Vasospastic Angina and Microvascular Angina are Differentially Influenced by PON1 A632G Polymorphism in the Japanese

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Background  Ethnicity and smoking are well-known risk factors for the pathogenesis of coronary vasospasm. Oxidative stress induced by smoking plays a crucial role in coronary vasospasm, but is not enough to account for the pathogenesis of coronary vasospasm, indicating that genetic factors are strongly involved.

Methods and Results  The study group comprised 162 vasospastic angina patients (VSAs), 61 microvascular angina patients (MVs) and 61 non-responders (NRs) diagnosed by acetylcholine provocation test. Four polymorphisms of the oxidative stress related genes, cytochrome b-245, polypeptide gene (CYBA) C242T and A640G, paraoxonase 1 gene (PON1) A632G, phospholipase A2 group VII gene (PLA2G7) G994T were genotyped. Allele frequency of PON1 632-G was significantly higher in both the VSA with dominant fashion and the MVA with recessive fashion compared with NR. This association was strongly influenced by gender in the MVA only. There were no significant associations between the other polymorphisms and coronary vasospasm. In addition, the allele frequency of PON1 632-G in the Japanese was higher than in Caucasians.

Conclusions  There was a significant association between PON1 A632G polymorphism and MVA as well as VSA, but the impact of this on VSA and MVA is different in the Japanese. (Circ J 2005; 69: 1466 – 1471)

Key Words:  Coronary vasospasm; Oxidative stress; Polymorphism; PON1 (paraoxonase 1 gene); Smoking

Coronary vasospasm is a major cause of the ischemic heart disease1 and previous studies have demonstrated a significantly higher prevalence in the Japanese than in Caucasians, suggesting an important role of genetic factors in the pathogenesis2,3.

Smoking is also a crucial risk factor for coronary vasospasm.5 In humans it causes oxidative modification of biological molecules to form superoxide anions which injure the endothelium and inactivate nitric oxide (NO), a major biological vasodilator, by forming peroxynitrite, resulting in impaired endothelium-dependent vasorelaxation.6,7 Antioxidants, such as vitamins C and E, restrain coronary vasospasm8–10. However, smoking is not sufficient to account for the pathogenesis of coronary vasospasm because not all smokers suffer from coronary vasospasm11 indicating that genetic factors are strongly involved.

Recent progress in molecular genetics has made it possible to search for the genes responsible for coronary vasospasm. Oxidative-stress-related genes are attractive candidates in terms of the pathogenesis of coronary vasospasm and several genetic studies have been carried out. One study reported that cytochrome b-245, polypeptide gene (CYBA) C242T polymorphism is associated with coronary vasospasm12 and another reported that paraoxonase 1 gene (PON1) A632G polymorphism is also13. However, each study failed to confirm the others results. Phospholipase A2 group VII gene (PLA2G7) G994T polymorphism is also an attractive candidate, but no significant association with coronary vasospasm has been reported12,13.

Coronary vasospasm has 2 clinical entities: spasm occurring in angiographically visible epicardial coronary arteries (vasospastic angina: VSA) and that in angiographically invisible small coronary arteries (microvascular angina: MVA).14,15 The mechanisms of these are similar, but not identical16 and the clinical features also differ17. The difference in the pathogenesis of these 2 entities could be related to differences in genetic and non-genetic factors, but there have not been any studies addressing these issues by comparing VSA and MVA.

In this study, to elucidate genetic factors relating to oxidative stress in the pathogenesis of coronary vasospasm, genetic analysis was carried out for 4 polymorphisms of oxidative-stress-related genes (CYBA C242T and A640G, PON1 A632G, PLA2G7 G994T) among 3 groups: VSA patients, MVA patients and non-responders (NR) based on their response to intracoronary acetylcholine (ACh) administration.

