Lack of Association between the Glu298Asp Variant of the Endothelial Nitric Oxide Synthase Gene and the Risk of Coronary Artery Disease Among Taiwanese

Chiun-Li Wang, Lung-An Hsu, Yu-Shien Ko, Yu-Lin Ko, and Ying-Hsiung Lee

**Background and Purpose:** Endothelial nitric oxide synthase (eNOS) plays a key role in atherosclerosis, because its product, nitric oxide, possesses antitherogenic properties. Recent reports of molecular genetic analysis have suggested that genetic polymorphisms of the eNOS gene may be associated with coronary artery disease (CAD) or myocardial infarction (MI). However, some studies have reported discrepant results. The aims of this study were to assess whether any association exists between the Glu298Asp variant of the eNOS gene and the risk of CAD and/or MI among Taiwanese.

**Methods:** The subjects included 218 CAD patients and the same number of age- and sex-matched control subjects from Taiwan. Subjects’ DNA was extracted from their blood and genotypes were determined by polymerase chain reaction and restriction mapping using the restriction enzyme *Mbo*I. The allelic and genotypic frequencies were analyzed.

**Results:** The frequencies of the eNOS genotypes were similar for CAD patients (GG: GT:TT = 81.7%:17.4%:0.9%) and controls (81.2%:17.4%:1.4%; p = 0.904). No evidence of difference was found in the frequency of the T allele between CAD patients (9.6%) and controls (10.1%; p = 0.822), or between MI patients (7.5%) and controls (p = 0.322). Subjects with the GT or TT genotype did not demonstrate an increased risk of CAD compared with those with a GG genotype (p = 0.89; OR = 0.98; 95% confidence interval, CI, 0.76–1.27) in multivariate logistic regression, or when different subgroups of age, sex, or risk factors were analyzed.

**Conclusions:** In the present case-control study, we found no evidence of an association between the Glu298Asp variant of the eNOS gene and CAD/MI among Taiwanese.

Epidemiologic studies have shown that coronary artery disease (CAD) is associated with various risk factors, including hypertension, diabetes mellitus, cigarette-smoking, and hyperlipidemia. However, such classic risk factors explain less than half of the variability in the risk of CAD and evidence increasingly suggests that genetic factors may play an important and independent role in an individual’s predisposition to CAD and its associated thrombotic complications [1, 2].

Endothelium-derived relaxing factor (EDRF), a powerful vasodilator synthesized by the endothelium, has been identified as the nitric oxide radical or a sulfhydryl complex containing it [3]. In addition to its vasodilator action, endothelial nitric oxide inhibits smooth-muscle cell proliferation, suppresses platelet aggregation, inhibits platelet and monocyte adhesion to vascular endothelium, and limits the oxidation of atherogenic low-density lipoprotein [4–7]. All these
Endothelial Nitric Oxide Synthase Polymorphism

processes are important events during atherogenesis. It has been suggested that endothelial nitric oxide may play an important role in atheroprotection, and a deficit in, or the decreased formation of, endothelial nitric oxide may enhance atherosclerosis [8].

Nitric oxide is synthesized from L-arginine by the enzyme nitric oxide synthase [9, 10]. At least three isoforms of nitric oxide synthase have been identified: two constitutive isoforms: neuronal and endothelial nitric oxide synthase (eNOS), and an inducible isoform. In endothelial cells, nitric oxide is synthesized by eNOS, which is encoded by a 26-exon gene located on chromosome 7q35–36 [11]. Recent reports of molecular genetic analysis have suggested that eNOS gene polymorphisms may be associated with coronary atherosclerosis and/or myocardial infarction (MI). Hibi et al first reported in 1998 that patients with the TT genotype (Asp homozygosity) of the Glu298Asp variant of the eNOS gene were genetically predisposed to MI [12]. Hingorani et al studied subjects from England and demonstrated that the TT genotype of eNOS was associated with, and placed carriers at, a higher risk for angiographic CAD and MI [8]. Two other studies, however, found no consistent association between the Glu298Asp variant of eNOS and CAD and/or MI [13, 14]. To determine whether there is an association between the Glu298Asp variant of the eNOS gene and CAD, we analyzed the eNOS genotype of Taiwanese CAD patients.

Materials and Methods

Study population

This study included 218 patients with CAD and the same number of age- and sex-matched control subjects. They were admitted to Chang Gung Memorial Hospital, Taipei, from August 1994 through September 1996. All CAD patients had coronary angiographic evidence of more than 50% stenosis of at least one major coronary artery. Of the patients with CAD, 114 (52%) had experienced at least one previous MI attack that had been clinically verified by electrocardiography and evidence of left ventricular regional wall-motion abnormalities on left ventriculography.

Control subjects were recruited from patients undergoing routine health examination, and were individually matched for sex and age (within 2 yr) with CAD patients. Control subjects were excluded if they had any clinical evidence of CAD including: history of typical angina pectoris; abnormal Q wave or ST-T changes on electrocardiography; or positive Master exercise test results.

