Association Between Endothelial Nitric Oxide Synthase Polymorphisms and the Risk of Premature Coronary Artery Disease in Taiwan

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Abstract

Endothelial dysfunction is one of the initiating processes in atherosclerosis. Endothelium-derived nitric oxide, generated by endothelial nitric oxide synthase (eNOS), is important in endothelial function. Two polymorphisms in eNOS, 894 G→T in exon 7 and a 27-bp repeat in intron 4, have been proposed to be important in coronary artery disease (CAD). We hypothesized that eNOS polymorphisms might be associated with increased risk for premature CAD in Taiwan and susceptibility to endothelial dysfunction.

We enrolled 114 patients, including 108 males who were less than 45 years old with significant coronary stenosis, or below 55 years old with documented myocardial infarction (MI), and 6 premenopausal women with CAD or MI. Sixty-five age-matched subjects with patent coronary angiograms were included as controls. The eNOS polymorphisms were examined. Thirty subjects with patent coronary arteries underwent evaluation of brachial artery reactivity in response to hyperemia and sublingual nitroglycerin (NTG).

The distributions of 894 G→T in exon 7 and the 27-bp repeat in intron 4 genotypes did not differ significantly between the premature CAD and control groups. Male sex and hyperlipidemia were independent risk factors for premature CAD in Taiwan. Neither reactive hyperemia nor NTG-mediated vasodilatation showed any difference between genetic polymorphs. In terms of impaired flow-mediated vasodilatation, hypertension was the independent factor ($p=0.044$), and hyperlipidemia had a marginal effect ($p=0.056$) when we used a multivariate linear regression model.

Neither the rare eNOS T nor the 4a allele conferred an independent risk factor for premature CAD in Taiwan. In addition, they had little influence on the endothelial function assessed by flow-mediated vasodilatation. (J Intern Med Taiwan 2003;14:1-10)

Key Words：Premature coronary artery disease,Endothelial dysfunction, Nitric oxide synthase, Polymorphism

Introduction

Rapid discoveries of novel and unexpected di-sease-associated genes for atherosclerosis are anticipated as genomic maps become available. Although establishing an association or linkage of a marker locus to a susceptible gene is an important first step, the long-term goals should be aimed at defining the underlying functional mutations, exploring possible di-sease mechanisms, and facilitating early identification of individuals at risk 1,2.

Nitric oxide (NO) plays an important role in maintaining normal vascular functions 3-5, including relaxation of smooth muscle, inhibition of platelet aggregation and leukocyte adhesion, inhibition of
smooth muscle cells from migration and proliferation, and also LDL oxidation. Endothelial nitric oxide synthase (eNOS) is expressed in the endothelium, where it produces NO from L-arginine. Direct measurement of NO in atherosclerotic plaques confirmed a marked reduction in the expression of eNOS and increased production of the endogenous nitrate in patients with atherosclerosis 6.

The gene encoding eNOS is located on chromosome hands 7q35-36 and comprises 26 exons spanning 21 kb 7-9. A number of variable tandem repeats (VNTRs) and dinucleotide repeats ((CA)n) of the eNOS gene have been identified. Several studies suggest that 894 G→T in exon 7 is associated with coronary spasm 10,11, CAD 12, or acute myocardial infarction (AMI)13,14. Association between a 27-bp repeat in intron 4 (eNOS 4a/b) and plasma NO metabolite levels in man has been reported15. The eNOS 4a/b polymorphism has been also reported to be associated with CAD, AMI, and venous thromboembolism 16-19.

However, studies analyzing the function consequences of eNOS genotypes are limited. Many investigators showed patients with cardiovascular risk factors, such as hypertension, hypercholesteremia, smoking or diabetes, endothelium-dependent relaxation is impaired 20. It is possible that mutations in the eNOS genes might alter its expression or its function which may make carriers prone to develop endothelial dysfunction and in turn CAD.

Because the distribution of eNOS alleles differs among different populations, the significance of eNOS polymorphism may vary. Therefore, we investigated whether these two eNOS polymorphisms are independent risk factors for premature CAD in the population of Taiwan. We also tested whether flow-mediated vasodilatation functions in subjects with/and without these eNOS mutations, thereby defining the functional significance of eNOS polymorphism in the development of CAD at a young age.

