CARDIOVASCULAR MEDICINE

Association of endothelial constitutive nitric oxide synthase gene polymorphism with acute coronary syndrome in Koreans


Objective: To examine the effects of two polymorphisms of the endothelial constitutive nitric oxide synthase (ecNOS) gene, 4a/4b(A:B) located in intron 4 and Glu298Asp(G:T) located in exon 7, on the development of acute coronary syndromes (ACS).

Methods: 164 patients with ACS and 142 control participants were investigated for genotype and conventional risk factors. Genotype was determined by polymerase chain reaction and restriction fragment length polymorphism analysis.

Results: Genotype and allele frequencies of the A:B polymorphism in the ACS group (0.15:0.85 for AA+AB:BB, 0.09:0.91 for A:B) differed from those in the control group (0.26:0.74 for AA+AB:BB, 0.15:0.85 for A:B). However, genotype and allele frequencies of the G:T polymorphism in the ACS group (0.22:0.78 for TT+TG:GG, 0.11:0.89 for T:G) were similar to those in the control group (0.17:0.83 for TT+TG:GG, 0.09:0.91 for T:G). Multiple logistic regression analysis showed that the non-BB (AA+AB) and the non-BB+GG genotypes were significant protective factors against ACS (odds ratios 0.49 and 0.34, 95% confidence intervals 0.26 to 0.93 and 0.14 to 0.83, respectively). In addition, linear association analysis showed that the percentage of ACS patients was significantly lower in the genotype group non-BB than in the genotype group BB+GG (39.6% v 62.7%, p = 0.01).

Conclusions: The non-BB genotype of the ecNOS 4a/4b gene polymorphism is a protective factor against the development of ACS. The GG genotype of the ecNOS Glu298Asp polymorphism exerts a benefit in addition to the non-BB genotype in the Korean population.

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Patients who had smoked more than a third of a pack a day for more than one year were considered to be smokers.

**Gene analysis**

DNA was prepared from blood leucocytes by standard methods.\(^{10}\) Target regions of the ecNOS gene in extracted DNA samples were amplified by polymerase chain reaction according to previously reported protocols.\(^ {7,8}\) Briefly, for the ecNOS4a/4b polymorphism, the forward primer used was 5'-AGG CTC TAT GGT AGT GCC TTT-3' and the reverse primer 5'-TCT CTT AGT GCT GTG GTC AC-3'. For the Glu298Asp polymorphism, the forward primer was 5'-AGG CCA GGA GAC AGT GGA TGG A-3' and the reverse primer 5'-CTC TAA TTT GGT GAT CA-3'. A volume of 50 \( \mu \)l was used for each polymerase chain reaction, which contained 1 \( \mu \)M of each primer, 200 \( \mu \)M each of dNTP, 1.5 \( \mu \)M MgCl\(_2\), 50 \( \mu \)mol/L KCl, 10 \( \mu \)mol/L Tris-Cl at pH 8.3, and 1.6 U Taq polymerase (Boehringer Mannheim Biochemica, Mannheim, Germany) with 1 \( \mu \)g of genomic DNA. The conditions of amplification were denaturing at 94°C for five minutes, 35 cycles at 94°C for one minute, 56°C for one minute, 72°C for two minutes, and finally termination at 72°C for five minutes.

The polymerase chain reaction products of the ecNOS4a/4b polymorphism were electrophoresed in 3% agarose gel (NuSieve GTG agarose, FMC) and visualised by ethidium bromide staining. The polymerase chain reaction products of the Glu298Asp polymorphism were digested with the restriction enzyme BanII (Boehringer Mannheim). The digested products were also electrophoresed in 3% agarose gel (NuSieve GTG agarose, FMC) and visualised by ethidium bromide staining.