Methods

Study Population
The study population comprised 284 patients with VSA, MVA or NR who were admitted to Kyushu University Hospital between September 1994 and February 2005. All patients studied were Japanese, and had angina symptoms. All underwent coronary angiography followed by ACh provocation test for diagnosis as previously described15. Briefly, a graded dose of ACh (maximum 100 mg) was in-
fused into the left coronary artery, after which systemic arterial pressure, heart rate, and 12-lead electrocardiogram (ECG) trace and coronary cineangiogram were recorded. If ACh infusion into the left coronary artery did not induce angina, ECG changes, or coronary spasm, a further graded dose of ACh (maximum 50 mg) was infused into the right coronary artery. Whenever angina, ischaemic ECG changes, or coronary spasm were observed, the infusion was stopped and arterial pressure, 12-lead ECG trace, and coronary cineangiogram were recorded and 1 mg isosorbide dinitrate (ISDN) was infused into the coronary artery. VSA was defined as >75% diameter reduction compared with the post-ISDN infusion diameter; MVA was defined as induction of angina and ischemic ECG changes without coronary artery spasm; NR was defined as no induction of angina, ischemic ECG or coronary artery spasm. Patients with significant organic stenosis (>50% luminal diameter) after ISDN administration were excluded, as were patients with acute myocardial infarction, hypertrophic cardiomyopathy, severe valvular disease, severe angina, congestive heart failure or chronic renal failure requiring hemodialysis.

Other clinical parameters were collected: age, gender, body mass index, current cigarette smoking, hypertension, diabetes mellitus, and hypercholesterolemia (Table 1). Definition of current cigarette smoking was a patient who was currently smoking or had quit smoking within the past 2 years because the risk of coronary events in ex-smokers declines to the level of non-smokers within 2 years after quitting.18,19 Hypertension was defined as blood pressure greater than 140/90 mmHg or taking antihypertensive drugs. Diabetes mellitus was defined as blood glucose levels greater than 126 mg/dl at fasting, greater than 200 mg/dl at 2h in an oral glucose tolerance test, taking hypoglycemic drugs or using insulin. Hypercholesterolemia was defined as total cholesterol level equal to or more than 220 mg/dl, low-density-lipoprotein (LDL) level equal to or more than 140 mg/dl or taking lipid-lowering drugs.

The study protocol was approved by the Institutional Research Committee of Kyushu University. All patients gave written informed consent before enrolment.

DNA Extraction

A 10-ml sample of peripheral blood was collected from each subject, and immediately stored at 4°C until extraction of genomic DNA from peripheral blood leukocytes using the QIAamp DNA Blood Midi Kit (QIAGEN, Hilden, Germany).

Genotyping of Polymorphisms

Genotypes were determined by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) procedures according to previous reports with minor modification.20–22 PCR primers and restriction endonucleases used in this study are summarized in Table 2. After PCR-RFLP procedures, reaction mixtures were then electrophoresed on 5.0% agarose gel (2.0% Seakem agarose) and visualized by ethidium bromide staining.

Statistical Analysis

Continuous variables were compared by two-tailed unpaired t-tests. Categorical variables were compared by χ² analyses with Fisher's exact probability test. Odds ratios (ORs) were calculated as an index of the association of CYBA C242T (C/C type, C/T type, T/T type) and A640G (A/A type, A/G type, G/G type) polymorphisms, PON1 A632G (A/A type, A/G type, G/G type) polymorphism and PLA2G7 G994T (G/G type, G/T type, T/T type) polymorphism with the phenotypes of VSA and MVA. For each OR, we calculated two-tailed probability values and 95% confidence intervals (CIs). The effect of a mutant allele was assumed to be additive, dominant, or recessive. Values for additive effect were predicted by Hardy-Weinberg equilibrium.

Multiple logistic regression analysis was carried out using SPSS 9.0J for Windows (SPSS, Tokyo, Japan). Independent variables were coded as the following dummy variables: gender, 0 for female and 1 for male; age, 0 for <60 years and 1 for ≥60 years; body mass index, 0 for <26kg/m² and 1 for ≥26kg/m², current cigarette smoking, 0 for non-smokers and 1 for smoker; hypertension, 0 for
normotension and 1 for hypertension; diabetes mellitus, 0 for absence and 1 for presence; hypercholesterolemia, 0 for absence, 1 for presence. Statistical significance was defined as \( p < 0.05 \).