Materials

Study Population

One hundred and fourteen persons who had premature CAD were enrolled in the study. We defined premature CAD as occurring in men with clinical
evidence of AMI according to the World Health Organization criteria 21 for MI at or before the age of 55 years, or angiographic evidence of significant coronary stenosis (luminal stenosis≧

50%) at or before 45 years, or occurring in premenopausal women with a history of MI or a positive coronary angiogram. Coronary lesions were evaluated by on-line digital quantitative coronary angiograms (DCI-S Automated Coronary Analysis System, Philips Medical System, Eindhoven, The Netherlands).

As a control group, 65 age-matched subjects had coronary angiograms for reasons unrelated to possible CAD, including dilated cardiomyopathy before or after heart transplantation, valvular heart disease, and electrophysiologic studies. They were required to have normal coronary arteries as documented by angiography and to have neither a history of atherosclerosis nor clinical or laboratory evidence of atherosclerosis in other vascular beds. None of them had symptoms or signs of congestive heart failure.

A complete clinical history, including cardiovascular risk factors such as smoking, hypertension, and diabetes mellitus, was obtained. Serum lipids were measured after an overnight fast. The definition of hyperlipidemia is total cholesterol≧200 mg/dl, or LDL≧130 mg/dl, or triglyceride≧200 mg/dl, in combination with either a T-CHO/HDL ratio >5 or HDL <35 mg/dl.

DNA Extraction and Genotyping

Genomic DNA was prepared from whole blood by standard methods (QIAamp, DNA Blood Mini Kit, QIAGEN Inc.). Oligonucleotide primers for polymerase chain reaction (PCR) were designed by use of the published sequence of the human eNOS gene (GenBank/EMBL L10693-L10709). To detect the VNTR polymorphism in intron 4, we used primer pairs as follows: sense 5'-GTT ATC AGG CCC TAT GGT AGT GCC T -3' and anti-sense 5'-GCT TCT CTT AGT GCT GTG GTC ACA G -3'. The larger allele, 4b, contained 5 tandem repeats of the 27-bp consensus sequence [GAA GTC TAG ACC TGC TGC (A/G)GG GGT GAG], as previously reported. The first 3 repeats contained A and the last 2 contained G at the 19th base of the 27-bp repeat. The smaller allele, 4a, contained 4 repeats; the first 2 repeats had A and the last 2 had G at the 19th base of the repeat. Amplified fragments, 393 or 420 bp, were resolved on 2 % polyacrylamide gels, corresponding to the 4a and 4b alleles, respectively.

The 894G→T polymorphism in exon 7 was amplified with a primer set as follows: sense 5'-CAT GAG GCT CAG CCC CAG AAC -3', and anti-sense 5'- AGT CAA TCC CTT TGG TGC TCA C -3', then further digested by the restriction enzyme MboI at 37℃ for more than 16 hours. The amplified fragments were resolved on 2% polyacrylamide gels containing ethidium bromide stain. The 206 bp amplified product is cleaved into 119 bp and 87 bp fragments in the presence of a T nucleotide at 894.

Evaluation of Brachial-Artery Reactivity

Thirty subjects with patent coronary arteries as documented by angiograms refrained from smoking, physical activity, and food intake for two hours prior to the examination. After they had rested in the supine position for 10 minutes, the right brachial artery was scanned longitudinally; gray-scale settings were optimized to provide the clearest view of the lumen

and the vessel wall, and images were then magnified (Hewlett-Packard 5500, MA, USA, with a 11-13 MHz linear array transducer). Blood pressure and pulse rate were recorded before and after the examination, and the electrocardiogram was monitored continuously. The ultrasonographic protocol included an initial recording of the brachial artery diameter and Doppler blood-flow velocity, after which a blood-pressure cuff was placed around the right forearm and inflated to a pressure of 250 to 300 mmHg for 4.5 minutes and then suddenly deflated. The diameter of the brachial artery was recorded continuously in the reactive hyperemic phase until the maximal diameter was recorded, then blood flow velocity was recorded at the same time. At recovery phase, brachial blood flow and diameter were recorded at 10 minutes after the cuff was released. The subjects received sublingual NTG (0.6 mg), and then the brachial artery diameter was recorded continuously until the maximal diameter was recorded, with the blood flow velocity recorded at the same time. Flow-mediated and NTG-induced brachial artery dilatation were expressed as percentage change from the baseline value.

