Polymorphism of the NADH/NADPH Oxidase p22 phox Gene in Patients With Coronary Artery Disease

Nobutaka Inoue, MD; Seinosuke Kawashima, MD; Kenji Kanazawa, MD; Shinichiro Yamada, MD; Hozuka Akita, MD; Mitsuhiro Yokoyama, MD

Background—Oxidative stress in the vasculature has been implicated in the pathogenesis of coronary artery disease (CAD). NADH/NADPH oxidase is a key enzyme of superoxide production in the vasculature. p22 phox, an essential component of NADH/NADPH oxidase, has four types of polymorphism. The C242T polymorphism changes histidine-72 to tyrosine, located in the potential heme-binding sites, whereas A640G polymorphism is located in the 3′ untranslated region.

Methods and Results—We investigated whether these polymorphisms were associated with risk of CAD by use of restriction fragment length polymorphism (RFLP). The prevalence of the TC+TT genotype of the C242T polymorphism was significantly more frequent in control subjects (n=201) than in the patients with CAD (n=201). The odds ratio of the TC+TT versus CC genotype of the C242T polymorphism between control subjects and case patients was 0.49 (95% CI, 0.28 to 0.87) (P = .015). The prevalence of the genotypes of the A640G polymorphism was not different between groups. The association of C242T polymorphism of the p22 phox gene with CAD was statistically significant and independent of other risk factors.

Conclusions—The mutation of the potential heme-binding site of the p22 phox gene may reduce susceptibility to CAD. Our observations suggest that the C242T polymorphism of the p22 phox gene is a novel genetic marker that has a protective effect on coronary risk. (Circulation. 1998;97:135-137.)

Key Words: coronary disease ■ genes ■ risk factors ■ free radicals ■ stress

Oxidative stress in the vasculature induced by O_2^- has been implicated in the pathogenesis of CAD. The sources of O_2^- in the vasculature are diverse and include VSMCs, endothelial cells, and macrophages. Although NADPH oxidase was originally described in phagocytes, it has recently become evident that the NADH/NADPH oxidase system is an important enzymatic origin of O_2^- in nonphagocytic cells such as VSMCs and endothelial cells. A recent investigation shows that p22 phox, one of the electron transfer elements of NADPH oxidase in phagocytes, is expressed in VSMCs and is a critical component for O_2^- generation in VSMCs.

Four types of allelic polymorphisms in the p22 phox gene have been reported. Among them, C242T polymorphism of the p22 phox substitutes histidine-72 to tyrosine located in the potential heme-binding sites, and A640G polymorphism is located in the 3′ untranslated region. However, the clinical significance of these polymorphisms has never been examined. The present study was designed to investigate whether these polymorphisms were associated with risk of CAD by use of RFLP.

Methods

Subjects

The study population was composed of 201 case patients and 201 control subjects; all subjects enrolled were Japanese. Case patients, who had been admitted to Kobe University Hospital at the age of ≤70 years, were clinically diagnosed as having CAD, and in all case patients, significant coronary artery stenoses (>75%) were demonstrated by coronary angiography. The control subjects were selected from the inpatients of the hospital and matched with the case patients for sex, and they had not had any symptoms of CAD or peripheral atherosclerotic artery disease documented. Written consent was obtained from every patient after a full explanation of the study. The Ethics Committee of Kobe University approved this study.

Patients were considered smokers if their smoking index was >100. They were considered to have hypertension if they met the criteria of the World Health Organization or had already been treated with antihypertensive drugs. They were considered to have hypercholesterolemia if their fasting total plasma cholesterol level was >220 mg/dL or they had already been treated with cholesterol-lowering drugs. They were defined as having diabetes if they met the diagnostic criteria of the World Health Organization or were already under treatment for diabetes.

Determination of Polymorphisms of p22 phox by RFLP

C242T Polymorphism

Because the C→T mutation of the C242T polymorphic site produces the Rsa I digestion site, Rsa I RFLP was used to analyze the polymorphism of the p22 phox gene. The p22 phox gene containing this polymorphic site was amplified from genomic DNA isolated from subjects by PCR. Digestion of the PCR product (348 bp) by Rsa I...
Selected Abbreviations and Acronyms

- CAD = coronary artery disease
- \( \text{O}_2^- \) = superoxide
- RFLP = restriction fragment length polymorphism
- VSMC = vascular smooth muscle cell

makes 160- and 188-bp fragments in the C→T mutation, whereas Rsai I does not cut the PCR product in the wild type.

