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Analysis of Endothelial Nitric Oxide Synthase Gene Polymorphisms with Cardiovascular Diseases in Eastern Taiwan

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Abstract

A few studies have been carried out to address the correlation between the endothelial nitric oxide synthase (eNOS) gene polymorphisms and cardiovascular diseases (CVD) within the Taiwanese population. However, no report has documented the situations in eastern Taiwan, which has different ethnic groups from those in western Taiwan. In this study, we explored the relationship between polymorphic eNOS alleles and CVD in eastern Taiwan. DNA extraction and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis were employed for the detection polymorphism in exon 7 of the eNOS gene. A total of 198 subjects was included. The subjects were 120 patients with CVD such as hypertension, coronary artery disease (CAD), and stroke. Normal subjects (78) served as control. Analysis of the gene polymorphism revealed that the frequency of the eNOS gene variant containing a 27-bp repeat in intron 4 is similar between control subjects (aa : ab : bb = 0% : 21.8% : 78.2%), and patients with CVD (aa : ab : bb = 3.3% : 21.7% : 75.0%). The frequency of the Glu298Asp (894G → T) polymorphism in exon 7 of the eNOS gene was significantly different between control subjects (TT : GT : GG = 7.7% : 29.5% : 62.8%) and patients with CVD (TT : GT : GG = 5.0% : 74.2% : 20.8%). These results suggest that the Glu298Asp polymorphism in exon 7 of the eNOS gene is likely to be a risk factor for CVD in the eastern Taiwanese population.

Key Words: endothelial nitric oxide synthase, gene polymorphism, cardiovascular disease

Introduction

It has been well established that nitric oxide (NO) derived from the endothelial cells exerts various physiological functions (17). Endothelium-derived NO formed by endothelial nitric oxide synthase (eNOS) mediates endothelium-dependent vasodilation and antithrombotic action (19). Accordingly, it has been suggested that NO deficiency may be a risk factor for cardiovascular diseases (CVD) including hypertension, coronary artery disease (CAD), myocardial infarction (MI), and atherosclerosis in human subjects and/or experimental hypertension in animals (3, 4, 20, 23, 24). In human subjects, the eNOS gene is located on chromosome 7q 35-36 and is comprised of 26 exons spanning 21 kb, including a number of variable tandem repeats and dinucleotide repeats [(CA)n] (18, 25). Among the reported polymorphisms of the eNOS gene, a close association of the 4a/b allele (four/five 27-bp repeats) in intron 4

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Table 1. Demographic characteristics of study populations

Characteristic	CVD (n = 120)	Control (n = 78)
Age (yr)	64.4 ± 1.3*	45.3 ± 3.1
Gender (Male/Female)	73 (61%) / 47 (39%)	44 (56%) / 34 (44%)
Hypertension	51 (43%)*	0
CAD	107 (89%)*	0
Stroke	32 (27%)*	0
Smoking	60 (50%)*	33 (42%)
Drinking	50 (42%)*	45 (58%)
Cholesterol, mg/dl	212.3 ±47.6*	187.7 ± 35.2
Triglyceride, mg/dl	194.0 ± 100.3*	107.7 ± 42.9

Values are mean ± SD. CVD, cardiovascular diseases; CAD, coronary artery disease. *$P < 0.05$ compared to the corresponding values in control subjects.

and the Glu298Asp (894G → T) polymorphism in exon 7 with the incidence of CAD, hypertension or stroke in many populations has been reported (9, 16, 26, 33). On the contrary, these polymorphisms in other studies were not associated with a higher incidence of CVD (1, 2, 10, 14, 15, 30, 32).

Taiwan is separated into eastern and western parts by the Central Mountains. The eastern part is mountainous, less-developed, much less-populated and more indigenous people such as Ammis, Taruko, Bunun, Puyuma, and Atayal groups, as compared to the west. Lifestyle differences between the large indigenous population of eastern Taiwan and other demographic regions of Taiwan include an increased frequency of alcohol drinking and smoking beginning from younger ages. The prevalence of CVD such as hypertension, CAD, MI, and stroke and the mortality rate are higher in this indigenous population than in any other ethnic groups in Taiwan (13). Because the main composition of ethnic populations and the prevalence of CVD are different between western and eastern Taiwan, the purpose of the present study was to analyze whether an association exists between the exon 7 Glu298Asp or the intron 4 27-bp tandem repeat eNOS polymorphism and CVD among persons living in eastern Taiwan.

