Association of the Missense Glu298Asp Variant of the Endothelial Nitric Oxide Synthase Gene With Myocardial Infarction

YUKIO SHIMASAKI, MD, HIROFUMI YASUE, MD, MICHIHIRO YOSHIMURA, MD, MASAFUMI NAKAYAMA, MD, KIYOTAKA KUGIYAMA, MD, HISAO OGAWA, MD, EISAKU HARADA, MD, TAKENOBU MASUDA, MD,* WASAKU KOYAMA, MD,* YOSHIHIKO SAITO, MD,† YOSHIHIRO MIYAMOTO, MD,† YOSHIHIRO OGAWA, MD,† KAZUWA NAKAO, MD†
Kumamoto and Kyoto, Japan

Objectives. We examined the possible association between the missense Glu298Asp variant of the endothelial nitric oxide synthase (eNOS) gene and myocardial infarction (MI).

Background. Endothelium-derived nitric oxide (NO) plays a key role in the regulation of vascular tone. Recently, we reported that a missense Glu298Asp variant in exon 7 of the eNOS gene is a possible genetic factor involved in the pathogenesis of coronary spasm. Endothelium-derived NO also has vasoprotective effects by suppressing platelet aggregation, leukocyte adhesion and smooth muscle cell proliferation.

Methods. We screened 285 patients with an MI and 607 control subjects in Kumamoto Prefecture, Japan. Genotypes were determined by polymerase chain reaction–restriction fragment-length polymorphism analysis.

Results. The frequency of the missense Glu298Asp variant was significantly higher in the MI group than in the control group (21.1% vs. 13.3%, p = 0.003, odds ratio 1.73 for the dominant effect of the eNOS T allele). Multiple logistic regression analysis showed that the missense Glu298Asp variant was an independent risk factor for MI, as was diabetes mellitus, hypertension, cigarette smoking, hypercholesterolemia and body mass index.

Conclusions. There was a significant association of the missense Glu298Asp variant of the eNOS gene with MI. This marker–disease association may be due to the impaired effects of NO on the cardiovascular system: dysregulation of vascular tone, platelet aggregation and leukocyte adhesion and smooth muscle cell proliferation, all of which promote coronary atherosclerosis and thrombosis.

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Epidemiologic studies (1–4) have shown that hyperlipidemia, hypertension, cigarette smoking, diabetes mellitus, obesity and family history of ischemic heart disease are the risk factors for coronary heart disease. Although the accumulation of coronary risk factors often leads to acute myocardial infarction (AMI), its precise pathogenesis has not been elucidated. AMI may arise from interactions between environmental and genetic risk factors.

We recently identified a missense variant within exon 7 of the endothelial nitric oxide synthase (eNOS) gene in patients with coronary spastic angina—GA to GT substitution, which results in the replacement of glutamic acid by aspartic acid (Glu298Asp)—and found that this variant is significantly associated with coronary spasm (5). Coronary spasm is a cause of variant angina, or coronary spastic angina, and is also involved in the pathogenesis of AMI (6–10). The variant may be a possible genetic risk factor for AMI as well as coronary spasm.

eNOS is present in the vascular endothelium (11–15), platelets (16) and several other cell types that continuously produce modest amounts of nitric oxide (NO). Endothelium-derived NO plays a key role in the regulation of vascular tone (17–19) and has vasoprotective effects by scavenging superoxide radicals and suppressing platelet aggregation, leukocyte adhesion and smooth muscle cell proliferation (19–22). The impaired effects of NO on the cardiovascular system appear to be responsible for coronary atherosclerosis and thrombosis.

In the present study, we examined the possible association between the missense Glu298Asp variant and myocardial infarction (MI). We also investigated coronary risk factors, including the insertion/deletion (I/D) polymorphism of the angiotensin-converting enzyme (ACE) gene, which was recently identified as a possible genetic risk factor for MI (23–25).
amplify the 248-base pair (bp) fragment encompassing the PCR-RFLP analysis. A set of primers was designed to the missense Glu298Asp variant was determined by polymerase chain reaction–restriction fragment-length polymorphism. Blood leukocytes by established methods (26). The presence of length polymorphism.

