Independent Risk Factor for Moderate to Severe Internal Carotid Artery Stenosis: T786C Mutation of the Endothelial Nitric Oxide Synthase Gene

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Background: NO synthesized from L-arginine by the constitutive endothelial NO synthase (eNOS) plays a key role in the atherosclerotic process. We investigated whether common variants in the NOS3 gene (a T786C mutation in the 5’ flanking region and the polymorphism on exon 7 that produced the Glu298Arg polymorphism in the protein) are associated with an increased risk of moderate to severe internal carotid artery (ICA) stenosis.

Methods: We studied 88 patients consecutively operated for ICA stenosis and 133 healthy controls. A T786C mutation in the 5’ flanking region and the polymorphism in exon 7 that produces the Glu298Asp polymorphism in the protein were explored by PCR and fluorescent probe analysis.

Results: Genotype distribution was significantly different between patients and controls only for T786C, the CC genotype frequency being 26% and 13%, respectively [odds ratio (OR), 2.26; 95% confidence interval (CI), 1.14–4.46; P = 0.018]. Moreover, the CC genotype was significantly more frequent in a subgroup of patients with ulcerative plaques compared with patients with nonulcerative lesions (44% vs 17%; OR, 3.82; 95% CI, 1.79–8.14; P = 0.003). Multiple logistic regression analysis using the most frequent risk factors and the eNOS gene variant showed that the CC genotype is an independent risk factor for ICA stenosis (P = 0.023).

Conclusion: C allele homozygosity in position 786 of the eNOS promoter seems to be an independent risk factor for the development of moderate to severe ICA stenosis, especially ulcerative lesions.

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Atherosclerosis is responsible for one-half of the deaths in Western countries (1). Its pathogenesis, however, remains unclear. The known atherosclerosis risk factors, i.e., increased cholesterol, diabetes mellitus, hypertension, and smoking, are related to vascular dysfunction with underlying monocyte adhesion and invasion, smooth muscle proliferation and migration, platelet activation, and extracellular matrix formation. In both animal and human models, the endothelial NO pathway appears to be involved in atherosclerosis (2). Endothelium-derived NO is synthesized from L-arginine by endothelial nitric oxide synthase (eNOS) (3), which is encoded by the NOS3 gene on chromosome 7 (4). eNOS is present in the vascular endothelium, platelets, and several other cell types that continuously produce modest amounts of NO (5). NO relaxes vascular smooth muscle, inhibits platelet activation, and modulates migration and growth of vascular smooth muscle cells (6). Indirect evidence suggests that alterations in the NO pathway might be involved in endothelial dysfunction and atherosclerosis (7–9).

There are at least three major mechanisms in the NO pathway that may lead to endothelial dysfunction and atherosclerosis: (a) functional abnormalities of NOS attributable to substrate or cofactor deficiency; (b) increased breakdown of NO; and (c) reduced production of eNOS.

Several lines of evidence indicate a significant association between mutations of the NOS3 gene and cardiovascular disease (10, 11), with discrepant findings possibly related to racial differences (12, 13). Given the complexity...
of directly assessing a causative role of constitutive eNOS, it might be important to evaluate any indirect evidence provided by functional allelic polymorphism studies.

Recently, Nakayama et al. (14) identified a NOS3 gene polymorphism in the 5' flanking region (T786C) that was significantly associated with coronary spasm (14). As assessed by luciferase reporter gene assays, the T786C mutation produced a significant reduction in the NOS3 gene promoter activity. Nakayama et al. also demonstrated that a negative regulatory factor binds to the T786C sequence. These observations strongly suggest that the T786C mutation could be responsible for decreased eNOS production.

Conflicting reports have been published on the relationship between the polymorphism in exon 7 of the NOS3 gene that causes the Glu298Asp polymorphism in eNOS and carotid atheroma, although differences in population selection may account for the variability (15, 16).

To our knowledge there are no published data on the role of functionally active mutations in the 5' flanking region in carotid atherosclerosis. The aim of this study was to investigate whether the eNOS polymorphism is associated with the presence of the atherosclerotic plaques that cause moderate to severe stenosis of the human internal carotid artery (ICA).

**Participants and Methods**

We studied 88 consecutive patients with moderate to severe ICA stenosis presenting to our vascular surgery service and 133 healthy controls. This study was approved by our Institutional Ethical Committee, and informed consent was obtained from patients and controls.

All the 221 participants underwent basic vascular evaluation, including clinical vascular examination, a thorough color-coded echo flow imaging and duplex scanning of the accessible arterial tree, and an electrocardiogram at rest. Patients underwent additional neurologic evaluations and cerebral computed tomography to assess symptoms and/or cerebral infarction related to carotid disease. Controls were defined as individuals with no history of cardiovascular disease and who had no ICA stenoses (0% stenosis) detectable by ultrasound duplex scanning powered by color-coded echo flow imaging.

Hypertension was defined according to the 1999 Canadian recommendations for the management of hypertension (17). Smoker definition included both ex-smokers and active smokers. Hypercholesterolemia was defined as increased total serum cholesterol >5.16 mmol/L.

Carotid stenoses were assessed as moderate (50–69%) and severe (70–99%) by duplex scanning with color-coded echo flow imaging and confirmed by angiography, according to the guidelines from the North American Symptomatic Carotid Endarterectomy Trial and the European Carotid Surgery Trial (18). Preoperative evaluation included ultrasound assessment of stenosing plaque density and presence of ulceration.

All 88 patients in the study had ≥50% ICA stenosis, and the decision on surgery was made according to the American Heart Association guidelines (19). Patients with <50% ICA stenosis were excluded from this study.

Carotid endarterectomies were carried out by a standard eversion method, and the specimens were then processed and examined by standard optical microscopy.