Materials and Methods

Patients and Control Subjects

The study included a total of 198 subjects living in eastern Taiwan. As shown in Table 1, our investigation consisted of 120 patients with CVD such as hypertension, CAD, and stroke (73 males and 47 females) and 78 normal control subjects (44 males and 34 females). They were admitted to Tzu Chi General Hospital, Hualien, from March 2002 through October 2003. The criteria for CVD used for this study were as follows: [1] hypertension: those either with systolic blood pressure ≥ 160 mm Hg and diastolic blood pressure ≥ 95 mm Hg; [2] CAD: those with coronary angiographic evidence of more than 50% stenosis of at least one major coronary artery; or [3] stroke: those with either hemorrhagic or thrombotic cerebral vascular disorders. Control subjects were recruited from patients who had no history of hypertension, CAD, and stroke. Informed consent was obtained from each subject. The study was approved by the Institutional Board for Human Research of the Hospital and University.

Genomic DNA Extraction

Genomic DNA was obtained from EDTA anticoagualted peripheral blood using a commercially available DNA extraction kit (InstaGene™ Whole Blood Kit, Bio-Rad, Hercules, CA, USA). The extracted DNA was stored at 4°C for later analysis.

Analysis of the eNOS Exon 7 894G → T Polymorphism

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis was performed to detect the Glu298Asp (894G → T) polymorphism in exon 7 of the eNOS gene. Primer pairs for PCR were designed to amplify a part of the eNOS gene containing exon 7 as follows: sense 5'-AAGGCAGGAGACAGTGGATGGA-3' and antisense 5'-CCCAGTCAATCCCTTTGGTGCTCA-3' (5, 16). Samples were amplified by denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 66°C for 1 min, and extension at 72°C for 40 sec. The 248-bp PCR product was digested with 1 U of the restriction enzyme *Ban*II (New England Biolabs, Beverly, MA, USA) at 37°C for at least 2 h. *Ban*II digested the amplified fragments into smaller fragments (163- and 85-bp). A single *Ban*II

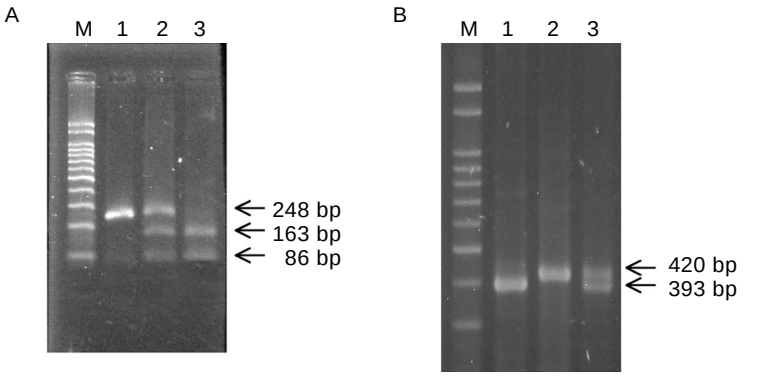

Fig. 1. Polymorphism of the eNOS gene. The exon 7 894G →T mutation detected by BanII digestion of the 248-bp PCR product (A). Lane M is the DNA marker; lane 1 shows restriction pattern corresponding to homozygosity for TT; lane 2, the heterozygote for GT; and lane 3, the homozygote for GG. The variable number of tandem (27-bp) repeats in intron 4 (B). Lane M, DNA marker; lane 1, aa homozygote with a fragment of 393 bp (four-repeats); lane 2, bb homozygote with a fragment of 420 bp (five-repeats); and lane 3, ab heterozygote with fragments of both 420 and 393 bp.

site was present in the wild type allele, and no *Ban*II site was found in the mutant allele. Therefore, digestion of the wild type (G/G) with *Ban*II yielded 163-bp and 85-bp fragments, and homozygous mutant type (T/T) results in a 248-bp fragment. In the case of the heterozygous mutant (G/T), digestion with *Ban*II results in three fragments of 248-bp, 163-bp, and 85-bp in size. Digested fragments were separated on a 3% agarose gel and visualized in UV light after ethidium bromide staining (Fig. 1A).

