C242T Polymorphism of NADPH Oxidase p22 PHOX Gene and Ischemic Cerebrovascular Disease in the Japanese Population

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Background and Purpose—Superoxide has been implicated in the pathogenesis of ischemic stroke and atherosclerosis. NADPH oxidase, a major source of superoxide generation in neutrophils and the vascular system, plays a critical role in ischemic injury and atherogenesis. Recently, an association between the C242T polymorphism of p22 PHOX, an essential component of NADPH oxidase, and coronary artery disease (CAD) has been reported in several studies. To investigate the relationship between the C242T polymorphism of p22 PHOX and ischemic cerebrovascular disease (CVD), we conducted a case-control study.

Methods—We recruited 226 CVD patients (atherothrombotic infarction, lacunar infarction, and transient ischemic attack) and 301 control subjects and analyzed C242T polymorphism of p22 PHOX by detection of restriction fragment length polymorphism.

Results—The TC TT genotype frequencies in the CVD group and control group were 21.7% and 13.3%, respectively, and the prevalence of the TC TT genotype was significantly higher in the CVD patients ($\chi^2 = 6.477, P = 0.01, \text{OR } 1.81, 95\% \text{ CI } 1.15 \text{ to } 2.86$). Analysis by CVD subtypes showed that the OR for the TC TT genotype was higher in the CVD patients with atherothrombotic infarction than in those with lacunar infarction and transient ischemic attack.

Conclusions—The C242T polymorphism of the NADPH oxidase p22 PHOX gene is a novel pathogenetic risk factor for CVD. (Stroke. 2000;31:936-939.)

Key Words: cerebrovascular disorders ■ oxygen radical ■ polymorphism (genetics) ■ risk factors ■ stroke, ischemic

It is now widely accepted that oxygen free radicals contribute to the pathogenesis of ischemic cerebrovascular disease.1–4 Reperfusion of the ischemic brain results in a massive increase in oxygen free radicals and can exacerbate ischemic injury. The antioxidant enzymes superoxide dismutase and catalase reduce ischemic damage, and overexpression of superoxide dismutase in transgenic mice protects neurons from ischemic/reperfusion injury.1–4 Although several distinct oxygen free radicals can be generated from a number of different sources, neutrophils and phagocytes, which are observed in ischemic brain tissue, are particularly able to produce oxygen free radicals and have thus been implicated in ischemic injury.5 Recently, the NADPH oxidase system was reported to be essential for superoxide formation in neutrophils and phagocytes and to play a major role in mediating ischemic injury in the brain.6

In addition, it was reported that the major source of superoxide in the vascular system, composed of vascular smooth muscle cells (VSMCs) and endothelial cells, is the NADPH oxidase system, and this enzyme system is involved in atherogenesis.7–9 A recent study indicated that p22 PHOX, which is a component of NADPH oxidase, is expressed in VSMCs and serves as a critical component of superoxide-generating vascular NADPH oxidase and regulates vascular hypertrophy.10

The NADPH oxidase system is a group of plasma membrane–associated enzymes, comprising 5 components: p40 PHOX, p47 PHOX, p22 PHOX, and p91 PHOX.11 Three genetic polymorphisms have been reported in the coding sequence of the p22 PHOX gene.12 Among them, the C242T polymorphism results in an amino acid dimorphism (His/Tyr) at residue 72, which is located in putative heme-binding sites. Because the histidine residue is a candidate for the coordinating ligand of the heme prosthetic group of cytochrome b, this polymorphism has been suggested to be directly associated with the function of p22 PHOX.

There is considerable controversy about whether the C242T polymorphism is associated with a risk of thrombotic risk. Originally, Inoue et al13 reported that the T allele of this polymorphism is associated with a reduced risk of coronary...
artery disease (CAD) and is a novel genetic risk factor for CAD. However, recent large studies14,15 found no evidence of any association between this polymorphism and CAD. In contrast, an Australian group16 found that the TT+TC genotype is associated with an increased risk for CAD in a young male population.