**A640G Polymorphism**

The A→G mutation of this polymorphic site loses the Dra III digestion site. The digestion of the PCR product (258 bp) by Dra III makes 227- and 31-bp fragments in the wild type, whereas Dra III does not cut the PCR product in the A→G mutation.

The resulting fragments were separated by agarose gel electrophoresis and identified by ethidium bromide staining (Fig). The results were confirmed by at least two investigators, who did not know the origin of the genomic DNA.

**Statistical Analysis**

Data on age are presented as mean±SD, and the difference in age was analyzed by unpaired Student’s t test. The differences in frequencies of smoking, hypertension, hypercholesterolemia, diabetes mellitus, and \( p22 \) phox genotypes were analyzed by Fisher’s exact test. Hardy-Weinberg equilibrium was assessed by \( x^2 \) analysis. Multivariate analyses were conducted with multiple logistic regression methods, and an estimation of conditioned relative risk and 95% CI was done.

**Results**

The characteristics of the subjects are summarized in Table 1. There was no significant difference in age between the groups. The coronary risk factors examined, ie, smoking, hypercholesterolemia, hypertension, and diabetes, were significantly pronounced in case patients (Table 1).

The distribution of genotypes and the frequency of alleles of the polymorphisms of the \( p22 \) phox gene are summarized in Table 2A. The allele frequencies in all subjects were obeyed according to Hardy-Weinberg’s law. The T allele frequencies of C242T polymorphism in control subjects were 0.13 and 0.08, respectively, and the prevalence of the TC genotype of the C242T polymorphism between control subjects and case patients. The odds ratio of the TC genotype of the C242T polymorphism between case patients and control subjects was 0.49 (95% CI, 0.28 to 0.87) \((P = .015)\). The association of this polymorphism with CAD was statistically significant and independent of the A640G polymorphism and other risk factors when subjected to logistic regression analysis (Table 2B).

**Discussion**

The present study is the first clinical investigation on the molecular identity and clinical significance of the A640G polymorphism of the \( p22 \) phox gene. DNA fragment containing A640G polymorphic site was amplified from genomic DNA by PCR with sense primer 5’-TGGTTGT-GGGTAAAAACCGGCGTGGTG-3’ (F1) and antisense primer 5’-AACACTGAGTAGGTCGGGGGTGC-3’ (R1), then PCR product was digested with Rsai I. C→T mutation at nucleotide 242 creates an Rsai I digestion site, which digests 348-bp fragment into 160- and 188-bp fragments. Bottom, Representative of 2% agarose gel electrophoresis on Rsai I RFLP of p22 phox gene. Lane 1, heterozygote of GC; lanes 2 and 3, homozygotes of CC; lane 4, homozygote of TT; B, Dra III RFLP for A640G polymorphism. DNA fragment containing A640G polymorphic site was amplified from genomic DNA by PCR with sense primer 5’-AGCAGTGACCCATCGAGCCCA-3’ (F2) and antisense primer 5’-CGCTGCGTTATTGAGGCGGGG-3’ (R2), then PCR product (258 bp) was digested with Dra III. Dra III digestion of PCR product makes 227- and 31-bp fragments in wild type, whereas Dra III does not cut PCR product in A→G mutation. Bottom, Representative of 2% agarose gel electrophoresis on Rsai I RFLP of p22 phox gene. Lane 1, homozygote of GG; lane 2, heterozygote of AG; lane 3, homozygote of AA.

NADPH oxidase. In contrast to the phagocytic NADPH oxidase, the molecular identity and clinical significance of the vascular NADH/NADPH oxidase system are poorly understood. \( p22 \) phox mRNA is expressed in phagocytes as well as nonphagocytic cells, whereas other components are restricted to phagocytic cells and are hardly detectable in nonphagocytic cells.\(^7,8\) Ushio-Fukai et al\(^4\) demonstrated that stable transfection of a nearly full-length antisense fragment of the \( p22 \) phox gene into VSMCs markedly decreased \( \text{O}_2^- \) production, indicating that the \( p22 \) phox gene was a critical component of \( \text{O}_2^- \) production in this cell type.
TABLE 1. Characteristics of Case Patients and Control Subjects

