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# Aging Affects the Association Between Endothelial Nitric Oxide Synthase Gene Polymorphism and Acute Myocardial Infarction in the Korean Male Population.

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**Objectives :** *The aging process affects responsiveness and other functions of endothelium and vascular smooth muscle cells, predisposing the old vessels to the development of atherosclerotic lesions. Endothelial nitric oxide synthase (ecNOS) gene polymorphisms were shown to affect the occurrence of acute myocardial infarction (AMI). We hypothesized that aging may affect the association between the ecNOS gene polymorphism and AMI.*

**Methods :** *We investigated the age-related distribution of the ecNOS gene a/b polymorphism in 121 male AMI patients and 206 age-matched healthy male controls.*

**Results :** *The aa, ab and bb genotypes were found in 1, 49 and 156 cases among the control subjects and 5, 23 and 93 cases among the AMI patients, respectively. There was a significant correlation between the ecNOS polymorphism and AMI ($p=0.045$). When the correlation was analyzed by age, the significance remained only in the group below the age of 51 ($p=0.009$). The proportion of smokers was increased in the young patients when compared to the old patients ($p=0.033$), indicating that smoking also has greater effect on the younger population. The incidences of hypertension and diabetes mellitus, however, were similar in both populations.*

**Conclusion :** *Our work provides the first evidence that links ecNOS polymorphism to the risk of AMI in relation to age. Young persons who smoke or have ecNOSaa genotype may have an increased risk of developing AMI. The functional as well as structural changes associated with aging in the vascular endothelium may mask the effect of the ecNOS polymorphism in the development of AMI in old persons.*

**Key Words :** *Myocardial infarction; Nitric oxide synthase; DNA polymorphism; Smoking; Aging.*

## INTRODUCTION

Factors influencing endothelial dysfunction in relation to AMI are not completely understood except for smoking which causes reduction in nitric oxide (NO) production by the endothelium and, thus, reduces the NO-dependent vasodilation. NO is the major molecule responsible for vascular regulation. NO mediates endothelium dependent vasodilatation, inhibits smooth muscle cell proliferation and adhesion of platelets and monocytes to endothelium, and plays the role of an

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oxygen radical scavenger. These essential roles of NO in vascular regulation suggest that a derangement in endothelial NO synthesis may lead to the development of atherosclerosis. It has been reported that the *ecNOS* gene a/b polymorphism, caused by four (allele ecNOSa) or five (allele ecNOSb) repeats of a 27 base pair sequence in intron 4 of the *ecNOS* gene, is associated with the risk of coronary artery disease (CAD) and AMI. It has also been suggested that the effect of cigarette smoking on the severity of CAD is greater in ecNOSa homozygotes than in heterozygotes or ecNOSb homozygotes. Although it is not known whether the ecNOS a/b polymorphism is associated with the variation in the expression level or functional activity of the enzyme, a recent report by Tsukada *et al.* demonstrated that the presence of ecNOSa allele is associated with the decrease in the level of plasma NO metabolites.

Increasing numbers of experimental and clinical data demonstrate that endothelial dysfunction is encountered in the normal physiological process of aging as well as in the disease state, such as atherosclerosis, hypertension, heart failure or diabetes. Various studies employing aged human and experimental animals indicated that the aged vascular endothelium has a reduction in the responsiveness to external stimulus resulting in an impairment of the NO-mediated vasodilation . Structural changes associated with the aging process also result in endothelial dysfunctions. Aged vasculature have accumulation in *the* extracellular matrix, increase in collagen content, disorganization of elastin fibers that lead to distension of the lumen diameter, kinking of the artery and increase in the rigidity of the arterial wall.

Since smoking is a major risk factor for AMI in young patients more than in old patients , we expected that any genetic predisposition associated with the hazard of smoking would be relatively easy to identify in young AMI patients. We chose to analyze the *ecNOS* gene which is involved in the endothelium-dependent vasodilation through the generation of NO. Our data indicate that there is a significant association between the ecNOSa/b polymorphism and the risk of AMI, and the association is affected by age.

## MATERIALS AND METHODS

### 1. Selection of patients and control population

A total of 121 unrelated Korean AMI patients (out of 243 acute myocardial infarction patients who had been questioned for participation in the study), from age 20 - 80 years who were consecutively referred to the Samsung Medical Center (SMC) for coronary angiography from 1994 to 1997, were entered in the study. During the recruitment, greater emphasis was put on AMI patients under age of 51, because these patients constituted only 24% of the total AMI patients. As a result, AMI patients under the age 51 constituted 46% (55 out of 121) of the study population. The diagnosis of AMI was based on the typical electrocardiographic changes and the clinical and laboratory data. The blood samples were taken at the time of a coronary angiography in the cardiac catheterization laboratory. The control subjects, selected from individuals who came to the health promotion center for periodic medical check-up, were healthy individuals without any history or diagnosis of coronary artery diseases. They were genetically unrelated and selected randomly for the current study after obtaining an informed consent. The investigation conforms with the principles outlined in the Declaration of Helsinki.

