3% and 4.2% patients were having 4ab and 4aa genotypes respectively. Among controls the genotypic distribution was: 76.2% for 4bb, 20.8% for 4ab and 2.9% for 4aa genotype, with the minor allele frequency being 13.4%. No significant difference was observed between cases and controls with respect to genotypic (p=0.785) and allelic frequency (p=0.465) distribution. Also, none of the genetic models suggested any association of eNOS 4ab polymorphism with ischemic stroke outcome.

To study the association between ischemic stroke and possible combinations of the eNOS polymorphisms (G894T and 4ab), we performed haplotype analysis presented in Table III. The result of haplotype analysis did not reveal any significant risk of ischemic stroke in any combination. Although the proportion of GT-4bb genotype combination was higher in cases (21.7%) as compared to controls (15.8%), the difference between two groups was noted to be statistically non-significant (p=0.13).

In line with observations of a significant disparity in the distribution of eNOS variants among various populations, the influence of ethnicity on the prevalence of gene polymorphisms is observed in our study. We carried out comparative analysis of rare allele frequencies in our control population with the reported frequencies from other ethnicities which indicated marked difference in the prevalence of minor alleles of the two variants, when comparing the North Indian population with the documented frequencies in other ethnicities (Table IV). The T allele frequency of G894T polymorphism in North Indian population was 9.4% that varied extensively from Caucasians (27.4–43.8%) and African-Americans (15–15.5%) However, it was comparable with Japanese (10.2%), Koreans (8.7–9%) and Chinese (7.9–11.49%). There was also a prominent contrast in the frequencies of 4a allele in our healthy controls (13.4%) from African-Americans (27–29.2%), Chinese (7.8–9.6%) and even from South Indians (21.5%), but it was quite similar to Koreans, Japanese and Caucasians.

DISCUSSION

We conducted a case-control study to demonstrate the association of eNOS G894T and intronic 4ab vari-
nants with the risk of ischemic stroke. It was hypothesized that exonic G894T and intronic 4ab polymorphism could have an important impact on ischemic stroke predisposition through different genetic models.

Various investigators around the world have carried out the studies regarding the contribution of eNOS G894T and 4ab polymorphisms to ischemic stroke pathogenesis, but the outcome of these studies remained inconclusive. In the present case-control study, we investigated the genotypic and allelic frequencies of G894T polymorphism and the findings suggested that there are indeed significant differences between cases and controls with regard to genotypic and allelic distribution. Odds ratio suggested that carriage of 894TT variant of eNOS increases the ischemic stroke risk of an individual by 1-fold. 894T allele was found to be associated with increased risk of developing ischemic stroke in North Indians, as the difference of allelic frequency distribution between patients and controls was highly significant (p=0.014). Our data was consistent with the findings of various studies that reported the strong association of G894T variant with ischemic stroke in Bahrain (Saidi et al. 2010) Asian (Wang et al. 2013a, Guo et al. 2014), French Caucasian (Elbaz et al. 2000), Moroccan (Diakite et al. 2014) and Chinese (Lv et al. 2004) populations. The positive association between this polymorphism and cerebral small vessel disease has also been reported by Hassan et al. (2000) and Henskens et al. (2005). Similarly, genotypic analysis in Italian population found G894T polymorphism as an independent risk factor for carotid atherosclerosis (Lembo et al. 2001). Conversely, some studies did not notice any significant difference of allelic and genotypic frequency between ischemic stroke patients and controls (Cheng et al. 2008, Ellul et al. 2011), thus indicating no association between G894T polymorphism and ischemic stroke. Similarly, some studies also indicated lack of contribution of G894T polymorphism to ischemic stroke susceptibility in Singapore (Moe et al. 2008), Turkish (Guldiken et al. 2009), Hungarian (Szolnoki et al. 2005) and in white (Markus et al. 1998) population.

Due to functional relevance of G894T variant and published reports on its association with ischemic stroke, several studies positively correlated G894T polymorphism with various cardiovascular diseases including ischemic heart disease (Casas et al. 2004), coronary artery dysfunction (Nakayama et al. 1999),

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Genotypes n (%)</th>
<th>Alleles n (%)</th>
<th>P value</th>
<th>OR (95% CI)</th>
</tr>
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<tbody>
<tr>
<td>Cases (n=120)</td>
<td>87 (72.5)</td>
<td>28 (23.3)</td>
<td>5 (4.2)</td>
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</tr>
<tr>
<td>Controls (n=101)</td>
<td>77 (76.2)</td>
<td>21 (20.8)</td>
<td>3 (2.9)</td>
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<table>
<thead>
<tr>
<th>Study Group</th>
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<th>P value</th>
<th>OR (95% CI)</th>
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<tr>
<td>Cases (n=120)</td>
<td>87 (72.5)</td>
<td>28 (23.3)</td>
<td>5 (4.2)</td>
</tr>
<tr>
<td>Controls (n=101)</td>
<td>77 (76.2)</td>
<td>21 (20.8)</td>
<td>3 (2.9)</td>
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<table>
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<tr>
<th>Study Group</th>
<th>Co-dominant model (4ab vs 4aa or 4ab vs 4bb)</th>
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<th>OR (95% CI)</th>
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<td>Cases (n=120)</td>
<td>87 (72.5)</td>
<td>28 (23.3)</td>
<td>5 (4.2)</td>
</tr>
<tr>
<td>Controls (n=101)</td>
<td>77 (76.2)</td>
<td>21 (20.8)</td>
<td>3 (2.9)</td>
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<tr>
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<th>Recessive model (4bb/4ab vs. 4aa)</th>
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<th>OR (95% CI)</th>
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<td>28 (23.3)</td>
<td>5 (4.2)</td>
</tr>
<tr>
<td>Controls (n=101)</td>
<td>77 (76.2)</td>
<td>21 (20.8)</td>
<td>3 (2.9)</td>
</tr>
</tbody>
</table>

