Polymorphism of the NADH/NADPH Oxidase \textit{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. \textit{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 \textit{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 \textit{p22 phox} gene may reduce susceptibility to CAD. Our observations suggest that the C242T polymorphism of the \textit{p22 phox} gene is a novel genetic marker that has a protective effect on coronary risk. (\textit{Circulation}. 1998;97:135-137.)

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

Oxidative stress in the vasculature induced by \(\cdot \text{O}_2^-\) has been implicated in the pathogenesis of CAD.\(^1\) The sources of \(\cdot \text{O}_2^-\) 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 \(\cdot \text{O}_2^-\) in nonphagocytic cells such as VSMCs and endothelial cells.\(^2,3\) A recent investigation shows that \textit{p22 phox}, one of the electron transfer elements of NADPH oxidase in phagocytes, is expressed in VSMCs and is a critical component for \(\cdot \text{O}_2^-\) generation in VSMCs.\(^4\)

Four types of allelic polymorphisms in the \textit{p22 phox} gene have been reported.\(^5,6\) Among them, C242T polymorphism of the \textit{p22 phox} substitutes histidine-72 by 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 \(\leq70\) 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 \textit{p22 phox} by RFLP

\textit{C242T Polymorphism}
Because the C\(\rightarrow\)T mutation of the C242T polymorphic site produces the \textit{Rsa I} digestion site, \textit{Rsa I} RFLP was used to analyze the polymorphism of the \textit{p22 phox} gene. The \textit{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 \textit{Rsa I}
Selected Abbreviations and Acronyms

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

makes 160- and 188-bp fragments in the C→T mutation, whereas \( Rsa \) 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 \( \chi^2 \) 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 and case patients were 0.13 and 0.08, respectively, and the prevalence of the TC genotype of the \( C242T \) polymorphism between case patients and control subjects was 0.49 (95% CI, 0.28 to 0.87)

1

...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.

1

...the mutation of the potential heme-binding site in the \( p22 \) phox gene was significantly more frequent in control subjects than in CAD patients, indicating that the mutation of the potential heme-binding site in the \( p22 \) phox gene might have a protective effect on coronary risk.

Two kinds of NADH/NADPH oxidase system have been proposed to exist, ie, phagocytic and vascular NADH/

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 al3 demonstrated that stable transfection of a nearly full-length antisense fragment of the \( p22 \) phox gene into VSMCs markedly decreased \( \cdot \text{O}_2^- \) production, indicating that the \( p22 \) phox gene was a critical component of \( \cdot \text{O}_2^- \) production in this cell type.

\[ \text{A} \]

\[ \text{B} \]
phism 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 \( p_{22} \) 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 \( p_{22} \) 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.

**Acknowledgment**

We are grateful to Kiyoko Matsui for her skillful technical assistance.

**References**


**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>.015</td>
</tr>
<tr>
<td>Hypercholesterolemia, n (%)</td>
<td>23 (11.4)</td>
<td>55 (27.4)</td>
<td>.001</td>
</tr>
<tr>
<td>Diabetes status, n (%)</td>
<td>15 (7.5)</td>
<td>69 (34.3)</td>
<td>.001</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>33 (16.4)</td>
<td>71 (35.3)</td>
<td>&lt;.001</td>
</tr>
</tbody>
</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.

**TABLE 2. Polymorphism of the NADH/NADPH Oxidase \( p_{22} \) phox Gene**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control Subjects</th>
<th>Case Patients</th>
<th>( P )</th>
</tr>
</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±80 (79.1)</td>
<td>83±82 (82.1)</td>
<td></td>
</tr>
<tr>
<td>G allele frequency</td>
<td>0.59</td>
<td>0.61</td>
<td></td>
</tr>
</tbody>
</table>

A. **Prevalence of genotypes and allele frequencies of \( p_{22} \) phox polymorphism**

B. **Odds ratios of C232T polymorphism and major coronary risk factors**

<table>
<thead>
<tr>
<th></th>
<th>Odds Ratio</th>
<th>95% CI</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT+TC</td>
<td>0.49</td>
<td>0.28–0.87</td>
<td>.015</td>
</tr>
<tr>
<td>Smoking status</td>
<td>2.36</td>
<td>1.52–3.67</td>
<td>.0001</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>2.19</td>
<td>1.23–3.90</td>
<td>.0079</td>
</tr>
<tr>
<td>Diabetes status</td>
<td>5.05</td>
<td>2.71–9.45</td>
<td>.0001</td>
</tr>
<tr>
<td>Hypertension</td>
<td>2.22</td>
<td>1.33–3.73</td>
<td>.0023</td>
</tr>
</tbody>
</table>

Odds ratios and 95% CIs were calculated by multiple logistic regression analyses.
Polymorphism of the NADH/NADPH Oxidase p22phox Gene in Patients With Coronary Artery Disease
Nobutaka Inoue, Seinosuke Kawashima, Kenji Kanazawa, Shinichiro Yamada, Hozuka Akita and Mitsuhiro Yokoyama

_Circulation._ 1998;97:135-137
doi: 10.1161/01.CIR.97.2.135

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1998 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/97/2/135

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/