Association of endothelial nitric oxide synthase polymorphisms with coronary artery disease in Korean individuals with or without diabetes mellitus

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Abstract. Polymorphisms of the endothelial nitric oxide synthase (*eNOS*) gene have been implicated in various diseases, but their roles as risk factors in type 2 diabetes mellitus (T2DM) with regard to coronary artery disease (CAD) are largely unknown. Therefore, we investigated the association of the genotypes and haplotypes of *eNOS* polymorphisms in CAD with T2DM. A case-control study was performed to evaluate the genotypes and haplotypes of the *eNOS* polymorphisms (-786T>C, 4a4b and 894G>T) in 192 CAD patients and 196 controls. The same population was also re-organized upon the status of T2DM. The genotypes of *eNOS* -786T>C, 4a4b and 894G>T polymorphisms were determined by polymerase chain reaction-restriction fragment length polymorphism. We found that *eNOS* -786TC+CC and 4a4b+4a4a genotypes were significantly prevalent in the diabetic controls and the diabetic CAD patients compared to the non-diabetic controls or non-diabetic CAD patients, respectively. The frequency of the -786C-4a-894G haplotype was significantly greater in the diabetic CAD patients (p=0.001) and diabetic controls (p=0.023) compared to the non-diabetic controls, whereas the haplotype of -786T-4b-894G was less prevalent in the diabetic CAD patients compared to the non-diabetic controls (p=0.018). Significant associations of the genotypes and the haplotypes were consistently observed in the T2DM group compared to non-DM group, regardless of CAD status. Our finding suggests that the *eNOS* -786T>C and 4a4b polymorphisms and the -786C-4a-894G haplotype are risk factors for T2DM, whereas the haplotype of -786T-4b-894G has a protective effect against the development of T2DM.

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Introduction

Endothelial dysfunction plays a crucial role in the initiation and progression of atherogenesis, and the presence of endothelial dysfunction predicts the presence of coronary artery disease (CAD) and provides prognostic information (1,2). Factors including smoking, diet, aging and various diseases affect the proper function of the endothelium (3), and genetic predisposition also imposes considerable risk for the development of atherosclerosis (4), as polymorphisms of an increasing number of genes have been associated with cardiovascular diseases.

The endothelium plays an essential role in maintaining vascular tone and blood pressure through its production of nitric oxide (NO). The cardioprotective roles of NO include the inhibition of platelet aggregation, leukocyte adhesion and smooth muscle cell proliferation, the prevention of low-density lipoprotein (LDL) oxidation and its antioxidant effects (5-9). Thus, reduced bioavailability of NO is common to CAD, and defects in NO production and function correlate well with the incidence of CAD. NO is produced from l-arginine by endothelial nitric oxide synthase (eNOS), and decreased expression of eNOS has been observed in human atherosclerotic vessels (10).

Polymorphisms of the *eNOS* gene have been observed in various populations. A single nucleotide polymorphism (SNP), -786T>C, involving a substitution of thymine (T) to cytosine (C) was identified in the 5'-flanking region of *eNOS* at a locus 786 base-pairs (bp) upstream of the first exon (11). Another common variant of *eNOS* has also been described with a G-to-T transversion at nucleotide position 894 (894G>T) leading to a change of amino acid 298 (Glu298Asp) (12). A 27-bp repeat polymorphism in intron 4 of the *eNOS* gene (*eNOS* 4a4b) has also been reported (13).

Numerous studies have analyzed the association of *eNOS* polymorphisms and cardiovascular diseases, but no solid conclusions have been drawn, partly due to the discrepancies

Table I. Demographic and clinical characteristics of coronary artery disease (CAD) patients and controls with or without diabetes mellitus (DM).

Characteristics	Subjects without CAD			Subjects with CAD		
	Controls non-T2DM (n=161)	Subjects T2DM (n=35)	p-value[a]	Controls non-T2DM (n=138)	Subjects T2DM (n=54)	p-value
Male, n (%)	78 (48.4)	22 (62.9)	0.138	79 (57.2)	32 (59.3)	0.871
Age[b] (years)	59.78±11.04	61.29±11.09	0.467	59.70±12.53	62.02±11.33	0.239
Smoking, n (%)	11 (6.8)	7 (20.0)	0.023	55 (39.9)	25 (46.3)	0.421
Hyperlipidemia, n (%)	19 (11.8)	15 (42.9)	<0.0001	23 (16.7)	14 (25.9)	0.157
Hypertension, n (%)	79 (49.1)	24 (68.6)	0.041	71 (51.4)	38 (70.4)	0.023