The presence of hypertension, diabetes mellitus, hyperlipidemia, or cigarette-smoking was determined by history-taking, previous medical results, current medication, or results of examination during hospitalization. Obesity was defined as a body mass index (BMI) of 26 kg/m² or greater. The hospital’s ethics committee approved the study protocol.

Genomic DNA extraction and genotyping

Approximately 10 mL of blood was drawn into heparinized tubes, and white blood cells were separated by centrifugation. Genomic DNA was extracted from peripheral blood leukocytes by a standard method using proteinase K digestion of nuclei. Phenol/chloroform extraction of DNA was followed by isopropanol precipitation. Laboratory personnel performed genotyping without knowledge of the angiographic data. Detection of the G894-to-T transition in the eNOS gene was performed via polymerase chain reaction (PCR) amplification of exon 7 with the flanking intronic primers 5’-CATGAGGCTCAGCCCCAGAAC-3’ (sense) and 5’-AGTCAATCCCTTTGGTGCTCAC-3’ (antisense), followed by MboI restriction endonuclease digestion for 16 hours at 37°C and resolution by electrophoresis on a 2.5% agarose gel [8].

Statistical analysis

Chi-square test and stepwise regression analysis were used for comparison of the allelic and genotypic frequencies using the SPSS program (SPSS 10.0, SPSS Inc, Chicago, IL, USA). The clinical characteristics of the case and control groups were expressed as mean ± standard error of the mean (SEM) and were compared using the unpaired Student’s t-test.

Results

The clinical characteristics of the study population are shown in Table 1. The frequencies of classic risk factors for CAD, such as hypertension, diabetes mellitus, hyperlipidemia, and smoking, were significantly greater among CAD patients than control subjects, as has been reported previously [15]. The genotypic and allelic frequencies of the eNOS gene Glu298Asp variant among CAD or MI patients and controls are shown in Table 2. The distributions of genotypes in the two groups were in agreement with those predicted by the Hardy-Weinberg equilibrium. Frequencies of the eNOS genotypes were similar for CAD patients and controls (p = 0.904). The T allele occurred at almost the same frequency among CAD patients and controls (p = 0.822). The distribution of genotypes among patients with MI was also similar to that
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Table 1. Clinical characteristics of the study population

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 218)</th>
<th>CAD patients (n = 218)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>61.29 ± 0.60</td>
<td>61.5 ± 0.60</td>
<td>0.987</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>168/50</td>
<td>168/50</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.34 ± 0.25</td>
<td>25.19 ± 0.25</td>
<td>0.785</td>
</tr>
<tr>
<td>Hypertension</td>
<td>31%</td>
<td>45%</td>
<td>0.002</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>8.7%</td>
<td>31%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>11%</td>
<td>22%</td>
<td>0.002</td>
</tr>
<tr>
<td>Smoking</td>
<td>42%</td>
<td>57%</td>
<td>0.003</td>
</tr>
</tbody>
</table>

CAD = coronary artery disease; BMI = body mass index.

Table 2. Specific endothelial nitric oxide synthase genotypes and their allelic frequencies in coronary artery disease (CAD) patients, CAD/myocardial infarction (MI) patients, and controls

<table>
<thead>
<tr>
<th>Genotype*</th>
<th>Controls (n = 218)</th>
<th>CAD patients (n = 218)</th>
<th>CAD/MI patients (n = 114)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>177 (81.2%)</td>
<td>178 (81.7%)</td>
<td>97 (85.1%)</td>
</tr>
<tr>
<td>GT</td>
<td>38 (17.4%)</td>
<td>38 (17.4%)</td>
<td>17 (14.9%)</td>
</tr>
<tr>
<td>TT</td>
<td>3 (1.4%)</td>
<td>2 (0.9%)</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Alleles†</th>
<th>%</th>
<th>%</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>89.9%</td>
<td>90.4%</td>
<td>92.5%</td>
</tr>
<tr>
<td>T</td>
<td>10.1%</td>
<td>9.6%</td>
<td>7.5%</td>
</tr>
</tbody>
</table>

Comparisons between CAD patients, CAD/MI patients with controls: *p = 0.804 (CAD), p = 0.832 (CAD/MI); †p = 0.003 (CAD/MI).

Discussion

In animal models, the inhibition of nitric oxide production accelerates atherosclerosis, whereas its administration reverses it [16]. Genetic contribution of the eNOS gene polymorphism to plasma nitric oxide levels has been previously demonstrated [17, 18]. Several different polymorphisms of the eNOS gene have been identified [19]; however, the purported association of these polymorphisms and CAD remains controversial. In the present case-control study, we found no evidence of an association between the Glu298Asp variant of the eNOS gene and the risk of either angiographically-defined CAD or MI. Our results suggest that this genetic variant of the eNOS gene is not an important risk factor for CAD or MI among Taiwanese.

