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Influence of eNOS Gene Polymorphisms on Carotid Atherosclerosis

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Introduction. *Nitric oxide (NO) is an endothelium-derived relaxing factor which plays a role in atherogenetic events. Polymorphisms in the endothelial NO synthase gene (eNOS) influences the functional activity of the enzyme and affect the susceptibility to atherogenesis. In this study we determined whether T-786C, G894T and 4a/4b eNOS genetic variants may increase the susceptibility to carotid atherosclerosis.*
Methods. *The study groups included 304 consecutive patients with severe carotid stenosis (≥70%) and 544 control subjects. The eNOS polymorphisms were analysed by molecular biology techniques.*
Results. *The genotype distribution and allele frequency of eNOS 4a/4b, but not T-786C and G894T, polymorphism was significantly different between patients and controls. Using logistic regression with adjustment for other risk factors, the 4a allele and the combined genotype 4a4a + 4a4b/894TT + GT and -786CC + TC/894TT + GT were associated with carotid stenosis (OR = 1.5, p = 0.02; OR = 1.8, p = 0.01; OR = 1.5, p = 0.04, respectively). In a subset of patients (30 of 304) with no traditional risk factors for atherosclerosis, a relatively high incidence of the 4a allele and 4a4a + 4a4b/-786CC + TC combined genotype was noted.*
Discussion. *Our findings suggest that the 4a allele and the eNOS combined genotypes are independent predisposing factors to carotid atherosclerosis.*

Key Words: eNOS gene polymorphisms; Carotid atherosclerosis; Vascular surgery.

Introduction

Atherosclerosis is a complex disease caused by an interaction between genetic and environmental factors. Endothelial dysfunction is a key step in both initiation and progression of atherogenesis.[1] Nitric oxide (NO) has an ability to modulate most of the steps believed to be important in atherogenesis. For example, NO inhibits platelet aggregation,[2] leukocyte adhesion,[3] smooth muscle cell migration and proliferation[4] and oxidation of atherogenic low-density lipoproteins.[5] Dysfunction in NO release or action may therefore promote atherogenesis by inhibiting these protective effects.

Common polymorphisms in gene encoding for endothelial NO synthase (eNOS) influence the expression and functional activity of the enzyme and, in turn, might affect the susceptibility to atherogenesis. A point mutation of guanine to thymine, G894T (also called Glu298Asp) in exon 7 of the eNOS gene has been described; it has been demonstrated that this polymorphism is associated with reduced basal NO production.[6] Evidence that this polymorphism is associated with carotid atherosclerosis has been reported[7] but controversial results were obtained in other Caucasian populations.[8–10]

Another variant, resulting from a thymidine being replaced by a cytosine at nucleotide -786 (T-786C), has been identified in the 5′-flanking region of the eNOS gene.[11] This variant, which results in a reduction in the eNOS gene promoter activity, has been associated with carotid artery stenosis in Italians.[10] Finally, a 27-base pair (bp) repeat polymorphism, 4a/4b, at intron 4 of the eNOS gene has been associated with altered plasma NO levels[12] and has been found to be responsible for variations in the genetic control of plasma nitrite and nitrate levels.[13] No association between this polymorphism and carotid atherosclerosis in asymptomatic Italians was observed.[7]

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The aim of the study was to investigate if there was an association between eNOS gene frequency and carotid atherosclerosis.

Methods

Subjects

The study population consisted of 316 unrelated consecutive patients admitted to the Department of Vascular Surgery of the University of Florence with severe carotid stenosis and 544 unrelated healthy subjects recruited from the staff of the University of Florence and from partners or friends of patients. We used a structured questionnaire to identify disease-free controls and to exclude subjects who were suspected of having any form of vascular disease.

Consanguineous subjects were excluded; patients and controls were Caucasian and drawn from the same area (Central Italy). All subjects gave informed consent and the study complies with the Declaration of Helsinki and was approved by the local ethics committee of the Faculty of Medicine, University of Florence.