**Statistical analysis**

Case-control differences in clinical and biological continuous variables were analysed with a general linear model. Categorical variables were evaluated with the \( \chi^2 \) test. The observed frequencies of the genotypes were compared with the frequencies expected under the Hardy-Weinberg equilibrium by \( \chi^2 \) tests. To evaluate the combined effects of the two polymorphisms, the genotypes were grouped as BB+non-GG (TT+TG), BB+GG, non-BB (AA+AB)+non-GG, and non-BB+GG. Linear association analysis was used to identify any relations between the genotype groups and the percentage of patients with ACS in each group. We hypothesised that the prevalence of ACS would be higher among patients with the BB and non-GG genotypes than among patients with the non-BB and GG genotypes. Multiple logistic regression analysis was used to estimate the contributions made by the ecNOS4a/4b and Glu298Asp polymorphisms to ACS. SPSS for Windows version 10.0 (SPSS Inc, Chicago, Illinois, USA) was used for statistical analysis and \( p < 0.05 \) was considered significant.

### RESULTS

#### Clinical characteristics

Table 1 summarises the clinical characteristics of patients enrolled in the study. Mean (SD) age, body mass index, the presence of hypertension, and mean (SD) plasma concentrations of total cholesterol, low density lipoprotein cholesterol, and high density lipoprotein cholesterol were similar between the ACS group and control group. There were significantly higher percentages of men, smokers, and patients with diabetes mellitus in the ACS group (\( p < 0.05 \)). The baseline triglyceride concentrations were higher in the ACS group (\( p < 0.05 \)).

#### ecNOS gene polymorphism and ACS

Table 2 shows the distribution of genotypes and the prevalence of alleles in the control group and the ACS group.

The prevalence of the ecNOS 4a allele was 15% and of the Glu298Asp T allele 9% in the control group, which was comparable with a previous report on Koreans.\(^ {11}\) When the control group and the ACS group were compared, a significant difference was found in the distribution of the ecNOS4a/4b genotype and allele prevalence (table 2); the ecNOS4a allele was more common in the control group (15% \( v \) 9%, \( p = 0.019 \)). In the analysis of the dominant effects of the ecNOS4a (A) allele, the prevalence of the non-BB genotype (AA+AB) was found to be significantly higher in the control group than in the ACS group (26% \( v \) 15.2%, \( p < 0.05 \)). However, the genotype distributions and allele prevalences of the Glu298Asp polymorphism were not significantly different between the control and ACS groups. When the genotypes of the two polymorphisms were combined as BB+non-GG (TT+TG), BB+GG, non-BB (AA+AB)+non-GG, and non-BB+GG, a significant difference was found in genotype distribution and a significant linear trend was observed in terms of the percentage of patients with ACS between the combined genotype groups. The proportion of patients with ACS was significantly lower in the genotype group non-BB+GG than in the genotype group BB+non-GG (39.6% \( v \) 62.7%, \( p = 0.01 \)) (table 3, fig 1).

To determine whether the polymorphisms were independent risk factors or protective factors of ACS, we performed multiple logistic regression analysis with conventional risk factors. The results showed that the non-BB (AA+AB) genotype was an independent protective factor against ACS (odds ratio 0.49, 95% confidence interval 0.26 to 0.93;
The Hardy-Weinberg equilibrium (distribution of the study group (CAD patients) deviated from 0.170/0.830 in the control group; \( p < 0.05 \)), thus showing a further benefit in terms of protection against ACS. In this model, other independent risk factors of ACS were smoking, diabetes mellitus, male sex, hypercholesterolaemia, and increasing age.

**DISCUSSION**

Our study shows that the ecNOS4a/4b polymorphism is of pathophysiological significance in ACS, at least in this Korean population. The results show that the non-BB genotype of the ecNOS4a/4b polymorphism has a protective effect against the development of ACS and the GG genotype of the ecNOS Glu298Asp polymorphism exerts an additive beneficial effect on the non-BB genotype. These findings imply that polymorphisms of the ecNOS gene may have significant roles in the pathogenesis of ACS.