Statistical Analysis

The results are reported as means±SD unless otherwise indicated. Comparisons between subjects with premature coronary artery history and controls were made by use of Student's t-test and chi-square analysis for continuous and categorical variables, respectively. Odds ratios (within 95 percent confidence intervals) for premature CAD were determined by multiple logistic-regression analysis. We used linear regression models to estimate the effects of risk factors on the reactivity of the brachial artery. Statistical significance was set at $p < .05$. All analyses were conducted with the use of statistical software (Stata for Windows, version 7, Stata Press, College Station, Texas).

Results

The clinical characteristics of the subjects with premature CAD and of the subjects with patent coronary arteries are shown in Table 1. There were significant differences ($p<0.05$) between the two groups with regard to male sex, hypertension, smoking, and plasma concentrations of HDL, LDL, and triglyceride. The frequencies of the GG, GT, and TT genotypes in exon 7 for the premature CAD group were 79.8%, 20.2%, and 0%, and for the control group they were 81.5%, 15.4%, and 3.1%, respectively ($p=0.134$). The frequency of the T allele was 10.1% in the premature CAD group and 10.8% in the control group ($p=0.134$). The distribution of 4a/b (i.e., aa, ab, and bb genotypes) was 0.9%, 28.9%, and 70.2% in the premature CAD group, and 0%, 23.1%, and 76.9% in the control group, respectively ($p=0.511$). In multivariate logistic regression, male sex and hyperlipidemia were independent risk factors for premature CAD in Taiwan. Neither the rare T allele in exon 7 nor the 4a allele in intron 4 conferred an independent risk factor for premature CAD (Table 2).

The baseline characteristics of the subjects who underwent flow-mediated vasodilatation are

shown in Table 3. The results of ultrasonographic studies of brachial-artery reactivity to hyperemia and NTG are shown in Table 4. Hypertension was the independent factor in impaired flow-mediated reactivity (p=0.04), and hyperlipidemia had a marginal effect (p=0.056). A significant inverse correlation (p=0.004) between smoking and NTG-mediated vasodilatation was also noted in multivariate linear regression model. Neither flow nor NTG-mediated vasodilatation showed difference between different eNOS polymorphisms.

Discussion

As previously reported, endothelial dysfunction is a possible early contributor to atherosclerosis, and flow-mediated vasodilatation is defective in individuals with atherosclerotic risk factors or advanced atherosclerosis 22-26. However, the molecular mechanism by which eNOS gene polymorphism interacts with the development of CAD is unclear.

Among reported eNOS polymorphisms, associations between genotypes and cardiovascular diseases are not consistent. The eNOS 4a/b gene was correlated with a smoking-dependent risk of CAD in an Australian population 16. An 894G->T in exon 7 was also correlated with increased risks of coronary spasm 10,11, CAD 12, or AMI 13-14. Three of these studies 10,13,14 were carried out in Japan. In this study, we enrolled only patients with premature CAD to minimize the confounding of environmental components. The low frequencies of the T allele in exon 7 at 894 and a allele of intron 4 VNTR are similar between Taiwan and Japan 10,13,14,17. However, we failed to detect any association between eNOS polymorphisms and the risk of premature CAD in Taiwan. Our results were not totally unexpected, as other studies 27-34 yielded similar findings, including lack of association between eNOS polymorphisms and premature CAD in three studies 32-34. The inconsistent results in different populations may suggest an interaction between other genetic or environmental factors and eNOS genotypes (Table 5).

There are, however, some limitations on the current study. eNOS polymorphisms may have a role in early atherosclerosis, but CAD may be asymptoma-tic until it is quite advanced. Our study population was relatively old (50.5±13.2 years). In this age group, long-term exposure to known environmental risk factors may be predominant for CAD. A selection bias might exist in this hospital-based, case-controlled
study, especially in the limited sample size for the rare 894T and 4a allele.