Analysis of the eNOS 4a/b Polymorphism

Oligonucleotide primers flanking the 27-bp repeat region in intron 4 of eNOS were used. The sequence of the forward primer was 5'-AGGCCCTATGGTAGTGCCTTT-3'. This primer was located at position 5111 to 5130 bp of the genomic sequence of eNOS. The reverse primer sequence was 5'-TCTCTTAGTGCTGTGGTCAC-3' and its position within the genomic sequence of eNOS was 5530 to 5511 bp (21, 31). Using this PCR strategy, the wild-type allele (allele b) generated a 420-bp band (including five copies of a 27-bp repeat). The mutant allele (allele a) generated a 393-bp band (four copies of the same repeat). PCR conditions were denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 40 sec, annealing and extension at 66°C for 1 min, and final extension at 72°C for 4 min. The PCR products were resolved on a 3% agarose gel and visualized in UV light after ethidium bromide staining (Fig. 1).

Statistical Analysis

Data were expressed as means ±SD, percentage or range. χ^2 test was used for comparisons between patients with CVD and control subjects. Allele frequencies were calculated from the genotypes of all subjects by the gene-counting method and compared using the χ^2 analysis. Hardy-Weinberg equilibrium was assessed by the χ^2 test. Odds ratios (ORs) and their 95% confidence intervals (95% CIs) were also calculated. P values < 0.05 were considered statistically significant.

Results

Characteristics, Genotype and Allele Frequencies in All Study Subjects

There were 120 patients with CVD and 78 control patients without any symptom related to CVD. Table 1 shows the basic characteristics of the CVD patients and control subjects. Patients with CVD were older, and the serum cholesterol and triglycerides were higher than control. In all study subjects, the genotype frequencies of eNOS exon 7 G/G, G/T, and T/T were 37.4%, 56.6%, and 6.0%, respectively; the allele frequencies of G and T were 66.2 and 33.8%, respectively. In addition, the genotype frequencies of eNOS intron 4 a/a, a/b, and b/b were 2.0%, 21.7%, and 76.3%, respectively, and the allele frequencies of a and b were 13.1 and 86.9%, respectively.

Comparison of Genotype and Allele Frequencies in Exon 7 894G →T eNOS Variants between Patients with CVD and Control Subjects

As shown in Table 2, the frequencies for the eNOS exon 7 894G → T genotypes G/G, G/T, and T/T were 20.8%, 74.2%, and 5.0%, respectively, in

Table 2. Genotypes and alleles of eNOS 894G →T variant in cardiovascular diseases (CVD) and control group

	CVD (n = 120)	Control (n = 78)	OR	95% CI	*P*
Genotype					
GG	25 (20.8%)	49 (62.8%)	–	–	0.004
GT	89 (74.2%)	23 (29.5%)			
TT	6 (5.0%)	6 (7.7%)			
Allele					
G	70 (58.3%)	61 (78.2%)	0.39	0.20-0.75	0.005
T	50 (41.7%)	17 (21.8%)			

CVD, cardiovascular diseases; OR, odds ratio; CI, confidence interval.

Table 3. Genotypes and alleles of eNOS 4b/a 27-bp repeats variant in cardiovascular diseases (CVD) and control groups

	CVD (n = 120)	Control (n = 78)	OR	95% CI	*P*
Genotype					
a/a	4 (3.3%)	0			
a/b	26 (21.7%)	17 (21.8%)	–	–	0.616
b/b	90 (75.0%)	61 (78.2%)			
Allele					
a	17 (14.2%)	9 (10.9%)	1.27	0.53-3.0	0.593
b	103 (85.8%)	69 (89.1%)			

CVD, cardiovascular diseases; OR, odds ratio; CI, confidence interval.

CVD subjects (n = 120), and the allele distributions of G and T were 58.3 and 41.7%, respectively. In the control group (n = 78), the genotype frequency was 62.8% for G/G, 29.5% for G/T, and 7.7% for T/T, and the frequency of either the G or T allele was 78.2 and 21.8%, respectively. There were significant differences in genotype and allele frequencies between the patients with CVD and the control group ($P = 0.004$ for genotype distribution; $P = 0.005$ for allele distribution, OR = 0.39, and 95% CI 0.20-0.75).