Carotid stenosis was assessed as severe ($\geq$70%) by duplex scanning with colour coded echo flow imaging and confirmed by angioCT, according to the NASCET criteria.[14] Preoperative neurological symptoms, defined as ipsilateral cerebrovascular events during 180 days within the intervention, were recorded in 112 patients; the remaining 192 patients were asymptomatic. Patients with non-hemispheric symptoms were considered to be asymptomatic. All patients underwent a clinical cardiological evaluation, ECG and echocardiogram and peripheral arteries echo color Doppler analysis. In patients with symptoms suggestive of ischemic heart disease, further investigations were performed (ECG on exercise stress testing, myocardial scintigraphy, and coronary angiography). Twelve patients have been excluded because of having abdominal aortic aneurysm. The study group included 304 patients.

Controls were defined as subjects who had no carotid stenosis detectable by ultrasound duplex scanning powered by colour-coded echo flow imaging.

Hypertension was defined as systolic pressure $\geq$140 mmHg and/or diastolic pressure $\geq$90 mmHg according to The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure;[15] dyslipidemia was defined according to the Third report of the National Cholesterol Education Program (NCEP).[16] Diabetes was defined in agreement with the American Diabetes Association.[17]

Genetic analysis

eNOS polymorphisms were analysed after genomic DNA extraction from peripheral blood leukocytes using a QIAmp Blood Kit (QIAGEN, Hilden, Germany). The eNOS T-786C polymorphism was analysed by PCR-RFLP analysis as previously described.[11] The eNOS 4a/4b polymorphism was analyzed by PCR-amplification according to Gonzalez-Ordonez *et al*.[18] The eNOS G894T polymorphism detection has been performed by real-time fluorescence PCR through Light Cycler instrument (Roche Diagnostics).[19]

Statistical analysis

Statistical analysis was performed using SPSS (Chicago, USA) for Windows (Version 11.5, Copyright© SPSS Inc.). The χ^2-test was used to test for deviation of genotype distribution from Hardy–Weinberg equilibrium. Established risk factors for atherosclerosis were identified by univariate logistic regression analysis. The relationship between genetic variables and carotid atherosclerosis was determined by multivariate logistic regression analysis after adjustment for traditional risk factors. A p-value <0.05 was considered to indicate statistical significance. The analysis was based on a dominant model of inheritance.

Results

Demographic and clinic characteristics and the prevalence of the atherosclerotic traditional risk factors in patients and controls are reported in Table 1. Genotype distributions were in Hardy–Weinberg equilibrium in patients and controls for all eNOS polymorphisms. The genotype distribution and allele frequencies of the eNOS gene T-786C, G894T and 4a/4b polymorphisms in patients and controls are shown in Table 2. No significant difference in eNOS T-786C and G894T polymorphisms genotype distribution and allele frequency was observed between patients and controls (Table 2). The eNOS 4a/4b genotype distribution and allele frequency were significantly different between patients and controls (Table 2). When we assumed a dominant model of inheritance, an association between the 4a allele and an increased predisposition to the disease was observed (OR 4a4a + 4a4b vs. 4b4b = 1.55 95% CI 1.15–2.08, $p = 0.004$); no association between the -786C and 894T allele and carotid stenosis predisposition was found (OR-786CC + TC

Table 1. Demographic and clinical characteristics of study population

Variable	Patients ($n = 304$)	Controls ($n = 544$)
Age (years)*	72 (33–80)	71 (24–81)
Males, *n* (%)	212 (70)	372 (68)
Females, *n* (%)	92 (30)	172 (32)
Hypertension, *n* (%)	182 (60)	164 (30)
Diabetes, *n* (%)	56 (18)	22 (4)
Dyslipidemia, *n* (%)	170 (56)	106 (19)
Smoking habit, *n* (%)	198 (65)	108 (20)
BMI (kg/m^2) (%)†	25.8 ± 3.5	24.8 ± 3.8
Familial history for cardiovascular disease *n* (%)	114 (37)	42 (8)

BMI, body mass index.
*Median and range.
†Mean ± standard deviations.

vs. -786TT = 1.03 95% CI 0.72–1.46, $p = 0.9$; OR894TT + GT vs. GG = 1.02 95% CI 0.72–1.43, $p = 0.9$, respectively).