### Results

#### Clinical Parameters of the Study Population

Clinical parameters of each patient group are summarized in Table 1. Each parameter was compared between the VSA and NR groups as well as between the MVA and NR groups. In the comparison of the VSA and NR groups, the frequency of smoking was higher in the VSA, which was compatible with previous studies.\(^4,17\) In the comparison of the MVA and NR groups, the frequency of females was significantly higher (\( p < 0.01 \)), and the frequency of current smoking status was significantly lower (\( p < 0.05 \)) in the MVA than in the NR, which was also compatible with results from a previous study.\(^17\)

#### Genetic Analysis of the Relationship of Oxidative-Stress-Related Gene Polymorphisms With Coronary Vasospasm

Polymorphisms of 4 oxidative-stress-related genes, *CYBA*, *PON1*, *PLA2G7*, and *PLA2G7* G994T, were genotyped for the 3 groups of patients (Table 3). There was a Hardy-Weinberg equilibrium in the allele frequencies of *CYBA C242T*, *PON1 A632G*, and *PLA2G7 G994T* polymorphisms, verified by chi-square analyses with Fisher’s exact probability test. In contrast, the allele frequency of the *CYBA A640G* polymorphism was significantly (\( p < 0.01 \)) lower in the MVA than in the NR, which was also compatible with results from a previous study.\(^17\)

#### Odds Ratio (OR) and 95% Confidence Interval (CI) was Calculated of the Each Genotypes With the Phenotype of the VSA and MVA, With the Effects of *CYBA C242-T*, *CYBA A640-G*, *PON1 632-G* and *PLA2G7 994-T* Allele as Being Additive, Dominant or Recessive

<table>
<thead>
<tr>
<th></th>
<th>VSA vs NR</th>
<th>MVA vs NR</th>
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<tbody>
<tr>
<td></td>
<td>OR (95% CI)</td>
<td>p-value</td>
</tr>
<tr>
<td><strong>CYBA C242T</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Additive</td>
<td>1.32 (0.69–2.55)</td>
<td>0.40</td>
</tr>
<tr>
<td>Dominant</td>
<td>0.87 (0.42–1.81)</td>
<td>0.72</td>
</tr>
<tr>
<td>Recessive</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>CYBA A640G</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Additive</td>
<td>1.03 (0.68–1.58)</td>
<td>0.88</td>
</tr>
<tr>
<td>Dominant</td>
<td>0.90 (0.30–2.66)</td>
<td>0.84</td>
</tr>
<tr>
<td>Recessive</td>
<td>1.17 (0.55–2.48)</td>
<td>0.68</td>
</tr>
<tr>
<td><strong>PON1 A632G</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Additive</td>
<td>1.56 (1.02–2.40)</td>
<td>0.040</td>
</tr>
<tr>
<td>Dominant</td>
<td>2.24 (1.04–4.82)</td>
<td>0.035</td>
</tr>
<tr>
<td>Recessive</td>
<td>1.47 (0.81–2.67)</td>
<td>0.21</td>
</tr>
<tr>
<td><strong>PLA2G7 G994T</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Additive</td>
<td>0.75 (0.43–1.27)</td>
<td>0.28</td>
</tr>
<tr>
<td>Dominant</td>
<td>0.70 (0.37–1.29)</td>
<td>0.25</td>
</tr>
<tr>
<td>Recessive</td>
<td>0.75 (0.07–8.42)</td>
<td>0.61</td>
</tr>
</tbody>
</table>

See Table 1 for abbreviations.
was not in agreement with those predicted by the Hardy-Weinberg equilibrium for each group (p<0.05).