**ecNOS gene polymorphism and ACS**

Investigations into the relation between ecNOS gene polymorphism and CAD have given various and sometimes contradictory results. Uwabo et al. showed that the ecNOS4a allele is associated with essential hypertension and may be a genetic marker of the disease. Ichihara et al. showed that the ecNOS 4a allele is an independent risk factor of MI, and Wang et al. reported that the ecNOS 4a/4a homozygote is a smoking dependent risk factor of CAD. However, studies by Hibi et al. and Jeerooburkhan et al. found no association between the ecNOS 4a/4b polymorphism and acute MI.

Likewise, the Glu298Asp polymorphism of the ecNOS gene has been shown to be associated with acute MI in some studies but others found no association between the Glu298Asp polymorphism and premature CAD.

In the present study, we found that the ecNOS4a allele exerts a protective effect against ACS and that this effect is not attenuated but rather potentiated by the GG genotype of the Glu298Asp polymorphism, which by itself was not found to be a significant protective factor against ACS. To the best of our knowledge, this is a novel finding, which contradicts previous reports. The discrepancy between the results of previous studies and ours may be explained as follows. Firstly, the study populations in previous studies deviated from the Hardy-Weinberg equilibrium and therefore the results obtained could not be easily applied to the general population. In the report by Wang et al., the genotype distribution of the study group (CAD patients) deviated from the Hardy-Weinberg equilibrium (\( \chi^2 = 5.41, p = 0.035 \)). Furthermore, the observed prevalence of the 4a/4a genotype in the subgroup with no significantly diseased vessels was zero, while the expected prevalence under the Hardy-Weinberg equilibrium was 2.5. In addition, the allele prevalence and distribution of the patient group were not significantly different from those of the control group (4a/4b allele prevalence was 0.144/0.857 in patients with CAD and 0.170/0.830 in the control group; \( p > 0.05 \)). The study showed a difference in the genotype distribution within the patient group—that is, between patients with no significantly diseased vessels and patients with one or more significantly diseased vessels. However, even this was no longer significant when lifetime smoking dose was entered into the analysis. The only significant finding was that, in smokers, there was a significant correlation between those with one or more significantly diseased vessels and the ecNOS polymorphism. Secondly, the patient selection was different—that is, unstable angina, a very important and common disease that shares the same pathophysiological mechanism as MI, was not examined in the study group. In studies by Ichihara et al. and Hibi et al., the study groups consisted only of patients with MI. If ecNOS polymorphism contributes significantly to the pathogenesis of MI, as previous investigators think it does, it should contribute similarly to the development of unstable angina. Since previous studies did not examine unstable angina, the data in these studies cannot be generalised to patients with ACS.

In contrast, not only was the genotype distribution of the ACS group and the control group in our study within Hardy-Weinberg equilibrium, but our results also showed a significant difference in both genotype distribution and allele prevalences between the two groups. Furthermore, the study group not only consisted of MI patients but also included patients with unstable angina. In addition, although our findings seem new and in some ways contradictory to previous results, a number of studies support our findings indirectly. For example, in white patients, nitric oxide metabolites were found to be significantly higher in the 4a/4a genotype, which suggests a higher concentration of basal nitric oxide release in the 4a/4a genotype. Moreover, a reduction in basal nitric oxide release has been reported to predispose to hypertension, thrombosis, vasospasm, and atherosclerosis. In Koreans, the 4a allele has also been associated with higher concentrations of nitric oxide metabolites, although the association was significant only in hypertensive patients with CAD in one study and associated with higher nitric oxide metabolite concentrations in smokers in another study. The effect of the Glu298Asp polymorphism on nitric oxide concentrations is also unclear. Although no previous study has documented the exact role of the polymorphism on the plasma concentration of nitric oxide metabolites, previous studies have shown that the T allele is associated with impaired endothelial function and thus is suspected to be associated with a reduction in nitric oxide production.

The exact mechanism by which the ecNOS4a/4b gene polymorphism affects the pathogenesis of ACS is not known.