### 2. Identification of ecNOS polymorphism

The DNA was extracted from the WBCs by a method described by Miller *et al.* . The extracted DNA was stored at 4o℃ until the analysis. The DNA samples were subjected to amplification by the polymerase chain reaction (PCR). The oligonucleotide primers and PCR reactions were performed, as described previously . The PCR products were separated by electrophoresis in 2% agarose gel and visualized by ethidium bromide staining.

### 3. Statistical analysis

The frequencies of the alleles and genotypes among different subgroups were compared by the Chi-square test and the clinical data were compared by the Mann-Whitney test in the Prism statistical program (Graph Pad Software).

## RESULTS

We excluded the female patients because more than 95% of the female CAD patients were older than 50. The demographic and clinical features of the study subjects are shown in Table 1. When compared to the control subjects, the patient population had a higher incidence of

**Table 1. Characteristics of the study population**

| Group | Control (n=206) | AMI group (n=121) | p* |
|---|---|---|---|
| Age (yrs) | 51.0±9.5 | 53.5±11.6 | ns** |
| BMI (kg/m$^2$) | 24.0±2.6 | 24.4±3.2 | ns |
| Total Cholesterol (mg/dl) | 195.3±34.7 | 189.3±37.2 | ns |
| Current Smokers | 100(48.5)† | 90(74.4) | <0.0001 |
| Hypertension | 15(7.3) | 41(33.9) | <0.0001 |
| Diabetes Mellitus | 11(5.3) | 25(20.7) | <0.0001 |

Age, BMI, and total cholesterol levels are expressed as mean ± SD.
* values represent comparison of incidences between the patient and control group.
** not significant.
† number(%).

smoking and a higher proportion of individuals with a history of diabetes mellitus and hypertension.

In the control population, genotype frequencies for ecNOS aa, ab and bb were 0.005, 0.238 and 0.757, respectively. The distribution of the genotypes was in the Hardy-Weinberg equilibrium (p=0.166). In the patient population, however, genotype frequencies for ecNOS aa, ab and bb were 0.041, 0.190\ and 0.769, respectively. There was a significant deviation from the Hardy-Weinberg equilibrium (p=0.034). When the patients were compared with the control population, there was an excess of ecNOSa/a homozygotes and fewer ecNOSa/b heterozygotes, which is in agreement with the genotype distribution pattern in CAD patients in the Australian study. We found a significant association between the ecNOS polymorphism and the risk of AMI (p=0045)(Table 2). The odds ratio of the aa versus ab plus bb genotype between the AMI and the control group was 8.84 (95% CI: 1.02~76.55) (p=0.018). The difference in the allele frequencies for ecNOSa (0.136) and ecNOSb (0.864) were not statistically significant (p=0.631).

Then, we investigated the association between the ecNOS gene polymorphism and AMI in different age groups (those aged below 51 and above 50). The distribution of ecNOS a/b genotypes in the young age group was significantly different from that of the old age group (Table 2). When compared with the control group, only the younger patient group had significant alteration in the ecNOS a/b genotype distribution. These observations suggest that the effect of ecNOS gene polymorphism in the development of AMI is greater in the younger population than in *the* older male population in Korea.

Then we investigated whether other risk factors of AMI are associated with age. We divided the AMI population into different age groups and compared smoking status or the incidences of hypertension or diabetes mellitus. We found that 83.6% of the AMI patients below the age of 51 are current smokers, while 66.7% of the AMI patients

**Table 2. Distribution of ecNOSa/b allele in AMI patients and the control group. Comparisons were made both in total cases and in different age groups.**

| | | | Genotype Frequency | | | |
|---|---|---|---|---|---|---|
| Group | Age | n | aa | ab | bb | p* |
| AMI | Total | 121 | 5(4.1)† | 23(19.0) | 93(76.9) | 0.045 |
| Control | Total | 206 | 1(0.5) | 49(23.8) | 156(75.7) | - |
| AMI | ≤50 | 55 | 4(7.3) | 7(12.7) | 44(80.0) | 0.009 |
| Control | ≤50 | 104 | 0(0) | 24(23.1) | 80(76.9) | - |
| AMI | >50 | 66 | 1(1.5) | 16(24.2) | 49(74.2) | 1,000 |
| Control | >50 | 102 | 1(1.0) | 25(24.5) | 76(74.5) | - |

*values represent comparison of genotype frequency between the AMI patients and control group.
† number(%).

above the age of 50 are current smokers (p=0.033) (Table 3). This is in agreement with previous observations indicating that smoking, as the major risk factor of AMI, has a greater effect in the young population . The incidence of diabetes was not significantly different in the age groups. The incidence of hypertension was slightly increased in older patients but failed to reach a statistical significance.