<table>
<thead>
<tr>
<th></th>
<th>Control Subjects (n=201)</th>
<th>Case Patients (n=201)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y, mean±SD</td>
<td>54.9±10.0</td>
<td>59.8±7.6</td>
<td>NS</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>140 (69.7)</td>
<td>140 (69.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Smoking status, n (%)</td>
<td>79 (39.3)</td>
<td>128 (63.7)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Hypercholesterolemia, n (%)</td>
<td>23 (11.4)</td>
<td>55 (27.4)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Diabetes status, n (%)</td>
<td>15 (7.5)</td>
<td>69 (34.3)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>33 (16.4)</td>
<td>71 (35.3)</td>
<td>&lt;.001</td>
</tr>
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</table>

*Student’s t test (for continuous values) and Fisher’s exact test (for discrete variables) were used to compare the values for case patients and control subjects.

Four types of polymorphism of the p22 phox gene are reported, that is, C242T, C549T, G508A, and A640G. The C242T and C549T polymorphisms affect amino acid sequence, whereas the G508A polymorphism does not because of the degeneracy of the genetic code, and the A640G polymorphism is located in the 3’ untranslated region. p22 phox, a heme-binding protein, contains two histidine residues at amino acids 72 and 94, respectively, and these are the potential heme-binding sites. Because the C242T polymorphism substitutes the histidine-72 to tyrosine residues, this base substitution may have a direct functional role in the association between the C242T polymorphism and coronary risk. It is interesting to speculate that this mutation of the p22 phox gene might modulate the activity and regulation of NADH/NADPH oxidase, which leads to a decrease of oxidative stress in the vasculature, and it might, in turn, reduce susceptibility to CAD.

In conclusion, the prevalence of the TC+TT genotype of the C242T polymorphism of the p22 phox gene in control subjects was significantly more frequent than that in CAD patients. To confirm that this polymorphism is a novel genetic marker for CAD, investigations in a larger population and other ethnic populations are necessary. Although some antioxidants have been reported to have beneficial effects on CAD, the precise criteria for their use are not fully determined. Some method to distinguish patients who have genetically increased susceptibility to oxidative stress would be a great advantage in treatment for CAD. Genetic investigation of the genes related to oxidative stress, like this study, might provide clues for determination of patients genetically susceptible to oxidative stress.

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REFERENCES

TABLE 2. Polymorphism of the NADH/NADPH Oxidase p22 phox Gene

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control Subjects</th>
<th>Case Patients</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>C242T polymorphism</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC, n (%)</td>
<td>148 (73.6)</td>
<td>173 (86.1)</td>
<td>.002</td>
</tr>
<tr>
<td>TC+TT, n (%)</td>
<td>53±0 (26.4)</td>
<td>26±2 (13.9)</td>
<td></td>
</tr>
<tr>
<td>T allele frequency</td>
<td>0.13</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>A640G polymorphism</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA, n (%)</td>
<td>42 (20.9)</td>
<td>36 (17.9)</td>
<td>NS</td>
</tr>
<tr>
<td>AG+GG, n (%)</td>
<td>79±0 (79.1)</td>
<td>83±2 (82.1)</td>
<td></td>
</tr>
<tr>
<td>G allele frequency</td>
<td>0.59</td>
<td>0.61</td>
<td></td>
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</table>

A. Prevalence of genotypes and allele frequencies of p22 phox polymorphism

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control Subjects</th>
<th>Case Patients</th>
<th>P</th>
</tr>
</thead>
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<tr>
<td>Odds Ratio</td>
<td>0.49</td>
<td>0.28–0.87</td>
<td>.015</td>
</tr>
<tr>
<td>95% CI</td>
<td>2.36</td>
<td>1.52–3.67</td>
<td>.0001</td>
</tr>
<tr>
<td>1.23–3.90</td>
<td>5.05</td>
<td>2.71–9.45</td>
<td>.0001</td>
</tr>
<tr>
<td>1.33–3.73</td>
<td>2.22</td>
<td></td>
<td>.0023</td>
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B. Odds ratios of C232T polymorphism and major coronary risk factors

Odds ratios and 95% CIs were calculated by multiple logistic regression analyses.
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