

# C242T polymorphism of P22phox gene of the nox: a putative pathological risk factor for stroke in Kashmiri population

## Abstract

**Background and purpose:** NADPH oxidase (Nox) is a multicomponent enzyme responsible for the generation of reactive oxygen species (ROS). The *p22phox* is a key component of the cytochrome b558 of Nox and is essential for the activation of this enzyme, which by generating reactive oxygen species (ROS) is involved in the pathogenesis of many inflammatory diseases. The Nox-mediated excessive ROS production can damage tissue and represents an important cause of injury in many chronic inflammatory disorders including rheumatoid arthritis, atherosclerosis, lung injury and inflammation-associated cancer. An intensive expression of the *p22phox* has been reported in human atherosclerotic arteries. However, studies on the association of the C242T polymorphism in the *p22phox* gene with cerebrovascular disease (CVD) have produced conflicting results, and the relation of this polymorphism with CVD is not well known in a population with acquired risk factors enhancing the Nox-dependent superoxide production. We investigated the relevance of C242T polymorphism of *p22phox* gene to stroke, the maiden study directed in ethnic Kashmiri subjects.

**Methods:** The study included 100 cases suffering from stroke (both ischemic and hemorrhagic stroke) and 120 controls. DNA was isolated from peripheral leukocytes and the genomic analysis of C242T polymorphism was done by PCR-RFLP.

**Results:** We found T allele frequency of 38% in cases and 14% in controls ( $p < 0.0001$ ). Hemorrhagic stroke T allele frequency was 41% ( $p < 0.0001$ ) and Ischemic stroke T allele frequency was 34% ( $p < 0.0001$ ).

**Conclusion:** The presence of T allele was associated with higher risk of stroke. The prevalence of T allele was significantly higher in stroke patients ( $p < 0.0001$ ) irrespective of type which demonstrates that C242T polymorphism of *p22phox* gene plays an important role in the stroke in Kashmiri population.

**Keywords:** *p22phox* gene, Stroke, NADPH oxidase, C242T polymorphism

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**Abbreviations:** CVA, Cerebro Vascular Accident; ROS, Reactive Oxygen Species; VSMCs, Vascular Smooth Muscle Cells; SKIMS, Sher-I-Kashmir Institute of Medical Sciences

## Introduction

Stroke is a global health problem; it is the second commonest cause of death and fourth leading cause of disability worldwide.<sup>1</sup> In developed countries, stroke is the first leading cause for disability, second leading cause of dementia and third leading cause of death.<sup>2</sup> According to a worldwide assessment, roughly 2 crore people each year will suffer from stroke and of these 50 lakh will not survive.<sup>3</sup> Out of this, low and middle-income countries account for 85.5% of total stroke deaths worldwide and the number of disability-adjusted life years in these countries is projected to be seven times that in high-income countries.<sup>4</sup> In India, the ICMR evaluations put the stroke contributed deaths at 41% and disability adjusted life years amongst the non-communicable diseases at 72%.<sup>5</sup> Overall in India, the adjusted annual incidence per 100,000 persons of stroke is 124 in rural area<sup>6</sup> and 145 in urban area.<sup>7</sup> Reliable morbidity and mortality estimates for stroke from the North-Indian state of Jammu & Kashmir are limited and are confined to studies that suffer from frequent bias, small and variable sample sizes, and inconsistent diagnostic criteria. Nevertheless, the only documented prevalence rate reported that the crude and age adjusted prevalence rate was 143 and 244 per 100,000 of population.<sup>8</sup>

A stroke, or cerebrovascular accident (CVA), is a clinical syndrome with the rapid loss of brain function due to disturbance in the blood supply to the brain. The WHO clinically defines stroke as 'the rapid development of clinical signs and symptoms of a focal neurological disturbance lasting more than 24 hours or leading to death with no apparent cause other than vascular origin'.<sup>9</sup> Based on its pathophysiology, it can be due to ischemia (lack of blood flow) caused by blockage (thrombosis, arterial embolism), or a hemorrhage caused either by subarachnoid bleeding from one of the brain's arteries into the brain tissue or intra-cerebral arterial bleeding in the space between meninges. Ischemic stroke account for 50%-85% while hemorrhagic strokes comprise of 1%-7% due to subarachnoid bleeding and 7%-27% due to intra-cerebral arterial bleeding of all strokes worldwide.<sup>10</sup> The effects of a stroke depend on the site and severity of brain injury. A very severe stroke can cause sudden death.