The primary aim of this study was to determine whether the C242T polymorphism of the p22 PHOX gene is associated with cerebrovascular disease.

Subjects and Methods
We recruited 226 Japanese patients aged ≥70 years with symptomatic ischemic cerebrovascular disease (CVD) from Keio University Hospital in Tokyo and 301 control subjects. The CVD patients with cardioembolic cerebral infarction and cerebral hemorrhage were excluded. Control subjects were those who visited for regular check-ups; those who had a clinical history of cerebrovascular disease or myocardial infarction or peripheral vascular diseases were excluded. Informed consent was obtained from all subjects after a full explanation of the study. In all CVD patients, brain CT and/or MRI were performed. MR angiography and/or extracranial duplex ultrasonography were available in >80% of the CVD patients. On the basis of Classification of Cerebrovascular Diseases III, reported from the committee established by the National Institute of Neurological Disorders and Stroke,17 the CVD patients were divided into 3 clinical categories: atherothrombotic infarction, lacunar infarction, and transient ischemic attack (TIA).

Hypertension was defined as systolic blood pressure >140 mm Hg and/or diastolic pressure >90 mm Hg or current treatment with antihypertensive drugs. Smoking was defined as a current smoking. Hypercholesterolemia was defined as a cholesterol level ≥220 mg/dL or current treatment with a cholesterol-lowering drug.

DNA Procedure
Whole blood was collected into sodium citrate tubes. A direct DNA amplification kit (Shimadzu Co), which enabled us to amplify DNA from whole blood without DNA extraction steps, was used in this study. Amplification of the 353-bp fragment of the p22 PHOX gene was performed essentially as previously described,13 with the 5’ primer 5’-TGTCTCTGGGGTAAACCAAGG-3’ and 3’ primer 5’-GGAAAAACACTGAGGTAACTG-3’. A 0.5-μL quantity of whole blood, 25 pmol of each primer, 400 μmol/L of each deoxy nucleotide triphosphate, 2.5 μL of 5× Ampdirect, 2.5 μL of 5× Amp Addition-1, and 1.25 U Taq polymerase (TOYOBO Co) and water were added to the reaction to achieve a total volume of 25 μL. The polymerase chain reaction (PCR) consisted of 1 cycle of 15 minutes at 95°C and 3 minutes at 95°C, 40 cycles of 1 minute at 55°C, 1 minute at 55°C, and 1 minute at 72°C in a Gene Amp PCR system 2400 (Perkin Elmer). The 353-bp PCR product (4 μL) was cleaved in appropriate buffer with 10 U of Rsa 1 restriction enzyme (New England Biolabs). The DNA fragments were separated by electrophoresis through a 2% agarose gel containing 0.5 μg/mL of ethidium bromide and visualized under ultraviolet light. Digestion of the PCR products yielded bands of 353 bp in CC-homozygotes, 193 and 160 bp in TT-homozygotes, and all 3 bands in the heterozygotes.

Statistical Analysis
The differences in the frequencies of p22 PHOX genotypes and other risk factors were analyzed by χ² test. Mean ages between 2 groups were compared with the use of the Student t test. Multiple logistic regression methods were conducted to control for possible confounding factors. Associations and differences with values of P<0.05 were considered significant. All statistical analyses were performed with Statview (version 5.0 for Windows; SAS Institute).

Results
A total of 226 CVD patients and 301 control subjects were recruited for the study. Table 1 shows the prevalence of selected risk factors for CVD among the CVD patients and the controls. There were no significant differences in age or sex between the 2 groups. The risk factors hypertension, diabetes, and smoking were significantly more common in the CVD patients.

The distributions of genotypes and the frequencies of alleles of the p22 PHOX gene polymorphism in the control and CVD groups are shown in Table 2. The genotype frequencies in both groups were in Hardy-Weinberg equilibrium. The T allele frequency in the CVD group was 0.12 compared with 0.07 in the control group, and the prevalence of the TC+TT genotype was significantly higher in the CVD than in the control group (χ²=6.477, P=0.01). The crude OR of the CT+TT genotype versus the CC genotype between the CVD patients and control subjects was 1.81 (95% CI, 1.15 to 2.86; Table 3). In addition, analysis by CVD subtypes showed the OR of the TC+TT genotype to be highest in the CVD patients with atherothrombotic infarction, followed by those with lacunar infarction, and then TIA (2.22, 1.71, and 1.37, respectively).