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**Table 3** Combined analysis of occurrence of ecNOS4a/4b and Glu298Asp polymorphisms in the ACS and control groups

<table>
<thead>
<tr>
<th>Combined genotypes</th>
<th>ACS group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>BB+non-GG</td>
<td>32 (62.7%)</td>
<td>19 (37.3%)</td>
</tr>
<tr>
<td>BB+GG</td>
<td>107 (55.4%)</td>
<td>86 (44.6%)</td>
</tr>
<tr>
<td>Non-BB+non-GG</td>
<td>4 (4.4%)</td>
<td>5 (5.6%)</td>
</tr>
<tr>
<td>Non-BB+GG</td>
<td>21 (39.6%)</td>
<td>32 (60.4%)</td>
</tr>
</tbody>
</table>

Linear by linear association \( p = 0.01 \).

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**Figure 1** Linear by linear association of the combined genotypes of the endothelial constitutive nitric oxide synthase (ecNOS) polymorphism. Group 1: BB+non-GG genotype; group 2: BB+GG genotype; group 3: non-BB+non-GG genotype; group 4: non-BB+GG genotype. The proportion of patients with ACS was significantly lower in the genotype group non-BB+GG than in the genotype group BB+non-GG (\( p = 0.01 \)).

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Previous studies have shown that the \textit{ec}NOS gene polymorphism is responsible for variations in the genetic control of the plasma concentration of nitric oxide metabolites.\textsuperscript{17, 21} Moreover, nitric oxide can inhibit vascular smooth cell proliferation,\textsuperscript{1} which is responsible for the synthesis and assembly of the macromolecules that strengthen the fibrous cap. Therefore, there is a possibility that the inhibition of smooth muscle cell proliferation with changing \textit{ec}NOS activity determined by \textit{ec}NOS genotype contributes to the formation of a friable fibrous cap.\textsuperscript{22}

\textbf{Limitations of this study}

The first limitation concerns the sample size of the study. We examined the distribution of the \textit{ec}NOS gene polymorphisms in 142 control subjects and 164 patients, which is not a large sample. However, the genotype prevalences of the polymorphisms in each group were in Hardy-Weinberg equilibrium. A limitation is that patients in the controls were not healthy volunteers representative of the general population and they had various atypical symptoms. However, the control participants were confirmed by coronary angiography to be free of significant coronary stenosis. Lastly, the intermediate phenotypes of the polymorphism were not studied. Therefore, this study goes only as far as showing a significant association between \textit{ec}NOS polymorphism and ACS.

In conclusion, our study shows that the non-BB genotype of the \textit{ec}NOS 4a/4b gene polymorphism is a protective factor against the development of ACS and that the GG genotype of the \textit{ec}NOS Glu298Asp polymorphism exerts an additive beneficial effect on the non-BB genotype in the Korean population. These findings imply that, although the mechanism underlying the association between the \textit{ec}NOS gene polymorphism and ACS has so far remained elusive, the genetic background controlling nitric oxide may be associated with the pathogenesis of ACS.

\textbf{ACKNOWLEDGEMENTS}

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\textbf{REFERENCES}


\begin{table}[h]
\centering
\caption{Multiple logistic regression analysis of the effect of the \textit{ec}NOS gene polymorphism on ACS} \label{tab:10}
\begin{tabular}{|l|c|c|c|}
\hline
 & Odds ratio & 95\% CI & \textit{p} Value \\
\hline
Smoking & 2.781 & 1.458 to 5.305 & <0.05 \\
Diabetes mellitus & 2.651 & 1.335 to 5.262 & <0.05 \\
Sex (male/female) & 2.215 & 1.130 to 4.431 & <0.05 \\
Hypercholesterolemia & 1.785 & 1.051 to 3.032 & <0.05 \\
Age & 1.480 & 1.118 to 1.960 & <0.05 \\
Hypertension & 1.116 & 0.687 to 1.979 & NS \\
non-B/B & 0.489 & 0.257 to 0.928 & <0.05 \\
non-B=B-G/G & 0.340 & 0.140 to 0.827 & <0.05 \\
\hline
\end{tabular}
\footnote{Total cholesterol > 4.15 mmol/l; odds ratio to B/B
non-G/G genotype.}
\end{table}

\textit{CI}, confidence interval.
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