NADPH oxidase/Nox is a multicomponent enzyme responsible for the generation of reactive oxygen species (ROS); the catalytic core-Nox2 of the NADPH oxidase first identified in phagocytes along with several homologues (Nox1 to Nox5, Duox1 and Duox2) characterized in various non-phagocytic cells have distinct features in tissue distribution, expression regulation, and physiological functions. In the resting state, Nox2 associated *p22phox* to the membrane; both proteins constituting the cytochrome b558. Upon stimulation, the cytosolic components (p47phox, p67phox, p40phox and Rac1/ 2) of the NADPH oxidase are recruited to the membrane where they assemble with the cytochrome b558 to form the active enzyme.<sup>11</sup>

Oxidative stress plays critical roles in cardiovascular physiology and in the pathogenesis of vascular disease.<sup>12</sup> Vascular and cardiac tissues are rich sources of reactive oxygen species (ROS), including superoxide (O<sub>2</sub><sup>-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and nitric oxide (NO<sub>2</sub>). Virtually every cell type in the vascular wall has been shown to produce and be regulated by ROS.<sup>13,14</sup> The ischemic brain reperfusion produces a massive increase in oxygen free radicals triggering exacerbate ischemic injury. Initial work identified xanthenes oxidase as a prime source of endothelium-dependent O<sub>2</sub>-production.<sup>15,16</sup> Superoxide dismutase and catalase- the antioxidant enzymes shrink ischemic damage as over expression of superoxide dismutase in transgenic mice are shown to extend protection to neurons from ischemic/ reperfusion injury.<sup>17-20</sup>

While there are diverse sources of free radicals generation, numerous distinct oxygen species from neutrophils and phagocytes that are observed in ischemic brain tissue, are primarily able to produce oxygen free radicals and thus have been associated with ischemic injury.<sup>21</sup> NADPH oxidase-mediated ROS production are involved not only in the killing of invading microorganisms but are also used as a second messenger for signal transduction in the endothelium and plays a major role in mediating ischemic injury in the brain.<sup>22</sup> However, an excessive ROS production can damage tissue and represents an important cause of injury in many chronic inflammatory disorders. Given the importance of the Nox in the pathophysiology of inflammatory diseases, it appears that its activity needs to be firmly controlled by different mechanisms that ensure an appropriate physiological regulation.

NAD(P)H oxidase is present in vascular smooth muscle cells (VSMCs) and endothelial cells. A component of Nox, *p22phox* is expressed in VSMCs and serves as a critical component of regulating vascular hypertrophy,<sup>23</sup> and seems to be a major source of superoxide production in animal models of vascular disease<sup>24</sup> and in human atherosclerosis.<sup>25,26</sup> The *p22phox* subunit of the Nox multi-subunit enzyme complex is required for oxidase activity in smooth muscle cells, and is expressed in human coronary arteries.<sup>27</sup> Much interest has been focused on the possible association of polymorphisms in the *CYBA* gene, which encodes *p22phox*, with atherosclerosis.<sup>28</sup> A putatively functional polymorphism of the *CYBA* gene encoding the *p22phox* subunit of Nox has been described; the C242T variant results in the substitution of tyrosine for histidine within one of the two haem-binding sites that are thought to be essential for the stability of the protein.<sup>29</sup>

Ethnicity is an important determinant of genetic polymorphisms. Distribution of allelic variation in the gene for *p22phox* has been established for several ethnic groups in the world. The present study was sought to study the distribution of *p22phox* C242T polymorphism and its relevance to stroke in a community based sample drawn from ethnic north Indian population of Kashmir valley.

## Materials and methods

### Subject characteristics

The present study was conducted at Sher-i-Kashmir Institute of Medical Sciences (SKIMS), Srinagar. The study was approved by Institutional Ethics committee and written consent was taken from each patient. The participants included a total of 220 ethnic Kashmiri subjects comprising of 100 diagnosed stroke patients (without any modifiable risk factor) and 120 age and gender matched controls. One hundred above-mentioned stroke patients of both genders of age >45 years were enlisted for the present study except those with presence or history of hypertension, diabetes mellitus, hyperlipidaemia,

smoking, known or suspected coronary/ peripheral vascular disease, any significant chronic medical or surgical condition any acute illness within previous two weeks, atrial fibrillation or significant carotid stenosis. Routine laboratory investigations like complete haemogram with ESR, serum chemistry including plasma glucose, complete lipid profile, KFT, LFT, serum uric acid were measured on Olympus AU640 automated analyser. Electrocardiogram, Chest Roentgenogram, Neck Vessel Doppler and Echocardiography were also done.

### Genotyping analysis

5ml of peripheral blood was obtained from each subject and total genomic DNA was extracted using Phenol-Chloroform method. The 353-bp target region of *p22phox* gene was amplified by polymerase chain reaction with sense primer 5'-TGCTTGTGGGTAACCAAGG-3' and antisense primer 5'-GGAAAACACTGAGGT AAGTG-3' in a 25µl reaction volume. The PCR reaction consisted of an initial denaturation step of 2 minute at 95 °C, followed by 35 cycles of 3 minutes at 95 °C (denaturation), 1 minute at 56 °C (annealing) and 7 minutes at 72 °C (extension) and a final extension of 7 minutes at 72 °C. The 353 bp PCR product was cleaved with 1µl Rsa I restriction enzyme for 18-24 hrs at 37 °C. The restriction fragments were separated on a 2.5% agarose gel and visualized under a gel-documentation system. Digestion of the amplicon yield bands of 353bp in CC homozygous, 193bp & 160bp bands in TT homozygous and all the three 353bp, 193bp and 160bp in CT heterozygous.

