

# A genetic study of the NOS3 gene for ischemic stroke in a Chinese population

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**Abstract:** We recruited 560 unrelated patients with ischemic stroke and 153 unrelated controls to undertake a genetic analysis for association between the NOS3 gene and ischemic stroke. All the subjects were Chinese of Han descent. Because the NOS3 gene spans about 21 kb of DNA and contains 26 exons, we selected a single nucleotide polymorphism (SNP) rs3918181, an A to G base change located in intron 14 of the gene, as a DNA marker. PCR-based restriction fragment length polymorphism analysis was applied to genotype rs3918181 (RsaI site). The chi-square ( $\chi^2$ ) goodness-of-fit test showed that the genotypic distributions of the marker were not deviated from Hardy-Weinberg equilibrium in both the patient group ( $\chi^2 = 0.166$ ,  $p = 0.684$ ) and the control group ( $\chi^2 = 0.421$ ,  $p = 0.517$ ). The cocophase analysis showed allelic association of rs3918181 with ischemic stroke in males ( $\chi^2 = 4.04$ ,  $p = 0.044$ , OR = 1.43, 95% CI 1.01–2.02) and frequency of allele A was significantly higher in male patients than male control subjects. The  $\chi^2$  test revealed genotypic association between rs3918181 and ischemic stroke in males ( $\chi^2 = 4.26$ ,  $df = 1$ ,  $p = 0.039$ , OR = 1.61, 95% CI 1.02–2.53) but not in females. The present work suggests that rs3918181 is associated with ischemic stroke in male patients. This finding gives further evidence in support of the eNOS association with ischemic stroke in the Chinese population.

**Keywords:** eNOS, gene, single nucleotide polymorphisms (SNPs), ischemic stroke

## Introduction

Nitric oxide (NO) is known to have several important vasculo-protective effects (El-Mas et al 1997; Grdal et al 2005). Reduced production of NO may lead to atherosclerosis (Lscher and Vanhoutte 1990; Harrison 1997; Shimokawa 1999). The synthesis of NO from the amino acid, L-arginine, is catalysed by NO synthase (NOS). NO then plays a critical role in the relaxation of vascular smooth muscle cells (VSMCs). It is well known that NO is involved in reducing VSMC proliferation, adhesion of platelets and leukocytes (Moncada and Higgs 1993; Draijer et al 1995; Myers and Tanner 1998; Brocq et al 2008). NO is likely, therefore, to be an important contributory factor in the pathogenesis of some forms of stroke.

There are 3 distinct isoforms of NOS identified in humans: endothelial (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). eNOS is a highly regulated isoform that is expressed primarily in endothelial cells. It synthesizes very low levels NO under basal conditions (Lscher and Vanhoutte 1990; Harrison 1997; Shimokawa 1999). Because eNOS plays a central role in the maintenance of vascular homeostasis, including regulation of the cerebral circulation, a number of studies have been conducted to investigate the genetic association between the eNOS gene and the incidence of ischemic stroke. Howard and colleagues (2005) detected 3 single nucleotide polymorphisms (SNPs) in the promoter region, 1 in exon 7 (G894T) and 1 insertion/deletion polymorphism within intron 4 among 110 cases with ischemic stroke and 206 controls. They found strong associations of both the –922 G–A and –786 T–C SNPs with ischemic stroke.

Hou and colleagues (2001) were the first to report the genetic association between the eNOS gene and ischemic stroke in a Chinese population and a recent work has confirmed the initial finding (Berger et al 2007) although the eNOS association failed to be replicated in a Japanese population (Yahashi et al 1998). This study might have been hampered by poor replication, a common problem with genetic analysis. Linkage disequilibrium (LD) structure and sample power may be one of major reasons for poor replication of an initial finding. Therefore, the present study was designed to apply a large sample size to investigate genetic association between the eNOS gene and ischemic stroke.

## Materials and methods

The present study recruited 560 unrelated patients with ischemic stroke and 153 unrelated controls in order to undertake a genetic analysis for association between the NOS3 gene and ischemic stroke. All the subjects used for this study were Chinese of Han descent. Patients with ischemic stroke were admitted to the First Hospital of Jilin University, Changchun, China in the period between 2005 and 2006. They were diagnosed as having ischemic stroke independently by at least two well-trained neurologists based on strict neurological examination, computed tomography, or magnetic resonance imaging, which meet the *International Classification of Diseases* (NCHS 2007). Detailed information regarding their medical history, medication and clinical presentation was taken through either their close relatives or themselves. Of these 560 patients (398 males and 162 females), 426 were suffering from simple lacunar infarction, 246 from large-vessel infarction and 112 from both. Those with hemorrhagic stroke were not included in this study. The general information of the subjects recruited is given in Table 1.

All the subjects gave written informed consent for the genetic analysis. The whole blood sample was taken from them for extraction of genomic DNA. The study was granted

prior approval by the ethics committee of Jilin University, Changchun, China.