Proportion test was carried out next between the VSA and NR groups and between the MVA and NR groups. As summarized in Table 4, there was a significant association of PON1 A632G polymorphism in both comparisons. The genetic impact of PON1 632-G allele was dominant and additive (dominant: OR 2.24, 95% CI 1.04–4.82, p=0.035; additive: OR 1.56, 95% CI 1.02–2.40, p=0.040) in the comparison of the VSA and NR. In contrast, the genetic impact of PON1 632-G allele was recessive and additive in the comparison of the MVA and NR (recessive: OR 2.22, 95% CI 1.08–4.45, p=0.049; additive: OR 1.46, 95% CI 1.04–2.02, p=0.040) in the comparison of the VSA and NR.

In contrast, there were no significant associations with the CYBA C242T/A640G or PLA2G7 G994T polymorphisms in either comparison (Table 4).

**Detailed Analysis of Relationship of PON1 A632G Polymorphism With Coronary Vasospasm**

Multivariate analysis that included PON1 A632G genotype, age, gender, current smoking status, hypertension, diabetes, and hypercholesterolemia was carried out and is summarized in Tables 5 and 6. In the comparison of the VSA and NR, the G allele of the PON1 A632G polymorphism had a significant association with VSA (p=0.037), which was independent of current smoking status (p=0.44, chi-square analyses with Fisher’s exact probability test with Smoking and PON1 G allele), indicating that the effect of the PON1 A632G polymorphism was not influenced by smoking in the pathogenesis of VSA.

In the comparison of the MVA and the NR, the G allele of the PON1 A632G polymorphism also had a significant association with MVA (p=0.049). Interestingly, female gender had a more significant association with MVA (p=0.002), so multivariate analysis after correction by gender was carried out for the MVA and NR, and the G allele of the PON1 A632G polymorphism no longer had a significant association with MVA, indicating that its effect in the pathogenesis of MVA was significantly influenced by gender.

**Ethnic Difference in PON1 A632G Polymorphism**

The allele frequency of the PON1 A632G polymorphism in the general Japanese population has not previously been reported, so we genotyped 102 healthy volunteers. The PON1 632A/A, A/G, and G/G genotypes were observed in 10, 54, and 38 subjects, respectively, and based on this result, the estimated allele frequencies of PON1 632-A and 632-G are 0.363 and 0.637, respectively. In contrast, the allele frequencies in Caucasian are 0.726–0.826 and 0.174–0.274, respectively (NCBI dbSNP ss5111592 and ss5586846). This ethnic difference in the allele frequency of PON1 A632G polymorphisms may account for the difference in the prevalence of coronary vasospasm.

**Discussion**

Smoking is known to be a crucial risk factors for coronary vasospasm because it generates oxidative stress, such as oxygen free radicals, which inactivates NO and damages endothelial cells causing impaired endothelium-dependent vasodilatation that leads not only to VSA but also MVA. Based on this, it is plausible that the oxidative-stress-related genes are candidates for the pathogenesis of VSA and MVA. In the present study, 3 genes, CYBA, PON1 and PLA2G7, were studied based on their function and previous genetic studies of cardiovascular diseases.
CYBA encodes p22-phox, a critical component of the NADH/NADPH oxidase, which generates superoxide in both endothelial and smooth muscle cells within vascular tissue. CYBA C242T polymorphism results in amino acid substitution (H72Y) and CYBA with the 242-T allele reduces NADH/NADPH oxidase activity in human blood vessels, suggesting that this genetic variation plays a significant role in modulating superoxide production.

It has been reported that CYBA C242T polymorphism is associated with a reduced risk of coronary artery disease (CAD) in the Japanese.

PON1 protects LDL from oxidation by hydrolysis of biologically active liperoxides, which injures the blood vessel endothelium. PON1 A632G polymorphism results in amino acid substitution (Q192R) and PON1 with the 632-G allele reduces its antioxidant activity. It has been previously reported that there is a significant association between the PON1 632-G allele and VSA.

PLA2G7 encodes platelet-activating factor acetylhydro-lase, which retards the oxidation of LDL by preventing the generation of phospholipid hydroperoxides. PLA2G7 G994T polymorphism results in an amino acid substitution (V279F), and PLA2G7 with the 994-T allele loses its catalytic activity. It has been reported that PLA2G7 activity is associated with angiographic CAD.