We next analyzed whether the prevalences of well-established acquired risk factors for stroke (hypertension, diabetes mellitus, and smoking) were different among the genotypes of p22 PHOX. As shown in Table 3, there was no significant difference in the frequency of these factors be-

### Table 1. Clinical Characteristics of CVD Patients and Control Subjects

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=301)</th>
<th>CVD Patients (n=226)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male, %</td>
<td>76.6</td>
<td>77.4</td>
<td>NS</td>
</tr>
<tr>
<td>Age (mean±SD), y</td>
<td>59±4</td>
<td>58±8</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>25.5</td>
<td>56.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypercholesterolemia, %</td>
<td>32.6</td>
<td>39.4</td>
<td>0.11</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>6.6</td>
<td>24.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smoking, %</td>
<td>38.2</td>
<td>53.2</td>
<td>0.001</td>
</tr>
</tbody>
</table>

*χ² tests were used to compare the values for CVD patients and controls for all parameters except age, which was compared with the use of the Student t test.

### Table 2. Allele Frequencies of p22 PHOX Genotypes in CVD Patients and Control Subjects

<table>
<thead>
<tr>
<th></th>
<th>T Allele Frequency</th>
<th>CC, n (%)</th>
<th>TC+TT, n (%)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n=301)</td>
<td>0.07</td>
<td>261 (86.7)</td>
<td>38 + 2 (13.3)</td>
<td></td>
</tr>
<tr>
<td>CVD patients (n=226)</td>
<td>0.12</td>
<td>177 (78.3)</td>
<td>46 + 3 (21.7)</td>
<td>0.011</td>
</tr>
<tr>
<td>Atherothrombotic</td>
<td>0.13</td>
<td>47 (74.6)</td>
<td>16 + 0 (25.3)</td>
<td>0.015</td>
</tr>
<tr>
<td>Lacunar</td>
<td>0.11</td>
<td>111 (79.3)</td>
<td>26 + 3 (20.7)</td>
<td>0.046</td>
</tr>
<tr>
<td>TIA (n=23)</td>
<td>0.09</td>
<td>19 (82.6)</td>
<td>4 + 0 (17.4)</td>
<td>0.306</td>
</tr>
</tbody>
</table>

*χ² tests were used to compare genotype frequencies between controls and all CVD patients, or between controls and each group of CVD patients.
between CC and CT+TT genotypes, suggesting that the polymorphism is not directly linked to those risk factors. In the multiple logistic regression analysis, hypertension, diabetes mellitus, smoking, and this polymorphism were included as independent variables. The ORs and probability value were as follows: hypertension (OR 5.03, \(P<0.001\)), diabetes mellitus (OR 4.41, \(P<0.001\)), smoking (OR 2.27, \(P<0.001\)), and genotypes of p22 \textit{PHOX} (CC+CT, OR 1.88, \(P=0.02\)). Although the OR of this polymorphism was lower than those of well-established risk factors (hypertension, diabetes mellitus, and smoking), this analysis revealed that the presence of T allele was one of the independent risk factors for the development of CVD.

### Discussion

In this study, the frequency of the T allele was found to be significantly higher in the CVD patients than in controls, and among CVD subtypes, T allele frequency was highest in atherothrombotic infarction. These findings indicate that the polymorphism itself may be functionally involved in the increased risk or that it may be a marker of linkage disequilibrium with relevant functional changes. Our data also show the absence of any statistical association between TIA and this polymorphism. TIA is caused by several distinct mechanisms, including thrombosis, embolism, vasospasm, vasculitis, and hemodynamic crisis. Therefore, the association between TIA and this polymorphism may be lower than in other types of CVD. Another possibility is that the C allele in TIA may have a protective effect on ischemic injury and prevent progression to infarction.