The eNOS-4 allele was the first polymorphism shown to be associated with an enhanced risk of CAD in Australian smokers and Japanese individuals [20, 21]. In the Edinburgh artery study, the eNOS-4 allele was found to be related to the occurrence of CAD in the non-smoking group (OR = 2.47, 95% CI = 1.42–4.34, p = 0.02) [22]. Hibi et al found no evidence of a significant increase in the risk of acute MI or the severity of coronary atherosclerosis among individuals with the a/a genotype of the eNOS-4a/b polymorphism (p = 0.74), and in the same study, patients with the TT genotype (Asp homozygosity) of the Glu298Asp variant of the eNOS gene were suggested to be genetically predisposed to acute MI (p = 0.0085) [12].

The association between the Glu298Asp variant of the eNOS gene and coronary atherosclerosis and MI has been supported by other reports [8, 23]. A study in England by Hingorani et al demonstrated that homozygosity (TT genotype) for the Glu298Asp variant of the eNOS gene was associated with, and placed carriers at, an elevated risk for angiographic CAD (OR = 4.2; 95% CI = 2.3–7.9) and MI (OR = 2.5, 95% CI = 1.3–4.2) [8]. Significant association has been found between the TT/GT genotype of the Glu298Asp variant of the eNOS gene and MI in a Japanese sub-population (frequency in MI patients vs control group: 21.1% vs 13.3%, p = 0.003) [23]. In addition, in the analysis of 213 Japanese subjects (113 patients with coronary artery spasm, and 100 control subjects) in 1998, Yoshimura et al demonstrated a greater frequency of the TT and GT genotypes among subjects with coronary artery spasm (21.2%) than in controls (9.0%, p = 0.014). They postulated that the missense Glu298Asp variant of the

Table 3. Logistic regression analysis of the various risk factors pertaining to coronary artery disease

<table>
<thead>
<tr>
<th>Variable</th>
<th>OR</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension</td>
<td>1.32</td>
<td>1.07–1.63</td>
<td>0.020</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>2.14</td>
<td>1.60–2.86</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>1.45</td>
<td>1.10–1.92</td>
<td>0.052</td>
</tr>
<tr>
<td>Smoking</td>
<td>1.43</td>
<td>1.17–1.76</td>
<td>0.003</td>
</tr>
<tr>
<td>Obesity</td>
<td>1.14</td>
<td>0.91–1.41</td>
<td>0.216</td>
</tr>
<tr>
<td>eNOS genotype</td>
<td>0.98</td>
<td>0.76–1.27</td>
<td>0.890</td>
</tr>
</tbody>
</table>

OR = odds ratio; CI = confidence interval; eNOS = endothelial nitric oxide synthase.
The eNOS gene affects the function of the eNOS protein [24]. Philip et al reported in 1999 that patients who were allele 894T (TT and GT) carriers had an enhanced responsiveness to alpha-adrenergic stimulation ($p = 0.02$) [25], although the ECTIM (Etude Cas-Témoins sur l’Infarctus du Myocarde) study had found no consistent evidence of an association between this gene polymorphism and the risk of MI ($p > 0.05$) [13]. Poirier et al found a higher frequency of the G allele in French MI patients compared to previous studies [13]. Liyou et al reported that an association between the Glu298Asp variant of the eNOS gene and the risk of CAD was not apparent in 1,119 elderly residents of Dubbo, Australia (genotype frequency, $p = 0.54$; allele frequency, $p = 0.07$) [14]. In our study, we also found a lack of association between the Glu298Asp variant of the eNOS gene and the risk of CAD and MI among Taiwanese.

The discrepancies in these results and the resulting controversy may be related to a number of factors. It is possible that ethnic differences may be responsible for the discrepancies between the results of these various studies [8, 13, 14, 23]. It has been noted that the allele frequency for the Glu298Asp variant of the eNOS gene in Japanese and Caucasian populations differs markedly [8, 12, 13, 23]. The T allele frequency appears to be lower among the Japanese population (6.8–8.7%) [12, 23] than among the Caucasian population (31.2–38.9%) [8, 13]. The frequency of the T allele (10.1%) in our study is similar to that reported from studies in the Japanese population. It is also possible that our sample size may have been somewhat masked by their conventional risk factors for CAD and/or MI (Table 1). It is possible that these conventional risk factors for CAD and/or MI may exhibit an over-riding effect over the analogous effect attributed to genetic polymorphism. It has been suggested that genetic factors are more likely to affect younger rather than older people and that certain genetic factors may contribute to premature atherosclerosis [26]. Thus, for those subjects in our study exhibiting conventional risk factors for CAD and/or MI, their genetic contribution may have been somewhat masked by their conventional risk factors. A previous study has linked the smoking-dependent risk of CAD with polymorphism of the eNOS gene [20]. However, our analysis of the association between CAD and MI within a smoker subgroup with the Glu298Asp variant of the eNOS gene found no apparent increased risk of CAD and/or MI with this gene variant (data not shown). Furthermore, as in some previously reported studies of the relationship between the Glu298Asp variant and CAD [8, 13, 14], our study employed a case-control design and thus a potential survival bias could not be avoided. Therefore, a larger, prospective, and longitudinal study is needed to confirm the role of the Glu298Asp variant in CAD and MI.

In conclusion, this case-control study with age- and sex-matched controls found no evidence of an association between the Glu298Asp variant of the eNOS gene and CAD or MI among Taiwanese.

### References