### Statistical analysis

Statistical Package for Social Sciences (SPSS), version 16.0 for Windows (Chicago Illinois, USA) was used for data analysis. Descriptive statistics are presented as Mean±SD or frequency unless indicated otherwise. Relevant statistical details like Chi-square trend analysis, p values and Cramer's V tests have been included. p value of <0.05 was taken as the criterion of statistical significance.

## Results

The participants in this study included a total of 220 subjects. One hundred patients suffering from stroke (both Ischemic and Hemorrhagic stroke) and 120 healthy age/ gender matched controls. Mean age of the subjects was 55.18±6.825. In our study, patients with stroke had higher values of ESR (p<0.001) and SGOT (p=0.003), while as lower Na<sup>+</sup> (p=0.018) and K<sup>+</sup> (p=0.003) levels. The T allele frequency was 0.14 in controls and 0.38 in cases (Table 1). CC genotype was present in 96% of controls and 35% of cases. We studied allelic variation in both Hemorrhagic as well as Ischemic stroke patients. Hemorrhagic subjects exhibited higher frequency of T allele (0.41) in comparison to Ischemic subjects (0.34).

## Discussion

This is the first documented report of allelic variation in *p22phox* component of superoxide generating enzyme- Nox in the community based sample from Indian subcontinent. In the present study, presence of T allele was associated with higher risk of both hemorrhagic and ischemic stroke. The genotype frequency of T allele in our population is higher than Japanese and Chinese populations<sup>29-31</sup> but similar to that reported in various other populations.<sup>25,32</sup> The baseline characteristics of subjects were similar between the genotypes (CC, CT and TT) except ESR and Na<sup>+</sup> levels (Table 2). Trend towards higher ESR was seen both in hemorrhagic stroke (pp<0.001) and Ischemic stroke (p<0.001). This finding is consistent with previous study showing relation between ESR and risk of developing coronary heart disease.<sup>33</sup> Trend towards higher SGOT was also observed in both

hemorrhagic stroke ( $p=0.003$ ) and Ischemic stroke ( $pp<0.015$ ). The presence of C allele has been shown to have a protective effect against stroke in our population. Presence of T allele correlates directly with both hemorrhagic and ischemic stroke in our population. Our findings depict substantial statistical significant than the study on Japanese stroke patients<sup>34</sup> and/ or various genetic studies conducted on ischemic stroke patients in India.<sup>35,36</sup> Our study is indicative of a role of T allele in the pathogenesis of stroke in ethnic Kashmiri population of north India. Further, this is the first study which has explored allelic variations in the gene for *p22phox* component of the superoxide-

generating enzyme after excluding all modifiable risk factors for stroke patients. However, higher sample size needs to be studied in order to further corroborate our results. Also, more SNPs need to be evaluated to rule out the possibility of linkage equilibrium between these SNPs and one or more other functional polymorphisms. On the other hand, the present study avoids several limitations that might have affected the interpretation of many of the previous studies as we excluded all modifiable risk factors for hemorrhagic and ischemic stroke and also excluded stroke in young in which other factors could have convoluted their findings.

**Table 1** Genotype and allele frequencies of p22PHOX (C>T) polymorphism in stroke patients and controls

Subjects (n)	CC (n) %	CT (n) %	TT (n) %	P value ( $\chi^2$ -trend) (Cramer's V)	T allele Frequency
Controls (120)	96 (80)	14 (11.66)	10 (8.33)	<0.0001 (49.88)[0.476]	0.14
Cases (100)	35 (35)	53 (53)	12 (12)		0.38
Ischemic (43)	18 (41.86)	20 (46.51)	5 (11.62)	<0.0001(25.38)[0.395]	0.34
Hemorrhagic (57)	17 (29.82)	33 (57.89)	7 (12.28)	<0.0001(46.97)[0.515]	0.41

**Table 2** Clinical features among subjects with different genotypes

Variable	CC	CT	TT	P value
Age	54.32±5.68	55.63±8.53	58.43±2.37	0.28
Hb	13.03±1.12	12.66±1.51	13.77±0.73	0.08
HCT	41.21±5.89	38.71±9.03	41.34±3.06	0.23
ESR	12.34±10.60	21.35±13.20	25±10.73	0.000
SGOT	33.30±20.70	36.25±12.89	31.14±5.87	0.63
Na <sup>+</sup>	136.24±4.38	134.22±4.97	132.36±4.23	0.03
K <sup>+</sup>	4.82±0.36	3.34±0.47	3.52±0.37	0.3

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## Conflicts of interest

None.

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