[a]p-values were estimated using the independent t-test for continuous data and the Fisher's exact test for categorical data. [b]Values (years) are means ± SD.

among studies and the lack of a reliable marker of *eNOS* gene function in humans (14). At present, only a few reports are available that assess the predispositions of *eNOS* genotypes to type 2 diabetes mellitus (T2DM), although many have investigated their associations to diabetic complications. NO is known to modulate peripheral and hepatic glucose metabolism and insulin secretion (15), and diabetes mellitus (DM) is a known risk factor for CAD (16). Thus, in the present study, we assessed the *eNOS* genotype distribution of the three polymorphisms, -786T>C, 4a4b and 894G>T, in CAD patients and controls, with regard to T2DM status, and performed haplotype analyses of three-loci *eNOS* polymorphisms.

Materials and methods

Subjects. In this study, we enrolled 192 CAD patients (including 54 T2DM patients) and 196 control subjects without CAD (including 35 T2DM patients) (Table I). CAD patients, who presented at the Cardiology Department of the Bundang CHA Medical Center from November 2003 to June 2005 by consecutive referral, were recruited. During coronary angiography, patients with at least one major coronary artery showing >50% stenosis were categorized as CAD patients. T2DM was defined as a fasting plasma glucose >126 mg/dl (7.0 mmol/l) (17) and included patients taking diabetes medication. Hypertension was defined as systolic pressure >140 mmHg and/or diastolic pressure >90 mmHg on more than one occasion, according to the Joint National Committee (JNC 7) report guidelines (18) and the current ingestion of hypertension medication. Smoking indicates current smoking.

As control subjects, we selected gender- and age-matched healthy individuals from those who presented at the Bundang CHA Medical Center for a health examination during the same period; these individuals had no history of myocardial infarction or cerebrovascular disease. The Institutional Review Board (IRB) of Bundang CHA Medical Center approved this genetic study in October 2003. All patients and controls were Korean and gave informed consent prior to enrollment in the study.

Genotype analysis. Genomic DNA was extracted from peripheral blood leukocytes using the G-DEX blood extraction kit (Intron Inc., Seongnam, Korea). Nucleotide changes were determined by polymerase chain reaction (PCR)-restriction fragment length polymorphism analyses using the isolated genomic DNA as a template. The PCR for the -786T>C polymorphism was performed using the following primers: 5'-ATG CTC CCA CCA GGG CAT CA-3' and 5'-GTC CTT GAA TCT GAC ATT AGG G-3'. DNA was amplified for 35 cycles of denaturing at 94˚C for 30 sec, annealing at 51˚C for 40 sec and extension at 72˚C for 40 sec. The PCR products were detected by gel electrophoresis after incubation with a restriction endonuclease, *Ngo*MIV (New England Biolabs, Beverly, MA, USA) at 37˚C for 16 h. The primer sequences to detect the *eNOS* intron 4 polymorphism were 5'-AGG CCC TAT GGT AGT GCC TTT-3' and 5'-TCT CTT TAG TGC TGT GGT CAC-3'. DNA was amplified for 35 cycles of denaturing at 94˚C for 1 min, annealing at 49˚C for 40 sec and extension at 72˚C for 40 sec. The PCR products were visualized by gel electrophoresis. The forward and reverse primers used to detect the 894G>T polymorphism were 5'-CAT GAG GCT CAG CCC CAG AAC-3' and 5'-AGT CAA TCC CTT TGG TGC TCA C-3', respectively. PCR reactions were run for 35 cycles: 95˚C for 45 sec, 63˚C for 45 sec and 72˚C for 45 sec. The products were digested with the restriction endonuclease *Mbo*I (New England Biolabs) at 37˚C for 16 h and detected by gel electrophoresis.

Statistical analysis. The clinical characteristics were compared by the Student's unpaired t-test. The distribution of genotypes for each polymorphism was assessed for deviation from Hardy-Weinberg equilibrium, and differences in genotype frequency and allele frequency between groups were assessed using χ^2-tests. A value of $p<0.05$ was considered statistically significant. StatsDirect Statistical Software version 2.4.4 (StatsDirect Ltd., Altrincham, UK) was used to calculate the odds ratio (OR) and 95% confidence interval (CI). Haplotype analysis was performed using SNPAlyze ver.5.1 (Dynacom Co., Ltd., Yokohama, Japan).