n – number of subjects, Figs in parentheses represents frequency of each genotype; OR – odds ratio; CI – confidence interval; *p≤0.05 was considered significant.
hypertension (Periaswamy et al. 2008, Zhao et al. 2008) and ischemic stroke (Diakite et al. 2014). The functional consequence of G894T polymorphism has been suggested to be due to the observed genotype-driven alterations in basal and shear induced activation, altered localization of protein at caveolae and consequential impairment in the co-ordination of the regulatory cycle of eNOS enzyme (Joshi et al. 2007). The subjects with TT genotype of G894T variant have been reported to have low levels of NOX as compared to individuals with GT and GG genotypes (Akhter et al. 2014). Furthermore, Senthil and coworkers (2005) found that endothelial cells separated from patients and having TT of G894T or 4aa of intronic 4ab polymorphism expressed higher levels of eNOS mRNA, while lower level of eNOS protein, compared with GG or 4bb genotype. Similarly, a study conducted by Saini et al. (2011) on 60 patients of CAD also indicated that patients had significantly lower levels of NO compared with controls in all G894T genotypes; however, patients with 894GG genotypes had significantly higher levels of NO as compared to patients with 894GT genotypes. Also, the findings by Metzger and others (2005, 2007, 2011) in previous studies suggested that eNOS variants combined within a specific haplotype, significantly affected NO formation, thus considerably impacting in vivo NO bioavailability in healthy white and black subjects. These evidences suggested that accelerated degradation of eNOS is an important mechanism for G894T and VNTR polymorphism to influence eNOS activity.

The relationship of G894T polymorphism with ischemic stroke was further assessed through three genetic models (dominant, co-dominant and recessive). On analyzing these genetic models, dominant and co-dominant models revealed the significant association of G894T polymorphism with ischemic stroke, reflecting 1.98 and 1.93 fold increased risk of an individual predisposition to ischemic stroke respectively (p<0.05 for both). The recessive genetic model also deciphered some extent of association of G894T variant with ischemic stroke, but that was noted to be weak association (p=0.07). Thus, our study was in agreement with the reports published by Saidi and colleagues (2010) and Tao and Chen (2009) in which all the three models might act as a contributing factor in pathogenesis of ischemic stroke. Likewise, the two independent case-control studies in German population (Berger et al. 2007) and the comprehensive meta-analysis in Asians concluded that dominant and co-dominant models were associated with elevated risk of

<table>
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<tr>
<th>G894T-4ab</th>
<th>No. of patients n (%)</th>
<th>No. of controls n (%)</th>
<th>OR (95% CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG – 4bb</td>
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<td>60 (59.4)</td>
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<tr>
<td>GG – 4ab</td>
<td>23 (19.2)</td>
<td>20 (1.8)</td>
<td>1.23 (0.61–2.48)</td>
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</tr>
<tr>
<td>GG – 4aa</td>
<td>5 (4.2)</td>
<td>3 (2.9)</td>
<td>NC</td>
<td>NC</td>
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<tr>
<td>GT – 4bb</td>
<td>26 (21.7)</td>
<td>16 (15.8)</td>
<td>1.74 (0.84–3.58)</td>
<td>0.13</td>
</tr>
<tr>
<td>GT – 4ab</td>
<td>4 (3.3)</td>
<td>1 (0.9)</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>TT – 4bb</td>
<td>5 (4.2)</td>
<td>1 (0.9)</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>TT – 4ab</td>
<td>1 (0.8)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

n – number of subjects, Figs in parentheses represents frequency of each genotype; OR – odds ratio; CI – confidence interval; NC – not calculated
ischemic stroke for G894T polymorphism but not with recessive models and these results remained same in Chinese even after sub-group analysis (Wang et al. 2013b).

For eNOS 4ab intronic variant, the proportion of 4aa variant genotype in cases (4.2%) and controls (2.9%) was comparable; hence no correlation was observed between intronic 4ab polymorphism and ischemic stroke susceptibility, as presented in Table III. Similar observations have been made by several studies conducted across different populations that also reported no association between intronic 4ab polymorphism and ischemic stroke (Markus et al. 1998, Grewal et al. 2007, Moe et al. 2008, Tao and Chen 2009).