Methods

Study subjects. Patients. The study included 285 patients with an AMI (219 men, 66 women; mean age 57 years, range 15 to 70 at onset of MI) admitted to Kumamoto University Hospital and the university-affiliated hospitals in Kumamoto Prefecture from October 1994 to February 1997. Diagnosis of AMI was made by chest symptoms, electrocardiographic changes and serum creatine kinase-MB isoenzyme (CK-MB) elevations more than twice the upper level of normal. Cardiac catheterization analyses, including coronary angiography and left ventriculography, were performed in the acute phase. Coronary artery stenosis was defined as significant when the lumen diameter was >50% narrowed after nitroglycerin administration. One hundred thirty-seven (48.1%), 71 (24.9%) and 61 (21.4%) patients had single-, double- and triple-vessel disease, respectively; the remaining 16 (5.6%) had no organic stenosis.

Written informed consent was obtained from all patients studied in the hospital. The study was in agreement with the guidelines approved by the ethics committee of Kumamoto University School of Medicine.

Control subjects. The study also included 607 age-matched volunteers as control subjects (403 men, 204 women; mean age 56 years, range 24 to 78) living in Kumamoto Prefecture, Japan. They underwent a check-up examination at the Japanese Red Cross Kumamoto Health Care Center from April to June 1996 that included determination of plasma lipid levels, blood pressure, smoking habit, blood glucose levels after oral glucose loading and body mass index and acquisition of chest X-ray film and an exercise ECG. They had no chest symptoms, and none had an MI.

Screening for the missense Glu298Asp variant of the eNOS gene by polymerase chain reaction–restriction fragment-length polymorphism. Genomic DNA was prepared from blood leukocytes by established methods (26). The presence of the missense Glu298Asp variant was determined by polymerase chain reaction–restriction fragment-length polymorphism (PCR-RFLP) analysis. A set of primers was designed to amplify the 248-base pair (bp) fragment encompassing the missense Glu298Asp variant (the sense and antisense primers 5’-AAGGCAGGAGACAGTGATGGA-3’ and 5’-CCCAGTCAATCCCTTGGTGCTCA-3’, respectively). The PCR fragments were digested with the restriction enzyme Ban II, separated by electrophoresis using low melting temperature agarose gel (4%, NuSieve GTG AGAROSE, FMC) and visualized by ethidium bromide staining. The mutant allele (T) has no Ban II cutting site (Fig. 1).

Screening for the ACE insertion/deletion polymorphism by PCR amplification. Genotyping for the insertion/deletion (I/D) polymorphism of the ACE gene was examined by PCR amplification, as previously described (27–29). The reaction included 5% dimethyl sulfoxide (DMSO) to ensure that the I allele was amplified in all heterozygotes (28).

To avoid potential misclassification of the ID genotype, which may appear as DD because of the preferential amplification of the D allele in heterozygous samples, each sample found to have the DD genotype was subjected to the second, independent PCR amplification. The primer pair (5’-TGGGACCACAGCGCCCGGCACTAC-3’ and 5’-TCGCCAGCCTCCCATGCCCATATAA-3’) recognizes an insertion-specific sequence. The reaction yields a 335-bp fragment only in the presence of I allele and no product in samples homozygous for DD (29).

Evaluation of coronary risk factors. The coronary risk factors of all subjects were assessed by history and chart review. Hypertension was defined by history of several blood pressure measurements elevated either systolically (>160 mm Hg) or diastolically (>95 mm Hg). Diabetes mellitus was defined by elevated blood glucose levels after fasting (>7.8 mmol/liter [140 mg/dl]) or 2 h after 75 g of oral glucose loading (>11.1 mmol/liter [200 mg/dl]). Hypercholesterolemia was defined by elevated total serum cholesterol levels (>240 mg/dl). Subjects were also classified as smokers (current and ex-smokers) and nonsmokers.