Results

The demographic and clinical characteristics of the 192 CAD patients (including 54 T2DM patients) and the 196 controls

Table II. *eNOS* genotype distribution and allele frequencies of coronary artery disease (CAD) patients and controls with or without diabetes mellitus (DM).

Genotype	Controls without CAD			Cases with CAD			
	Non-T2DM (n=161)	T2DM (n=35)	AOR[a] (95% CI)	Non-T2DM (n=138)	AOR (95% CI)	T2DM (n=54)	AOR (95% CI)
eNOS -786T>C							
TT	139 (86.3)	25 (71.4)	1.00	111 (80.4)	1.00	38 (70.4)	1.00
TC	22 (13.7)	10 (28.6)	2.45 (0.939-6.404)	27 (19.6)	1.62 (0.832-3.163)	14 (25.9)	4.04 (1.630-10.013)
CC	0 (0.0)	0 (0.0)	-	0 (0.0)	-	2 (3.7)	-
TC+CC	22 (13.7)	10 (28.6)	2.45 (0.939-6.404)	27 (19.6)	1.62 (0.832-3.163)	16 (29.6)	4.39 (1.800-10.706)
eNOS 4a4b							
4b4b	138 (85.7)	23 (65.7)	1.00	110 (79.7)	1.00	38 (70.4)	1.00
4a4b	23 (14.3)	12 (34.3)	2.56 (1.006-6.528)	28 (20.3)	1.69 (0.874-3.265)	14 (25.9)	3.85 (1.561-9.473)
4a4a	0 (0.0)	0 (0.0)	-	0 (0.0)	-	2 (3.7)	-
4a4b+4a4a	23 (14.3)	12 (34.3)	2.56 (1.006-6.528)	28 (20.3)	1.69 (0.874-3.265)	16 (29.6)	4.20 (1.732-10.160)
eNOS 894G>T							
GG	131 (81.4)	32 (91.4)	1.00	114 (82.6)	1.00	43 (79.6)	1.00
GT	30 (18.6)	3 (8.6)	0.38 (0.099-1.438)	23 (16.7)	0.90 (0.466-1.746)	11 (20.4)	1.12 (0.449-2.808)
TT	0 (0.0)	0 (0.0)	-	1 (0.7)	-	0 (0.0)	-
GT+TT	30 (18.6)	3 (8.6)	0.38 (0.099-1.438)	4 (17.4)	0.91 (0.473-1.764)	11 (20.4)	1.12 (0.449-2.808)

[a]Odds ratios (OR) were adjusted for age, gender, hypertension, hyperlipidemia and smoking status.

Table III. Haplotype analyses of three polymorphisms (-786T>C, 4a4b and 894G>T) of the *eNOS* gene from subgroups of coronary artery disease (CAD) patients and controls with or without diabetes mellitus (DM).

Haplotype[a]	Controls		CAD		P1	P2	P3	P4
	Non-T2DM	T2DM	Non-T2DM	T2DM				
T-4b-G	0.8348	0.7857	0.8080	0.7315	0.326	0.393	0.018[b]	0.413
T-4b-T	0.0907	0.0429	0.0906	0.1019	0.187	0.997	0.729	0.153
C-4a-G	0.0627	0.1429	0.0978	0.1667	0.023[b]	0.112	0.001[c]	0.670
T-4a-G	0.0062	0.0286	0.0036	-	0.092	0.653	-	-
C-4b-G	0.0031	-	-	-	-	-	-	-
C-4a-T	0.0025	-	-	-	-	-	-	-

[a]Haplotype of *eNOS* (-786T>C, 4a4b, 894G>T). Significant p-values: [b]$p<0.05$ and [c]$p<0.005$. P1, p-value of controls without DM vs. controls with DM; P2, p-value of controls without DM vs. CAD patients without DM; P3, p-value of controls without DM vs. CAD patients with DM; P4, p-value of controls with DM vs. CAD patients with DM.

(including 35 T2DM patients) are described in Table I. No significant differences in gender and age existed between the subgroups. Hypertension was higher in the controls and the CAD group with DM ($p<0.05$). In addition, smoking and hyperlipidemia were significantly higher in the control group with DM compared to the control group without DM ($p<0.05$) in contrast to the comparison in the CAD subgroups.