The polymorphisms in the non-coding region of eNOS could also play a role in regulation of gene expression. Intronic 4ab polymorphism in non-coding region may also be involved in increased disease risk or could be a marker in linkage disequilibrium with relevant functional changes. It was reported that intronic 4ab eNOS locus has a substantial effect on the variance of plasma NO levels (Wang et al. 1996). Lower NO plasma levels were demonstrated for 4a allele carriers in Asians and African-Americans (Yoon et al. 2000, Salimi et al. 2008) and eNOS 4ab polymorphism has been correlated with ischemic stroke in some American, African and Asian studies (Yahashi et al. 1998, Hou et al. 2001, Howard et al. 2005, Majumdar et al. 2010, Munshi et al. 2010, Saidi et al. 2010). However, the results are still indecisive regarding the contribution of intronic 4ab variant in ischemic stroke predisposition. Various reports have observed the high fold increased risk in 4a allele carriers compared to individuals carrying 4b allele (Hou et al. 2001, Pu and Tao 2003, Zhang 2004, Guo 2014). On the contrary, Shi and others (2008) correlated the eNOS 4bb genotype with increased risk of ischemic stroke.

In genetic studies, haplotype analysis has been viewed as powerful strategy, eliminating the inconsistencies commonly found in studies analyzing single polymorphisms. Likewise, studies have reported the significant contribution of eNOS haplotypes to variation in NO formation, as C-4b-Glu haplotypes have been found to be more prevalent in subjects with low circulating concentrations of nitric oxide products (Metzger et al. 2005, 2007, 2011). Since, the present study did not evaluate the nitrite and nitrate concentrations in subjects, we could not perform such analysis. Conversely, haplotype based analysis of possible geno-
type combinations in the present study did not reveal any significant association of gene variants with ischemic stroke outcome.

The prevalence of eNOS polymorphisms has been established for Caucasians, African-Americans and Asian populations. However, limited information is available on Indian population. On performing comparative analysis of allele frequencies in our control population with the reported frequencies from other ethnicities, we observed marked variation in the prevalence of minor alleles of the two variants in North Indian population. The prevalence of 894T allele is low in Japanese and African-Americans than in Caucasians (27.4–43.8%). However, the frequency of 894T allele in our control population was closer to Asians (Japanese, Koreans and Chinese). Among Asians, 894T allele has been known to increase the risk of ischemic stroke (Wang et al. 2013b). Likewise, 894T allele in North Indian population was found to be associated with ischemic stroke. There was also a prominent contrast of 4a allele frequency in the studied population from African-Americans and even from South Indians. Among South Indians females, 4a allele has been reported to confer the increased risk of ischemic stroke (Majumdar et al. 2010).

There are number of drugs that may interact with eNOS polymorphisms and significantly affect the drug response and modify clinical outcomes. However, the present study could not carry out such interactive analysis, due to non-availability of sufficient data on usage of drugs by patients. There is substantial evidence that many of the therapeutic approaches to the treatment of stroke, such as statins, rosiglitazone etc. exert their effect by increasing eNOS stimulation and increased expression of eNOS (Endres et al. 2004, Laufs et al. 2000). The pleiotropic and beneficial effects of these drugs have been observed to be modulated by single nucleotide polymorphisms in eNOS gene. Previous in vitro and pharmacogenetic studies showed that T-786 C polymorphism in eNOS modulates the effects of atrovastatin on NO bioavailability and oxidative stress (Nagassaki et al. 2006). Similarly, the results from a clinical study suggested that eNOS polymorphisms apparently contribute to better responses to enalapril in hypertensives (Silva et al. 2013).

Though the data is available on various aspects of the development of stroke in different ethnic populations, the present study adds to current existing knowledge. However, our study has some limitations. The primary one is that the study lacked multivariate regression analysis to investigate the interaction of other independent variables with mutant alleles of studied eNOS gene variants. The analysis is being done including another widely studied T-786C polymorphism in promoter region of eNOS to be published later. The sample size of the study was small to reach the high statistical power. Also, the study did not evaluate NOx levels among patients and controls to correlate with G894T and 4ab polymorphism and to explore the functional impact of these eNOS variants. The patients were mainly elderly, representing only a particular age group of the population. Apart from the above mentioned eNOS variants, it is possible that multiple rare eNOS mutations might influence endothelial function measures.

CONCLUSION

The case-control association studies are powerful approaches to study the genetic basis of complex disorders like stroke, as they do not require family-based collections which is a challenging task considering the late-onset of diseases. The present study shows an impact of eNOS G894T polymorphism on ischemic stroke predisposition in North Indian population. However, no influence of intronic 4ab polymorphism was observed on ischemic stroke outcome. Furthermore, studies with large sample size are required to establish an effective association of these variants with ischemic stroke and these genetic effects in ischemic stroke pathogenesis are needed to be studied in conjunction with functional effect of these variants.

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REFERENCES