Numerous neutrophils and phagocytes have been observed within the parenchyma of ischemic brain tissue. These cells induce tissue damage by releasing proteolytic enzymes and generating free radicals. Walder et al. demonstrated that ischemic injury is reduced in mice whose central nervous system and peripheral leukocytes lack a functional NADPH oxidase, suggesting that superoxide generated by this enzyme system plays a major role in mediating ischemic injury in the brain. Furthermore, the NADPH oxidase system reportedly contributes to the pathogenesis of atherosclerosis and thrombotic disease. Recent findings have confirmed that NADPH oxidase is present in a variety of nonphagocytic cells, including endothelial cells and VSMCs. Mohazzab and colleagues have demonstrated by chemiluminescence that NADPH oxidase is a major source of superoxide in cultured endothelial cells. The hypotrophic agent angiotensin II increases superoxide production in VSMCs by activating NADPH oxidase. This enzyme appears to be the major source of superoxide in this cell type and plays an important role in vascular hypertrophy. Ushio-Fukai et al. demonstrated that inhibition of p22 \textit{PHOX} mRNA expression by stable transfection of antisense cDNA into VSMCs decreases superoxide production and angiotensin II-induced vascular hypertrophy, which suggests that p22 \textit{PHOX} is a critical component of the superoxide-generating vascular NADPH oxidase system and that it is involved in vascular hypertrophy.

Published data on the association between the C242T polymorphism of p22 \textit{PHOX} and the risk of CAD are conflicting. Inoue et al. first investigated the association of this p22 \textit{PHOX} polymorphism with CAD in 201 Japanese patients. They found the T allele in the C242T polymorphism to be significantly more frequent in the control subjects than in the CAD patients, which indicates that the T allele might have a protective effect in terms of coronary risk. Two more recent studies, however, have failed to show any association between this polymorphism and CAD. In contrast, an Australian study in which 689 Australian Caucasians were analyzed reported that the T allele tended to be more prevalent in the CAD patient and that the difference was statistically significant among young male patients aged ≤45 years.

The present study is the first to show evidence of an association between CVD and the C242T polymorphism of p22 \textit{PHOX}. We demonstrated a significant by higher frequency of the T allele in the CVD patients than in the controls. Our findings support the results of the Australian study of a young population, but conflicts with those of the Japanese study reported by Inoue et al. No studies have yet examined the functional effects of the C242T polymorphism on the activity and regulation of NADPH oxidase. This polymorphism of the putative heme-binding site in the p22 \textit{PHOX} gene may have different effects on NADPH oxidase in the central nervous system and the cardiovascular system and have opposite effects in CVD and CAD. Further studies that examine the functions of NADPH oxidase in different p22 \textit{PHOX} genotypes will establish whether this reported association is causal.

### Table 3. Odds Ratios for the C242T Polymorphism in p22 \textit{PHOX}

<table>
<thead>
<tr>
<th>Subtype</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>All CVD patients</td>
<td>1.81</td>
<td>1.15–2.86</td>
</tr>
<tr>
<td>Atherothrombotic</td>
<td>2.22</td>
<td>1.11–2.78</td>
</tr>
<tr>
<td>Lacunar</td>
<td>1.71</td>
<td>1.01–2.88</td>
</tr>
<tr>
<td>TIA</td>
<td>1.37</td>
<td>0.45–3.05</td>
</tr>
</tbody>
</table>

### Table 4. Prevalences of Acquired Risk Factors According to C242T Polymorphism in p22 \textit{PHOX}

<table>
<thead>
<tr>
<th></th>
<th>Control Subjects</th>
<th>CVD Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC</td>
<td>CT+TT</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>85.3</td>
<td>14.7</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>85.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Smoking, %</td>
<td>88.4</td>
<td>11.6</td>
</tr>
</tbody>
</table>

\(\chi^2\) tests were used to compare the prevalences of given risk factors between CC and CT+TT genotypes (2×2 contingency table).

### References

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