Three polymorphisms of the *eNOS* gene, -786T>C, 4a4b and 894G>T, were investigated. The genotype distributions in the CAD patients and controls are shown in Table II. The genotype frequencies for all of the polymorphisms were in accordance with Hardy-Weinberg equilibrium in the cases and control groups. We subgrouped the controls and CAD patients with regard to T2DM status and evaluated for any association with particular genotypes. Genotype distributions of -786T>C, 4a4b and 894G>T in the subgroups of the CAD patients and controls are shown in Table II. The distribution of the *eNOS* -786T>C genotypes and allele frequencies in both the CAD patients and controls showed significant associations between the -786T>C polymorphism and the risk of T2DM. Additionally, the allele frequencies of *eNOS* 4a4b of the controls and CAD patients showed significant differences

with regard to T2DM. No predisposition to T2DM was found for the 894G>T polymorphism in either the controls or CAD patients.

Furthermore, we performed haplotype analyses for the control and CAD patient subgroups according to the T2DM status, and the results are shown in Table III. The most common haplotype of all of the subgroups was -786T-4b-894G. The *eNOS* -786C-4a-894G haplotype was found more commonly in the DM subgroups from both the CAD patients and controls compared to the non-diabetic subgroups, suggesting that this haplotype has a significant association with T2DM. Furthermore, the association was stronger in the CAD patients than in the controls. Also, the haplotype frequencies of -786T-4b-894G were significantly different between the non-diabetic controls and diabetic CAD patients (p=0.018).

Discussion

In the present study, we analyzed the associations of the *eNOS* -786T>C, 4a4b and 894G>T polymorphisms with CAD and T2DM by subgrouping both the CAD patients and controls by T2DM status. Our findings suggest that the genotypes and haplotypes of these three *eNOS* polymorphisms are not independent predisposition factors to CAD, but are associated with T2DM in the Korean population. The allele frequencies of both -786C and 4a were significantly associated with T2DM, but not with CAD *per se*, whereas the *eNOS* 894G>T polymorphism was not an independent risk factor for either CAD or DM (Table II).

The -786T>C mutation in the promoter region of *eNOS* results in the inhibition of *eNOS* promoter activity (11), leading to endothelial dysfunction by reduced NO production in blood vessels. The intron 4 variable number of tandem repeats polymorphism, in which alleles contain either 4 repeats (4a) or 5 repeats (4b), also affects basal NO production in the blood vessels (19). The mutation of *eNOS* 894G>T produces a missense Glu298Asp *eNOS*, which may alter *eNOS* activity by a conformational change or by protease-mediated cleavage (20,21). Although numerous studies have examined the association of *eNOS* genotypes to cardiovascular diseases in different populations, the influences of *eNOS* polymorphisms on the risk of cardiovascular diseases are not consistent. In our previous study, we did not find associations of the *eNOS* polymorphisms, -786T>C, 4a4b and 894G>T, with CAD in the Korean population, but -786T>C and 4a4b had predispositions to CAD after adjusting for other conventional cardiovascular risk factors (22). Consistent with this, no significant difference in *eNOS* genotype distributions of -786T>C, 4a4b or 894G>T was observed with CAD in the present study among Koreans without adjusting for risk factors and neither did an additional analysis of haplotypes of three-loci polymorphisms produce any association with CAD (data not shown).

Since T2DM is a known risk factor for cardiovascular diseases associated with endothelial dysfunction (23), we further analyzed the genotype distributions of *eNOS* polymorphisms in the CAD patients and control subjects after dividing each group according to T2DM status. To our knowledge, this is the first report to determine the association of CAD with three *eNOS* polymorphisms by comparing four subgroups divided according to both CAD and T2DM. Zhang *et al* (24) examined *eNOS* variants and the risks of coronary heart disease among diabetic men in the US and found that the genotype distributions of -786T>C and 894G>T were not significantly different among the men with or without coronary heart disease, but non-diabetic samples were not included in their study. We observed that the allele frequencies of *eNOS* -786T>C, 4a4b and 894G>T were not significantly different between the non-diabetic CAD patients and the non-diabetic controls (Table II). However, statistically significant associations in the allele distributions of -786T>C and 4a4b were found between the diabetic control and non-diabetic control subjects, although the T2DM sample size was relatively small. In addition, heterozygotes and mutant homozygotes of *eNOS* -786T>C and 4a4b were significantly prevalent in the diabetic CAD patients compared to the non-diabetic control group. Furthermore, when we re-organized the same population regarding the presence of T2DM, the heterozygotes and the mutant homozygotes of *eNOS* -786T>C and 4a4b were also significantly frequent in the group with T2DM (-786TT vs. -786TC+CC; OR=2.32, 95% CI 1.299-4.137 and 4b4b vs. 4a4b+4a4a; OR=2.28, 95% CI 1.293-4.022). Therefore, our findings suggest that the *eNOS* -786T>C and 4a4b polymorphisms are associated with predisposition to T2DM, but not to CAD, while no correlation exists between the *eNOS* 894G>T genotype and CAD or T2DM.