Few studies have examined the relationship between different eNOS genotypes and endothelial function. Knockout of the eNOS gene resulted in an elevation of blood pressure 35-36, and over-expression of the eNOS gene caused declines in blood pressure and reduction in NO-mediated vasorelaxation 37. Naber CK et al 38. demonstrated that in subjects without CAD, eNOS 894T allele carriers had reduced
average peak velocity and increased coronary vascular resistance at rest. It might indicated an increased microvascular resting tone in eNOS 894T allele carriers. A study demonstrated that

the 4a allele had lo-wer levels of mRNA and protein, but higher enzyme activity of eNOS, and smoking reduced eNOS acti-vity only in the 4a allele 39. eNOS protein with the Asp (894T) variant has been reported to be more susceptible to intracellular protease cleavage than the Glu (894G) variant in vivo 40. In subjects with a parental history of premature MI, the structural and functional changes of arteries are observed at an early age 22,25,26. All of these findings suggest that eNOS polymorphisms might be associated with altered endothelium-dependent vasodilatation, and thus related to the risk of premature atherosclerosis. Our study, however, does not support a major effect of the eNOS genotypes, including 894G→T in exon 7 and 4a/b in intron 4, on flow-mediated vasodilatation. Another
study in Germany 41 which enrolled subjects with mild hypertension or mild hyperlipidemia also showed no differences across the 894G→T genotypes in forearm blood flow in response to intra-arterial infusion of acetylcholine, nitroprusside, or NG-monomethyl- L-arginine.
NO is important in maintenance of normal cardiovascular function. Loss of its anti-platelet, anti-mitogenic, and vasodilator properties occurs in diverse conditions and many confounding factors may mask the effects of eNOS genotypes on endothelial function 42. Recent study showed that environmental and dietary factors influence flow-mediated vasodilatation in 894T carriers 43. Large cohort studies beginning in early adulthood would be helpful in determining the importance of genotypes on atherogenesis or endothelial function, and their interactions with environmental factors. This is important to define the role of eNOS genotypes in pathogenesis of CAD and the complex interactions between environmental and other genetic factors.
An increasing prevalence of CAD and a younger age of onset have been noted in Taiwan in recent years. Several studies have focused on genetic factors in the Chinese population 30,44-51 (Table 6), and the findings are not always consistent with results for other populations. The development of CAD is a complex process and should not be attributed to a single mutation. A further search for other important genetic factors and, more importantly, the interactions between genetic components and environmental factors may help us to understand the pathogenesis of CAD, and it may provide an effective way of early identification of a high-risk group.
Our data confirmed that the traditional atherosclerotic risk factors play important roles in the risk of premature CAD in Taiwan, whereas there is a lack of association with these eNOS polymorphisms. Our functional study also suggests that eNOS polymorphisms have little influence on endothelial dysfunction as assessed by flow-mediated vasodilatation.
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TABLE 1. Clinical characteristics of the study subjects according to the presence or absence of a history of premature coronary artery disease

	Premature CAD (N=114)	Controls (N=65)	*p*-value
Age – yr	46.9±5.7	49.2±14.2	0.123
(Range)	(26-55)	(19-75)	
Male sex (%)	108 (95%)	53 (83%)	0.005
Hypertension (%)	43 (38%)	15 (23%)	0.044
Diabetes mellitus (%)	21 (18%)	7 (25%)	0.175
Smoking (%)	87 (76%)	20 (58%)	0.000
Serum cholesterol (mg/dl)			
Total	204.1±49.4	190.1±49.6	0.07
HDL	40.4±10.0	48.8±18.3	0.003
LDL	128.4±40.7	112.2±40.2	0.019
Serum triglyceride (mg/dl)	182.8±106.1	147.8±89.7	0.027
GT polymorphism (%)			
GG	91 (79.8%)	53 (81.5%)	0.134
GT	23 (20.2%)	10 (15.4%)	
TT	0 (0%)	2 (3.1%)	
Total frequency of T allele	10.1%	10.8%	
4 a/b polymorphism (%)			0.511
bb	80 (70.2%)	50 (76.9%)	
ab	33 (28.9%)	15 (23.1%)	
aa	1 (0.9%)	0 (0%)	
Total frequency of 4a allele	15.4%	11.5%	

Plus – minus values are means ± SD. CAD denotes coronary artery disease, HDL high-density lipoprotein, and LDL low-density lipoprotein.