We studied 4 polymorphisms, CYBA C242T and A640G, PON1 A632G and PLA2G7 G994T polymorphisms, and there was only a significant association between the PON1 A632G polymorphism and coronary vasospasm (Table 4). As it has been previously demonstrated that PON1 632-G allele causes lower antioxidant activity, PON1 could be a causal gene for coronary vasospasm.

There are common characteristics of VSA and MVA: (1) Ca-channel blockers are effective and (2) endothelial dysfunction is thought to be primary cause. Therefore, it is likely that a single gene, such as PON1, is involved in the pathogenesis of both entities, as demonstrated in this study.

On the other hand, there are also unique characteristics of each type of coronary vasospasm. VSA sometimes results in myocardial infarction, whereas the prognosis of MVA tends to be good. Angiotensin-converting enzyme inhibitors and statins are effective only in some cases of MVA.

There is a significant association between the PON1 A632G polymorphism and VSA.13,16,17 Therefore, it is also plausible that the mode of inheritance of the PON1 A632G polymorphism has an influence on the clinical expression of VSA and MVA.

Previous studies demonstrate that several factors, such as hypertension, hypercholesterolemia and smoking, contribute to the microvascular endothelial dysfunction that results in MVA. Recently, estrogen deficiency has been recognized as a cause of endothelial dysfunction in MVA and estrogen replacement therapy has been shown to restore the impaired vasodilator response in women with MVA. Interestingly, in this study the association between the G allele of PON1 A632G polymorphism and MVA was significantly influenced by female gender, but was not of the same level of significance with VSA, indicating that genetic impact of PON1 A632G polymorphism on MVA is strongly influenced by gender. It is highly likely that the pathogenesis of MVA with the PON1 A632G polymorphism could involve an X chromosome effect or some female-specific gene expression. There were no significant associations between the other polymorphisms and coronary vasospasm. Recently, Murase et al reported that there is a significant association between CYBA C242T polymorphism and VSA only in males; however, there are crucial differences between their study and ours. They used ergonovine (Erg) provocation to induce coronary vasospasm and used 90% stenosis as significant after Erg provocation, whereas we used ACh provocation and 75% stenosis was significant. More importantly, they used subjects without chest pain as controls and did not perform cardiac catheter examination.

**Study Limitations**

The study population is relatively small for this type of study, which could potentially lead to a false-positive result. However, the diagnostic procedure was very precise and accurate, and ethnic divergence relatively small because the study included only Japanese. Therefore, it is more likely to detect a true genetic effect. Regarding the significant association between PON1 A632G polymorphism and VSA, this study confirms previous results, suggesting that it is highly likely that this is a true positive. In contrast, the association between PON1 A632G polymorphism and MVA has to be confirmed by other studies.

In conclusion, the allele frequency of PON1 632G was significantly higher in both VSA and MVA patients compared with NR. This is the first study to identify a gene responsible for the pathogenesis of MVA. The genetic impact of PON1 A632G polymorphism on MVA was significantly affected by female gender. These results indicate that PON1 A632G polymorphism has an impact on the pathogenesis of coronary vasospasm, but mechanisms of its effect on VSA and MVA are different.

**Acknowledgments**

This study was supported by health science research grants from the Ministry of Health, Labor and Welfare, Tokyo, Japan, by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (16590696), by research grants from the Uehara Memorial Foundation, Tokyo, Japan, from Kanfa Foundation for Life & Socio-Medical Science, and from Japan Heart Foundation/Zeria Pharmaceutical for Research on Molecular Cardiology. We thank members of the cardiac catheterization laboratory at Kyushu University Hospital for diagnosing the subjects, Dr Makoto Usui for assisting with the blood sampling, and Dr Miyuki Tsuchihashi-Makaya for assisting with the statistical analysis.

**References**

8. Miwa K, Miyagi Y, Iwaga A, Nakagawa K, Inoue H. Vitamin E defi-