Statistical analysis. Chi-square analysis and an unpaired t test were used to compare the frequencies of the missense Glu298Asp variant and other coronary risk factors, including age, gender, body mass index, hypertension, diabetes mellitus,
hypercholesterolemia and cigarette smoking, between the MI and the control groups. The Hardy-Weinberg equilibrium was tested by chi-square analysis for the frequencies of the eNOS and ACE genotypes (29).

Odds ratios (approximating to relative risk) were calculated as a measure of the association between the eNOS genotype and the phenotype of MI, with the effects of the T (mutant) allele assumed to be additive (T allele vs. G allele) or dominant (TT and TG combined vs. GG). Odds ratios were also calculated for the ACE genotype, with the effects of the D allele assumed to be additive (D allele vs. I allele), recessive (DD vs. ID and II combined) or dominant (DD and ID combined vs. II) (29). For each odds ratio, we calculated two-tailed p values and 95% confidence intervals.

To determine the independent risk factors for MI, we performed multiple logistic regression analysis for the effect of the missense Glu298Asp variant, the ACE I/D polymorphism and other coronary risk factors for MI. The analysis was performed using SPSS Advanced Statistics 6.1 for Macintosh (SPSS Japan Inc., Japan). All independent variables were coded as dummy variables (age: 0 for <60 years, 1 for ≥60 years; gender: 0 for female, 1 for male; body mass index: 0 for <26 kg/m², 1 for ≥26 kg/m²; hypertension, diabetes mellitus and hypercholesterolemia: 0 for absence, 1 for presence; cigarette smoking: 0 for nonsmoker, 1 for current or ex-smoker; missense Glu298Asp variant: 0 for GG, 1 for TG and TT combined [dominant effect of the T allele]; ACE I/D polymorphism: 0 for II, 1 for ID and DD combined [dominant effect of the D allele]). We used forward stepwise selection (Wald criterion) in this analysis. Statistical significance was defined as p < 0.05.

Results

Screening for the missense Glu298Asp variant of the eNOS gene and univariate analysis. Figure 1 shows a representative agarose gel loaded with three PCR products after digestion with Ban II. The eNOS/TT, TG and GG genotypes were present in 1 (0.4%), 59 (20.7%) and 225 (78.9%) of 285 patients with an MI, respectively. In contrast, the eNOS/TT, TG and GG genotypes were found in 1 (0.2%), 80 (13.2%) and 526 (86.7%) of 607 control subjects, respectively. The genotype frequencies were in agreement with those predicted by the Hardy-Weinberg equilibrium. In each analysis of the additive, recessive and dominant effects of the eNOS T allele, each frequency was not different between the MI and control groups (p = 0.919, 0.838 and 0.818, respectively).

Table 1. Frequency of Genotypes of Endothelial Nitric Oxide Synthase Gene in Control Subjects and Patients With Myocardial Infarction

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control Group (n = 607)</th>
<th>MI Group (n = 285)</th>
<th>OR (95% CI)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS Glu298Asp var</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eNOS/TT</td>
<td>1/607 (0.2%)</td>
<td>1/285 (0.4%)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>eNOS/TG</td>
<td>80/607 (13.2%)</td>
<td>59/285 (20.7%)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>eNOS/GG</td>
<td>526/607 (86.7%)</td>
<td>225/285 (78.9%)</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Data presented are number (%) of patients, unless otherwise indicated. CI = confidence interval; eNOS = endothelial nitric oxide synthase; eNOS/GG = homozygous normal; eNOS/TG = heterozygous carriers of the eNOS Glu298Asp variant; eNOS/TT = homozygous carriers of the eNOS Glu298Asp variant; MI = myocardial infarction; OR = odds ratio; var = variant.

Screening for the ACE I/D polymorphism and univariate analysis. Figure 2 shows representative agarose gels loaded with the PCR products of the first and second amplifications for the ACE gene. The second PCR results completely matched with the first ones. The ACE/DD, ID and II genotypes were present in 32 (11.2%), 137 (48.1%) and 116 (40.7%) of 285 patients with an MI and in 71 (11.7%), 284 (46.8%) and 252 (41.5%) of 607 control subjects. The genotype frequencies were in agreement with those predicted by the Hardy-Weinberg equilibrium. In each analysis of the additive, recessive and dominant effects of the ACE D allele, each frequency was not different between the MI and control groups (p = 0.919, 0.838 and 0.818, respectively).