TABLE 2. Odds ratios for premature coronary artery disease according to multivariate logistic-regression analysis

Variable	Odds ratio (95% CI) *	*p*-value
Male sex	4.17 (1.41-12.38)	0.010

Hypertension	1.88 (0.93-3.85)	0.081
Smoking	1.11 (0.78-1.58)	0.860
Hyperlipidemia @	2.01 (1.05-3.85)	0.035

* The odds ratios are for each increase of 1 SD in the independent variables.
Age was deleted by design.
Only variables significantly related to premature coronary artery disease in the univariate analysis ($p \leq 0.05$) were entered in the model.
@The definition of hyperlipidemia is T-CHO ≥200mg/dl, or LDL ≥130mg/dl, or TG ≥200mg/dl in combination with either T-CHO/HDL>5 or HDL <35mg/dl.

TABLE 3. Characteristics of the study subjects who underwent functional measures of brachial artery (N=30)

Age-yr (range)	50.5±13.2 (19-72)
Male sex	25 (83%)
Hypertension	6 (20%)
Diabetes mellitus	4 (13%)
Smoking	18 (60%)
Serum cholesterol (mg/dl)	
Total	194.9±56.9
HDL	48.7±21.2
LDL	116.±44.7
Serum triglyceride (mg/dl)	157.4±95.3
eNOS polymorphisms	No. of individuals
GG+bb	18
GG+ab	5
GT+bb	5
TT+bb	2

Plus – minus values are means ± SD. CAD denotes coronary artery disease, HDL high-density lipoprotein, and LDL low-density lipoprotein.

TABLE 4. Functional measures of the brachial artery in subjects without premature coronary artery disease*

Variable	GG+bb (N=18)	GG+ab (N=5)	GT+bb (N=5)	TT+bb (N=2)	*p* value
Base-line diameter (mm)	4.26±0.68	4.29±0.51	3.93±0.83	3.75±0.05	0.6
Base-line flow velocity (cm/sec)	69.2±17.0	66.2±8.9	56.8±6.0	56.0±8.7	0.3
Max. diameter during reactive hyperemia (mm)	4.81±0.71	4.89±0.50	4.48±0.74	4.27±0.11	0.59
Peak flow velocity during hyperemia (cm/sec)	77.0±21.7	82.7±19.4	56.5±8.4	59.8±3.25	0.11
Diameter reactivity during hyperemia (%)	13.4±4.5	14.2±6.8	14.9±7.8	14.1±4.5	0.95
Max. diameter during NTG (mm)	4.86±0.69	4.90±0.59	4.52±0.61	4.24±0.04	0.46

Peak flow velocity during NTG (cm/sec)	85.1±28.3	80.6±32.3	64.3±10.9	56.3±2.6	0.29
Diameter reactivity during NTG (%)	14.7±4.9	14.1±3.6	16.6±10.5	13.2±0.4	0.88

Plus – minus values are means ± SD. NTG: sublingual nitroglycerin.
*p value was calculated by means of ANOVA.

Table 5. Frequencies of *eNOS* polymorphisms in normal populations among different ethnic groups

Ethnic groups	Frequencies of G/T genotypes (%)			Association	Ref.
	G/G	G/T	T/T		
Taiwan (n=65)	81.5	15.4	3.1	No	Present study
Japan (n=100)	91	9.0	0	Coronary spasm	10
Japan (n=357)	82.6	17.4	0	T/T in MI	13
Japan (n=607)	86.7	13.2	0.2	MI	14
UK (n=138)	47.8	42.0	10.2	CAD	12
Australia[#]	43.2(M) 40.2(F)	40.7(M) 48.1(F)	16.0(M) 11.7(F)	No	27
Northern Ireland (n=155)	37.4	46.5	16.1	MI	28
France (n=421)	35.2	52.0	12.8	No	28