To perform genetic analysis, we selected SNP rs3918181 (Rsa I site) as a genetic marker at the NOS3 locus. It is an A to G base change present in intron 14 of the gene. Polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) analysis was performed to genotype rs3918181. Genomic DNA used for PCR amplification was extracted from the whole blood sample using a DNA extraction kit (Promega, Beijing, China). PCR amplification was performed in a 25- $\mu$ l reaction volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.001% (w/v) gelatin, 200  $\mu$ M of each dNTP, 0.4  $\mu$ M of each primer, 1.0 unit of Taq DNA polymerase (Promega, Beijing, China), and 30–50 ng of genomic DNA. The conditions used for PCR amplification included an initial denaturation at 94 °C for 5 min, followed by 35–40 cycles of 94 °C for 45s, 55–60 °C for 1 min and 72 °C for 1 min, and a final elongation at 72 °C for 10 min. A 15- $\mu$ l aliquot of the PCR products was completely digested with 6–8 units of restriction enzymes. The digested PCR products were then separated on ethidium bromide-stained agarose gels.

The Hardy-Weinberg equilibrium for the genotypic distributions of rs3918181 was tested using the chi-square ( $\chi^2$ ) goodness-of-fit test. Applied to test sample power for individual alleles was tested using the SPSS SamplePower 2.0 software package (SPSS Inc., Chicago, IL, USA). The significance level was set at 5% (two-tailed). Allelic association was tested by the COCAPHASE module of the UNPHASED program (Dudbridge 2003) and genotypic association was tested by the SPSS program for windows 15.0 (SPSS Inc.).

## Results

The  $\chi^2$  goodness-of-fit test showed that the genotypic distributions of rs3918181 were not deviated from Hardy-Weinberg

**Table 1** The general information of patients and controls

	Patients with ischemic stroke			
	Controls (n = 153)	Large-vessel (n = 246)	Lacunar (n = 426)	Mixed (n = 112)
Age (y)	55.6 $\pm$ 5.7	58.2 $\pm$ 12.4	60.5 $\pm$ 10.8	60.4 $\pm$ 11.5
Men	93	170	303	75
BMI (kg/m <sup>2</sup> )	24.9 $\pm$ 3.3	24.3 $\pm$ 3.2	25.1 $\pm$ 3.6	24.8 $\pm$ 3.6
History of hypertension	45	152	301	79
History of diabetes	16	44	80	21
History of heart disease	19	32	60	12
History of smoking	22	86	141	36

equilibrium in both the patient group ( $\chi^2 = 0.166$ ,  $p = 0.684$ ) and the control group ( $\chi^2 = 0.421$ ,  $p = 0.517$ ). These 560 case and 153 control samples gave a power of 60% for detection of small effect size and 100% for detection of medium effect size. Analysis with the COCAPHASE program showed that rs3918181 was associated with ischemic stroke in males ( $\chi^2 = 4.04$ ,  $p = 0.044$ , OR = 1.43, 95%CI 1.01~2.02) and frequency of allele A of rs3918181 was significantly higher in male patients than male control subjects (Table 2). However, female patients did not show such an association (Table 2). As shown in Table 3, the  $\chi^2$  test revealed genotypic association between rs3918181 and ischemic stroke in males ( $X^2 = 4.26$ ,  $df = 1$ ,  $p = 0.039$ , OR = 1.61, 95% CI 1.02~2.53) but not the genotypic association was not observed in female subjects.

## Discussion

The gene coding for eNOS is mapped to the long arm of chromosome 7 (7q36) and spans ~21 kb of DNA, although it contains 26 exons. It should be sufficient to capture a linkage disequilibrium (LD) signal by genotyping a highly polymorphic DNA marker present in the middle of the gene. SNP rs3918181 is located in intron 14 of the gene and has heterozygosity of 0.43. It may be an ideal DNA marker for association studies in a Chinese population.

The present work showed that rs3918181 was associated with ischemic stroke in male patients. This finding gives further evidence in support of the eNOS association with ischemic stroke in the Chinese population, although female patients did not show such an association in our samples (Tables 2 and 3). It is worth investigating the gender differences with more samples in a further study.

We recruited a total of 153 control subjects in this study. The sample power test indicates that the study is

**Table 2** Allelic association of the NOS3 gene with ischemic stroke

Group	Allele	Patients (%)	Controls (%)	$X^2$	P-value
Male					
	A	279 (35.1)	51 (27.4)	4.04	0.044 <sup>a</sup>
	G	517 (64.9)	135 (72.6)		
Female					
	A	107 (33.0)	36 (30.0)	0.37	0.543
	G	217 (67.0)	84 (70.0)		
Total					
	A	386 (34.5)	87 (28.4)	4.02	0.045 <sup>b</sup>
	G	734 (65.5)	219 (71.6)		

Notes: <sup>a</sup>OR = 1.428 (95% CI 1.009~2.021); <sup>b</sup>OR = 1.324 (95% CI 1.006~1.742).

**Table 3** Genotypic association of the NOS3 gene with ischemic stroke

Group	Genotype	Patients (%)	Controls (%)	$X^2$	P-value
Male					
	AA + AG	231 (58.1)	43 (46.2)	4.25	0.039 <sup>a</sup>
	GG	167 (42.0)	50 (53.8)		
Female					
	AA + AG	90 (55.6)	30 (50.0)	0.54	0.461
	GG	72 (44.4)	30 (50.0)		
Total					
	AA + AG	321 (57.3)	73 (47.7)	4.49	0.034 <sup>b</sup>
	GG	239 (42.7)	80 (52.3)		

Notes: <sup>a</sup>OR = 1.608 (95% CI 1.022~2.532); <sup>b</sup>OR = 1.472 (95% CI 1.028~2.107).

not sufficiently powered at present for detection of small effect-size gene. However, we will continue to collect controls matched for age, sex and ethnicity in order to confirm the results and 80% of power will be needed for combined samples in our future study.

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