The odds ratios for the contemporary presence of the 4a and 894T alleles and of the -786C and 894T variants were 1.6 (95% CI 1.1–2.4, $p = 0.009$) and 1.2 (95% CI 0.9–1.6, $p = 0.1$), respectively. By using multivariate analysis, eNOS 4a allele and the combined genotype 4a4a + 4a4b/894TT + GT and -786CC + TC/894TT + GT were associated with carotid stenosis (Fig. 1). The 4a allele was associated with carotid stenosis (OR 4a4a + 4a4b vs. 4b4b = 1.9 95% CI 1.2–2.9, $p = 0.003$ and OR4a4a + 4a4b vs. 4b4b = 1.6 95% CI 1.1–2.3, $p = 0.008$, respectively) but not with symptoms.

In 30 out of 304 patients (9.8%) no traditional risk factors for atherosclerosis were found. In this sub-group a significantly higher percentage of patients were homozygous for the 4a variant in comparison to those with one or more traditional risk factors (16.6% vs. 3.6%, respectively, $p = 0.007$) and a higher eNOS 4a allele frequency (0.30 vs 0.22, $p = 0.12$) were observed. The eNOS 4a4a + 4a4b/-786CC + TC, but not the other eNOS combined genotypes, was more frequent in this subgroup of patients in comparison to patients with traditional risk factors (40% vs. 28.5%, respectively).

When patients with other localizations of atherosclerotic diseases (coronary artery disease and chronic obstructive peripheral arteriopathy) were excluded from the analysis, a significant difference in genotype distribution and allele frequency of eNOS 4a/4b, but not of eNOS T-786C and G894T polymorphisms between patients and controls was found (Table 3). From the univariate and multivariate analysis a significant association between the eNOS 4a allele and an increased predisposition to carotid atherosclerosis was observed (OR 4a4a + 4a4b vs. 4b4b = 1.68 95% CI 1.18–2.39 $p = 0.004$, OR 4a4a + 4a4b vs. 4b4b = 1.6 95% CI 1.02–2.4 $p = 0.04$, respectively).

Discussion

This is the first report in which eNOS T-786C, G894T and 4a/4b polymorphisms are analyzed in a Caucasian population affected by carotid atherosclerosis. Our findings document a high prevalence of the eNOS 4a allele and combined genotypes in patients who have carotid artery stenosis. The 4a/4b polymorphism is associated with altered plasma NO levels, influencing both NO and enzyme production[13] and data from literature reported that it accounted for 25% of the variance of NO circulating levels.[12] Impaired NO function may contribute to the development of atherosclerosis. NO inhibits vascular smooth muscle cells proliferation, platelet adhesion/aggregation and leukocyte and monocyte adhesion. Moreover, NO

Table 2. Genotype distribution and allele frequencies of the eNOS polymorphisms

Genotype	Allele	Patients ($n = 304$) *n* (%)	Controls ($n = 544$) *n* (%)	*p*-value
-786CC		56 (18)	84 (15)	
-786TC		147 (48)	260 (48)	
-786TT		101 (33)	200 (37)	0.42
	C	0.43	0.39	0.19
894TT		46 (15)	58 (11)	
894GT		130 (43)	248 (46)	
894GG		128 (42)	238 (44)	0.16
	T	0.37	0.33	0.20
4a4a		15 (5)	14 (3)	
4a4b		104 (34)	138 (25)	
4b4b		185 (61)	392 (72)	0.002
	4a	0.22	0.15	0.0004

-786CC, homozygotes for the -786C allele; -786TC, heterozygotes; -786TT, homozygotes for the -786T allele; 894TT, homozygotes for the 894T allele; 894GT, heterozygotes; 894GG, homozygotes for the 894G allele; 4a4a, homozygotes for the 4a allele; 4a4b, heterozygotes; 4b4b, homozygotes for the 4b allele.