[#]M: male, F: female

Ethnic groups	Frequencies of intron 4 genotypes (%)			Association	Ref.
	b/b	a/b	a/a		
Taiwan (n=65)	76.9	23.1	0	No	Present study
Japan (n=357)	79.6	19.0	1.4	MI	13
Japan (n=555)	81.0	18.0	1.0	MI	17
Japan (n=122)	84.4	15.6	0	No (in NIDDM)	29
Australia (n=153)	66.7	32.7	0.7	CAD in smokers	16
UK (n=300)	77.0	21.3	1.7	CAD in non-smokers	19
African-American* (n=185)	45.0	34.0	8.0	MI in < 45y/o group	18
Germany (n=413)	73.0	25.0	0.02	No	30

*Other rare variants: 13.0%

Table 6. Studies of genetic polymorphisms and coronary artery diseases in Chinese population

Genetic polymorphisms	Association with CAD	Ref.
Apo B (signal peptide polymorphism or 3' VNTR)	Yes	44,45
Factor V gene (R485K)	Yes	46
Thrombomodulin gene (G33A in promoter region)	Yes	47
Connexin 37 gene (C1019T)	Yes	48
Paraxonase 1 gene (Q192R)	Yes (in NIDDM)	49
(Codons 55 and 191)	No	50,51
β 3 *-adrenergic receptor* gene (Trp64Arg)	No	52
ACE gene (I/D polymorphism)	No	53-56
Angiotensinogen gene (T174M, M235T)	No	56
MTHFR gene (C677T)	No	57
eNOS gene (intron 4 VNTR)	No	31
(G894T and intron 4 VNTR)	No	Present study

第三型一氧化氮合成酶之多型態與臺灣地區早發性冠狀動脈疾病之關連性

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摘　　要

血管內皮功能失調是動脈粥狀硬化的先驅變化。血管內皮產生的 NO 乃經第三型一氧化氮合成酉每（NOS-III 或稱 eNOS）所合成，爲血管內皮功能之重要調控因子，和動脈粥狀硬化產生有關。近年來 eNOS 基因中，有數種多型態被發現和血管粥狀硬化及冠狀動脈疾病相關；例如，位於 exon 7 的 894G→T 及 intron 4 的 27 個鹼基重複（a 或 b 型）均被發現與高血壓或冠狀動脈疾病可能相關。我們假設第三型一氧化氮合成酉每基因多型態，可能會藉由改變血管內皮功能而影響個體對冠狀動脈疾病之感受性。

本研究將收集 114 位早發性冠狀動脈疾病患者，包括 108 位男性，其年齡小於四十五歲，經血管攝影證實具冠狀動脈粥狀硬化，或年齡小於五十五歲且罹患急性心肌梗塞，及 6 位患有冠心症或急性心肌梗塞的停經前婦女；控制組則由 65 位年齡相匹配之且冠狀動脈血管攝影爲正常者所組成。所有受試者均接受 eNOS exon 7 中 894G→T 及 intron 4 多型態的檢驗。其中 30 位冠狀動脈血管攝影爲正常的受試者，並接受杜卜勒超音波進行前臂動脈反應性充血（reactive hyperemia）的試驗，以評估其血管內皮的功能。

第三型一氧化氮合成酉每的多型態，包括 exon 7 中 894G→T：GG，GT，或 TT 及 intron 4 中 a/a，a/b 及 b/b 多型態的分佈在國人早發性冠狀動脈硬化疾病組及控制組中並無統計學上之差異。男性及高血脂症爲本研究中早發性冠狀動脈疾病之獨立危險因子。第三型一氧化氮合成酉每的 exon 7 中 894G→T 及 intron 4 多型態對於反應性充血造成之血管擴張反應均無統計學上之影響。以多變項回歸分析模型發現，高血壓及高血脂症會減損反應性充血造成之血管擴張反應，p 值分別爲 0.004 及 0.056。

在第三型一氧化氮合成酶的多型態中，無論是 exon 7 中 G/T 或 intron 4 中 27 個鹼基重複次數不同的 4a/b 型，均非臺灣地區居民早發性冠狀動脈疾病之危險因子。同時，它們對於反應性充血造成之血管擴張所代表的血管內皮功能，亦無顯著的影響。相對的，血脂異常爲早發性冠心症之預測指標之一，這可能和血管內皮功能失調相關。