Distributions of Genotype and Allele Frequencies of the 27-bp Tandem Repeat Polymorphism in Intron 4 (4a/b) of the eNOS Gene in Patients with CVD and Control Subjects

The genotype frequencies of 4a/b polymorphism in CVD group were 3.3% for a/a, 21.7% for a/b, and 75.0% for b/b, and alleles of b and a in CVD subjects were 85.8 and 14.2%, respectively. On the other hand, the genotype frequency was 0% for a/a, 21.8% for a/b, and 78.2% for b/b, and frequencies of alleles b and a were 89.1 and 10.9%, respectively, in the control group (Table 3). No significant difference in frequency of 4a/b polymorphism was observed between CVD and control subjects ($P = 0.616$ for genotype distribution; $P = 0.593$ for allele distribution, OR = 1.27, 95% CI 0.53-3.00).

Discussion

Endothelium-derived NO, produced by eNOS from L-arginine, is a major contributor to vascular regulation in health and in disease (3, 4, 20, 23, 24). There are many reports indicating a significant association between variations of the eNOS gene and the incidence of CVD, and discrepant findings may be attributable to racial differences. Regarding the distribution of the exon 7 Glu298Asp polymorphism, Miyamoto *et al*. (16) and Shoji *et al*. (27) demonstrated that allele frequencies differed significantly between hypertensive and normotensive individuals. In addition, many studies have shown a positive association of this polymorphism with acute myocardial infarction and coronary spasm in Japanese populations, and in the UK subjects (7, 8, 26, 33). However, Cai *et al*. (2) and Liyou *et al*. (15) reported no association of this polymorphism with CAD in two Caucasian populations, and Kato *et al*. (12) also showed no correlation with hypertension among Japanese population. In our study, the frequency of the G allele was 78.2% in control

subjects. This frequency was higher than the frequency of 56% observed in the French population by Lacolley *et al*. (14), and the frequency of 69% in the UK population reported by Hingorani *et al*. (8). In contrast, the analysis in Japan yielded an exceedingly high frequency of 92% (12, 16, 27). Compared with the results (10.1% for controls, 9.6% for CAD patients, and 7.5% for CAD/MI patients) of a previous report carried out in Taiwan (30), we observed a higher T allele frequency (21.8% for controls, and 41.7% for CVD group) in our study. In the analysis of the association between CVD and exon 7 genetic polymorphism, the homozygous genotype TT was significantly associated with an increased risk of CVD in the population of eastern Taiwan. However, a previous study from Chang Gung Memorial Hospital (Taipei, Taiwan) found no consistent evidence of an association between this gene polymorphism and the risk of CAD or MI among northern Taiwanese (30). This implies that exon 7 894G → T variants of the eNOS gene may be a risk factor of CVD for eastern but not for northern Taiwanese populations.

The 27-bp repeat allele in intron 4 of the eNOS gene was associated with a high risk of CAD in Australian smokers (31) and Japanese individuals (11), and also correlated with hypertension in Japanese population (29), acute myocardial infarction in Korean male population (22), and ischemic stroke in Chinese patients (9). On the contrary, Sigusch *et al*. (28) showed no association between this polymorphism with CAD in a German population, Hibi *et al*. (7) showed no association with acute myocardial infarction, and Yahashi *et al*. (32) revealed no correlation with ischemic stroke in the Japanese population. In the UK, Fowkes *et al*. (6) reported a slight association with CAD and peripheral arterial disease only in non-smokers or ex-smokers. In our study population, the genotype frequencies for eNOS intron 4 b/b and a/b plus a/a were 76.3% and 23.7%, respectively, which were similar to the frequencies previously reported in the Taiwanese population (77.8% and 22.2%) consisting of subjects from National Taiwan University Hospital, Taipei (10). In this study, we revealed no significant association between eNOS4a allele and CVD.

In conclusion, the Glu298Asp allele variants in exon 7, but not the 27-bp repeat polymorphism in intron 4 of the eNOS gene is significantly associated with CVD, in patients collected from Tzu Chi General Hospital at Hualien in eastern Taiwan. This study suggests that ethnic compositions may affect the association of the genetic risk factors with CVD.

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