**Table 3. Comparison of different age groups in the AMI patients**

| Age Group | ≤50 (n=55) | >50 (n=66) | p* |
|---|---|---|---|
| Current smoker | 46(83.6)† | 44(66.7) | 0.033 |
| Hypertension | 16(29.1) | 25(37.9) | 0.439 |
| Diabetes Mellitus | 12(21.8) | 13(19.7) | 0.658 |

*values represent comparison between the two age groups.
† number (%).

It is possible that the alteration in the distribution of the ecNOS a/b genotypes in young AMI patients is affected by other risk factors, such as smoking, hypertension and diabetes. So, we compared the distribution of the ecNOS a/b genotypes between those who have the risk factor and those who do not. As shown in Table 4, no significant difference in the ecNOS a/b genotypes was found. These data indicate that age dependent association of the ecNOS a/b polymorphism with the development of AMI is not affected by other risk factors, such as smoking, hypertension and diabetes.

## DISCUSSION

In healthy humans, total body NO production appears to be decreased in old age when measuring the serum levels of NO metabolites. Furthermore, NO-mediated vasodilation is also impaired in old age . Analysis of rabbits and rats indicated that the level of iNOS and ecNOS proteins was increased in the aged animals but the NO-mediated vasodilations were still impaired . More analysis revealed that the production of NO in aged endothelium, after either hyperemia or acetylcholine infusion, did not increase as efficiently as in the young endothelium, indicating that some alterations have occurred either in the signaling event that is responsible for the activation of ecNOS or in the ecNOS activity itself. Haas et al. reported that the reduction in the response after acetylcholine infusion was caused by the reduction of Ca++ influx after the stimulation in the aged endothelium rather than by any changes in the activity of ecNOS. This means that the reduction in the responsiveness of the aged artery is caused by the signaling pathway rather than the ecNOS activity, resulting in a less effect of ecNOS polymorphism in the development of AMI in the aged population.

An alternative explanation may be that the structural changes in the aged artery may mask the effects of ecNOS polymorphism. The changes in the vasculature associated with aging might significantly impair the endothelial responsiveness in such a way that the minor changes associated with ecNOS a/b polymorphism might not be significant in the old population.

The ecNOS gene Glu298Asp polymorphism has been implicated in the occurrence of variant angina, essential hypertension and AMI . We found no association of the ecNOS a/b polymorphism with hypertension in our AMI patients (Table 4). The relationship between the ecNOS a/b and Glu298Asp polymorphism needs to be studied in the future. We are currently investigating the Glu298Asp

**Table 4. Distribution of ecNOS genotypes in AMI patients divided by the smoking status or the presence of hypertention or diabetes mellitus.**

| | | Genotype Frequency | | | |
|---|---|---|---|---|---|
| Group | n | aa | ab | bb | p* |
| Current Smoker | 90 | 5(5.6)† | 17(18.9) | 68(75.6) | 0.406 |
| Ex-/Non-Smoker | 31 | 0(0) | 6(19.4) | 25(88.0) | - |
| With DM | 25 | 0(0) | 3(12.0) | 22(88.0) | 0.269 |
| Without DM | 96 | 5(5.2) | 20(20.8) | 71(74.0) | - |
| With HTN | 41 | 2(4.9) | 10(24.4) | 29(70.7) | 0.514 |
| Without HTN | 80 | 3(3.8) | 13(16.2) | 64(80.0) | - |

*values represent comparison of genotype frequency between the AMI patients and control group.
† number(%).

polymorphism in our study population.

The smoking associated risk of CAD is also affected by aging. Smoking has been known to be the major risk factor of atherosclerosis. In our analysis, we found a significant increase in the smoking population in the patient group (Table 1). The smoking-related occurrence of AMI also appears to be affected by age in our patient population since we found a significant increase in the proportion of smokers in the young age patient group when compared to the old age patient group (Table 3). It is possible that the smoking-dependent occurrence of AMI is affected by the changes in the vasculature associated with aging, as is the case with the ecNOS polymorphism-dependent occurrence of AMI.

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