Results regarding the role of *eNOS* polymorphisms in the predisposition to T2DM are limited and conflicting. Pulkkinen and colleagues (25) found that the 4a4b and 894G>T polymorphisms were not associated with T2DM or coronary heart disease in Finnish individuals. By contrast, associations of 4a4b (26) and 894G>T (27) polymorphisms with T2DM were demonstrated in Polish and Italian populations, respectively. In a recent publication by Rittig *et al* (28), the independent association of the 4a4b, but not 786T>C and 894G>T polymorphisms, with disturbed endothelial function in a German population with increased risk to develop T2DM was reported. Our present study suggests an association of *eNOS* -786T>C and 4a4b, but not 894G>T, genotypes with T2DM. Therefore, further comprehensive studies with uniformly standardized criteria devoid of undetected confounding factors are required to establish a firm conclusion.

Recently, haplotype analysis of single nucleotide polymorphism (SNP) markers of genes has been recognized to provide better information than SNP analysis (29). Rios *et al* (30) analyzed the haplotypes of two loci, -786T>C and 894G>T, with the CAD risk and reported that the frequency of the wild-type haplotype, -786T-894G, was decreased in CAD patients. A recent report studying the effects of *eNOS* polymorphisms and smoking on CAD in Brazilians showed that the frequency of the -786C-4a-894G haplotype was higher in CAD patients, whereas that of -786T-4a-894G haplotype was lower, but associations were not present for non-smoking CAD patients (31). In the present study, three-loci haplotypes of *eNOS,* -786T>C, 4a4b and 894G>T, were analyzed for their associations with CAD and T2DM, and we found that no haplotype had any predisposition for CAD (data not shown). Therefore, these genotype and haplotype analyses suggest that the *eNOS* polymorphisms are unlikely to exhibit significant additive effects on predisposition to CAD in the Korean population. However, we cannot exclude the possibility that a marginal association

of *eNOS* polymorphisms with CAD is masked by other unadjusted risk factors.

Two haplotypes of *eNOS* polymorphisms affected the susceptibility to T2DM (Table III). The frequency of wild-type haplotype, -786T-4b-894G, was significantly lower in the diabetic CAD patients compared to the non-diabetic controls. A positive association of the -786T-4b-894G haplotype between the non-diabetic and diabetic control groups was also observed after re-organizing the population in regard to T2DM (data not shown). In addition, we observed that the frequency of the -786C-4a-894G haplotype varied significantly between the groups. Notably, a significant association was present between the non-diabetic and diabetic controls, indicating that this haplotype is susceptible to T2DM, and the association was even stronger when we compared the non-diabetic controls to the diabetic CAD patients. The haplotype frequency of -786C-4a-894G was also significantly different between the non-diabetic and diabetic groups (data not shown), indicating that this haplotype is indeed susceptible to T2DM. Therefore, the -786C-4a-894G haplotype is likely an independent risk factor for T2DM. While further studies with a larger sample size and with a random population are required to reach a firm conclusion, as far as we know, this is the first study to identify susceptible and protective haplotypes of *eNOS* loci to DM *per se*, although *eNOS* haplotype analyses for diabetic complications are available (32,33).

In conclusion, our finding suggests that the *eNOS* -786T>C and 4a4b polymorphisms and the -786C-4a-894G haplotype are risk factors for T2DM particularly in CAD patients, whereas the haplotype of -786T-4b-894G has a protective effect against the development of T2DM. Further studies involving larger and varied populations would be of great value to firmly conclude the correlation between *eNOS* polymorphisms and T2DM and to establish an intervention in the development of diabetes for those with the mutant *eNOS* genotypes.

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