Comparison of MI and control groups for coronary risk factors. We compared the MI and control groups for frequencies of coronary risk factors, including age, gender, hypercholesterolemia, cigarette smoking, hypertension, diabetes mellitus and body mass index. There were significant differences in

Figure 2. Use of PCR amplification to screen for the ACE I/D polymorphism. Shown are representative agarose gels loaded with three DNA samples of DD, ID and II after the first (A) and second (B) PCR amplification, respectively. The second PCR amplification was performed to avoid misclassifying the ID genotype as DD, with use of the insertion-specific primers. The primers create a product (335 base pairs [bp]) only in the presence of the insertion.
Table 2. Clinical Characteristics of Study Subjects

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control Group (n = 607)</th>
<th>MI Group (n = 285)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>56 ± 10</td>
<td>57 ± 10</td>
<td>0.121</td>
</tr>
<tr>
<td>Men/women</td>
<td>403/204</td>
<td>219/66</td>
<td>0.002</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.3 ± 2.9</td>
<td>24.0 ± 3.3</td>
<td>0.002</td>
</tr>
<tr>
<td>HTN</td>
<td>101/607 (17%)</td>
<td>114/285 (40%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>DM</td>
<td>51/607 (8%)</td>
<td>85/285 (30%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Hyperchol</td>
<td>121/607 (20%)</td>
<td>110/285 (39%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Cig smoker</td>
<td>283/607 (47%)</td>
<td>202/285 (71%)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Data presented are mean value ± SD or number (%) of patients. BMI = body mass index; Cig = cigarette; DM = diabetes mellitus; HTN = hypertension; Hyperchol = hypercholesterolemia; MI = myocardial infarction.

the frequencies of gender (p = 0.002), body mass index (p = 0.002), hypertension (p < 0.001), diabetes mellitus (p < 0.001), hypercholesterolemia (p < 0.001) and cigarette smoking (p < 0.001) between the MI and control groups, as shown in Table 2.

Multiple logistic regression analysis. Table 3 shows the results of multiple logistic regression analysis with forward stepwise selection (Wald) using all independent variables. Exp(β) is represented by odds ratios in Table 3. The analysis revealed that the independent risk factors for MI were diabetes mellitus (p < 0.001), hypertension (p < 0.001), cigarette smoking (p < 0.001), hypercholesterolemia (p < 0.001), body mass index (p = 0.022) and the missense Glu298Asp variant (p = 0.039).

### Discussion

**Present study.** We found a significant difference in the frequency of the missense Glu298Asp variant of the eNOS gene between the MI and control groups: The missense Glu298Asp variant (heterozygote [TG] and homozygote [TT] combined) was present in 21.1% of patients with an MI but in only 13.3% of control subjects. The frequency of the variant was significantly higher in the MI group than in the control group (p = 0.003). Multiple logistic regression analysis to determine the independent risk factors for MI revealed that the missense Glu298Asp variant was one of the independent risk factors for MI. Thus, the present study shows that the missense Glu298Asp variant is significantly associated with MI.

In such an association study between genetic marker and disease, population stratification should be controlled, and genotyping methods should be appropriate. In our study, we applied the following approach 1) We recruited 285 patients with a definite diagnosis of MI by chest symptoms, ECG changes and CK-MB elevations more than twice the upper level of normal. 2) The control group included subjects with no MI from the same general population as the patients and with a similar age distribution (56 ± 10 vs. 57 ± 10 years). 3) The distribution of the missense Glu298Asp variant and ACE I/D polymorphism was matched to the Hardy-Weinberg equilibrium. This approach suggests that there were no apparent genotyping errors and that the genetic background of the study subjects was homogeneous.