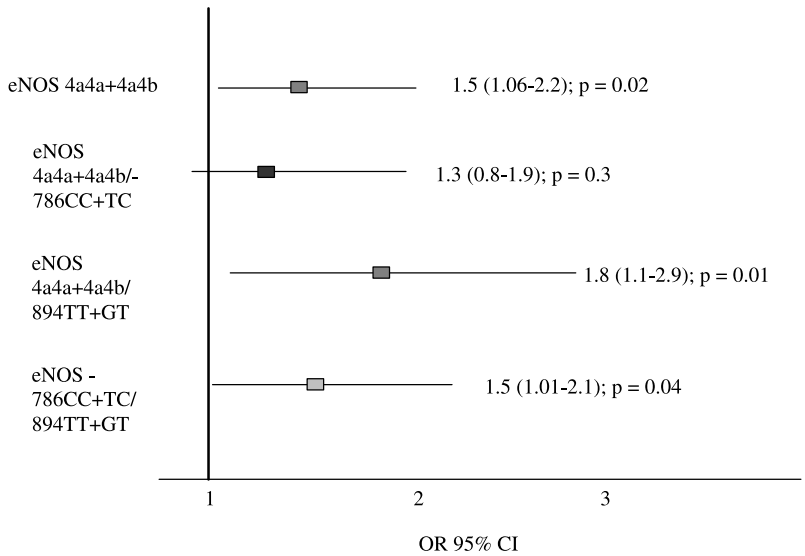

Fig. 1. Odds ratios for eNOS combined genotypes (Multivariate analysis). Adjusted for age, sex, hypertension, dyslipidemia, diabetes, smoking habit and BMI.

induces tissue factor expression and ability of lymphocyte to form coaggregates with platelets. The presence of rare variants in the eNOS gene may contribute to reduce NO levels; infact, it has been demonstrated that the polymorphisms investigated alter NOS activity and reduce basal NO production, contributing in plaque progression and atherothrombosis.

The presence of any DNA rare variant, such as eNOS 4a allele, which affects NO levels or NOS activity might promote pathological changes in vessel wall morphology associated with carotid atherosclerosis. The observation that the eNOS 4a4b polymorphism affects carotid stenosis also in patients with no traditional risk factors, supports the evidence of a genetic contribution to the pathogenesis of the disease.

Our results are at variance with some previous studies,[7,10] but in keeping with others,[8–9,20] which demonstrated no association between the G894T and T-786C polymorphisms and carotid atheroma. These different results are likely a consequence not only of different sample size, but also, most importantly, of different selection criteria adopted for patients and controls, in particular clinical presentation, extent of disease, age, race, geographical area, and concomitant environmental risk factors. In this study we investigated patients with severe carotid stenosis whereas, Lembo *et al*.[7] focused on characterization of the early vascular manifestations of the atherosclerotic disease

Table 3. Genotype distribution and allele frequencies of eNOS polymorphisms in patients with atherosclerosis localized at carotid artery (n = 180)

Genotype	Allele	Patients (n = 180) n (%)	Controls (n = 544) n (%)	p-value
-786CC		30 (17)	84 (15)	
-786TC		85 (47)	260 (48)	
-786TT		65 (36)	200 (37)	0.9
	C	0.40	0.39	0.7
894TT		26 (14)	58 (11)	
894GT		76 (42)	248 (46)	
894GG		78 (43)	238 (44)	0.4
	T	0.36	0.33	0.5
4a4a		9 (5)	14 (3)	
4a4b		62 (34)	138 (25)	
4b4b		109 (61)	392 (72)	0.01
	4a	0.22	0.15	0.002

-786CC, homozygotes for the -786C allele; -786TC, heterozygotes; -786TT, homozygotes for the -786T allele; 894TT, homozygotes for the 894T allele; 894GT, heterozygotes; 894GG, homozygotes for the 894G allele; 4a4a, homozygotes for the 4a allele; 4a4b, heterozygotes; 4b4b, homozygotes for the 4b allele.

and involved a high percentage of hypertensive patients.

In conclusion, the findings of this study suggest that the eNOS polymorphisms may influence the predisposition to carotid atherosclerosis. In this study we have found that eNOS 4a rare variant was associated with carotid atherosclerosis in patients without other risk factors for atherosclerosis. Similar prevalence of this polymorphism has been observed in studies considering coronary artery disease by Hwang J.J.[21] and by us.[22]

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