**Previous studies.** Recently, some studies showed (23–25) a significant association between the ACE D allele and MI in low risk subgroups, whereas others found no such association (29,31,32). In the present study, there was no significant association between the ACE I/D polymorphism and MI in both the total cohort and low risk subjects with low levels of total serum cholesterol (<6.21 mmol/liter) and a low body mass index (<26 kg/m²) (data not shown). In the patients and control subjects of the present study, the distributions of the ACE genotypes were in agreement with the Hardy-Weinberg equilibrium, although other studies in Japanese (33) have shown distributions that deviated from it.

The association between the missense Glu298Asp variant of the eNOS gene and MI is not understood at present. It has been shown that endothelium-derived NO plays a key role in the regulation of vascular tone (17–19) and that it scavenges superoxide radicals and suppresses platelet aggregation, leukocyte adhesion and smooth muscle cell proliferation (19–22). Dysregulated NO production by the mutant eNOS may promote coronary atherosclerosis and thrombosis.

Wang et al. (34) have recently reported that a smoking-dependent risk of ischemic heart disease is associated with a polymorphism ecNOS4a/b in intron 4 of the eNOS gene. In this regard, the data of the present study have added another example of an association between a polymorphism/mutation of the eNOS gene and cardiovascular disorders.

**Study limitations.** We examined the distribution of the missense Glu298Asp variant in 607 volunteer control subjects, representative of the general population of Kumamoto Prefecture, and found that the frequency of the variant was 13.3%. However, it is possible that some volunteers were highly health conscious and underwent a check-up examination because of a family history of ischemic heart disease. Furthermore, we cannot exclude that myocardial ischemia might have been present in some control subjects. The frequency of the mutation may be lower than 13.3% in the actual general population. These volunteers with the mutation should be evaluated in further, prospective studies.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Beta-Coef</th>
<th>SE</th>
<th>p Value*</th>
<th>OR† (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM</td>
<td>1.331</td>
<td>0.215</td>
<td>&lt; 0.001</td>
<td>3.7 (2.1–6.6)</td>
</tr>
<tr>
<td>HTN</td>
<td>1.213</td>
<td>0.184</td>
<td>&lt; 0.001</td>
<td>3.3 (2.1–5.2)</td>
</tr>
<tr>
<td>Cig smoker</td>
<td>1.183</td>
<td>0.172</td>
<td>&lt; 0.001</td>
<td>3.2 (2.1–4.8)</td>
</tr>
<tr>
<td>Hyperchol</td>
<td>0.783</td>
<td>0.178</td>
<td>&lt; 0.001</td>
<td>2.1 (1.6–2.8)</td>
</tr>
<tr>
<td>BMI</td>
<td>0.455</td>
<td>0.199</td>
<td>0.022</td>
<td>1.5 (1.3–1.8)</td>
</tr>
<tr>
<td>Glu298Asp var‡</td>
<td>0.437</td>
<td>0.212</td>
<td>0.039</td>
<td>1.5 (1.2–1.8)</td>
</tr>
<tr>
<td>Constant</td>
<td>-2.397</td>
<td>0.177</td>
<td>&lt; 0.001</td>
<td>—</td>
</tr>
</tbody>
</table>

*One degree of freedom. †Exp(beta). ‡eNOS/TT (homozygous carriers of eNOS Glu298Asp variant) and eNOS/TG (heterozygous carriers of the eNOS Glu298Asp variant) combined. Coeff = coefficient; other abbreviations as in Tables 1 and 2.
The functional significance of the missense Glu298Asp variant of the eNOS gene has not yet been demonstrated. Our computer analysis revealed that glutamic acid at position 298 is in an alpha-helix and that the missense Glu298Asp variant results in a tight turn (35) (unpublished data), suggesting that the missense Glu298Asp variant may affect the function of eNOS protein. However, X-ray diffraction by protein crystals is required to determine the exact three-dimensional structure and gain an understanding of the molecular basis of the protein’s function.

Conclusions. The present study found a significant association between the missense Glu298Asp variant of the eNOS gene and MI. Thus, we suggest that the presence of the missense Glu298Asp variant of the eNOS gene is a possible genetic risk factor for MI.

References