**Molecular Analysis**

Whole blood (3 mL) from patients and controls was collected into potassium EDTA. DNA was prepared with the Istagene Matrix extraction reagent set (Bio-Rad Laboratories). The PCR reaction for T786C and Glu298Asp was carried out in a 25-μL final volume and contained 12.5 μL of TaqMan 2× Universal PCR Master Mix (AmpliTaq Gold polymerase, Amperase uracil-N-glycosylase, dUTP, dGTP, dATP, dCTP, 6-carboxy-x-rhodamine dye, Tris-HCl, KCl, and MgCl2), and 2–20 ng of genomic DNA.

The primers and probes were designed with the Primer Express software. The T786C probes and primers were as follows: 100 nmol/L wild-type allele probe (5'VIC-CATCAAGCTCTTCCCCGGC-TAMRA3'), 50 nmol/L mutant allele probe (5'FAM-CATCAAGCTCTTCCCCGGC-TAMRA3'), where FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine, 50 nmol/L forward primer (5'-CACCCTGATCTGGAAGTGA-T3'), and 300 nmol/L reverse primer (5'-GGCGCATAGGAC-GAGACG-3'); the bold font indicates the site of the mutation. For Glu298Asp, the probes and primers were as follows: 100 nmol/L mutant allele probe (5'VIC-TGGGGGATCATCTGCGGCC-TAMRA3'), 50 nmol/L wild-type allele probe (5'FAM-TGGGGGATCATCTGCGGCC-TAMRA3'), 50 nmol/L forward primer (5'GCGCATCTGACTGCCCAGA-3'), and 300 nmol/L reverse primer (5'ACCGGTGAGCAGCCG-3').

The thermocycler program included 1 cycle at 50 °C for 2 min to activate uracil-N-glycosylase, which was added to prevent carryover contamination; 1 cycle at 95 °C for 10 min to activate the AmpliTaq Gold polymerase; and then 40 cycles of 95 °C for 15 s for denaturing and 62 °C for annealing/extension. After the PCR was finished, allelic discrimination was performed on the PCR product. The 7700 SDS collected fluorescence data on the samples for 5–5 s, and SDS software analyzed the fluorescence, which was visualized in easy-to-read graph form. This method detected four potential clusters of points, corresponding to the TT, CT, or CC genotypes or to no amplification. If there was fluorescence from the reporter (VIC) for the wild-type allele, then the sample was typed as TT. Fluorescence from only the FAM reporter indicated homozygosity for the mutant allele, and it was genotyped as CC. Intermediate fluorescence from both reporters (FAM and VIC) represented the heterozygous population (CT).

Differences between groups were examined by the χ² test or an unpaired Student t-test when appropriate. Odds ratios (ORs; approximate relative risk) were calculated as an index of the association of the eNOS genotypes (786TT,
CT, or CC) with each phenotype. For each OR, two-tailed probability values and 95% confidence intervals (CIs) were calculated.

Multiple logistic regression analysis was used to calculate the OR of ICA stenosis and its 95% CI in individuals exposed to specific risk factors. Only the factors that were significantly associated with the development of ICA stenosis on univariate analysis were included in the logistic regression analysis (diabetes mellitus was excluded from calculation; see below). All statistical analyses were two-sided and were performed with Stata Statistical Software (Stata Corporation).

Results

The allele frequencies of the two polymorphisms in each group were distributed according to the Hardy–Weinberg equilibrium. The control group had a different age distribution (P = 0.01), being slightly younger [mean (SD) ages: patients, 68 (7) years; controls, 64 (7) years], whereas gender frequency was similar (P = 0.433; Table 1). As shown in Table 2, the allele frequency for the polymorphism in exon 7 that produces the Glu298Asp polymorphism in the protein was comparable between patients and the control group.

For the T786C polymorphism, the genotype distribution was significantly different between cases and controls (P <0.02). As shown in Table 3, the frequency of the homozygous CC genotype was twice as high in patients compared with controls (26% vs 13%). Among the 88 patients with ICA stenosis, the CC genotype was significantly higher in patients with ulcerative compared with patients with nonulcerative plaques (CC, 44% vs 17%; OR for CC vs CT + TT, 3.82; 95% CI, 1.79–8.14; P = 0.0033). No differences in genotype distribution and allele frequency were found between patients with moderate or severe ICA stenosis. The incidence of stroke or transient ischemic attack in the CC genotype subgroup of patients with ICA stenosis was not different from that of the CT and TT genotype subgroups.

Multiple logistic regression analysis was performed to determine the independent risk factors for ICA stenosis among patients and controls. The following variables were considered: hypertension, cigarette smoking, hypercholesterolemia, and C allele homozygosity (Table 4). Diabetes mellitus was excluded from the multiple logistic regression analysis because there was no difference in the distribution of this variable between patients and controls in this series (Table 1).

Although there was a statistically significant difference in the number of individuals with hypercholesterolemia between patients (66 of 88 patients; cholesterol concentrations, 3.46–13.34 mmol/L) and controls (78 of 130 controls; cholesterol concentrations, 2.94–8.10 mmol/L; Table 1), this variable was not an independent risk factor for ICA stenosis in our series (OR, 1.57; 95% CI, 0.82–3.03; P = 0.171; Table 4). The other three variables were found to be independent risk factors: hypertension (OR, 2.16; 95% CI, 1.19–3.94; P = 0.011), cigarette smoking (OR, 2.12; 95% CI, 1.13–3.96; P = 0.018), and C allele homozygosity (OR, 2.61; 95% CI, 1.14–6.00; P = 0.023). Interestingly, C allele homozygosity was the most powerful independent predictor of ICA stenosis in our series, with an OR of 2.61.

Discussion

Our findings suggest that homozygosity for C in the T786C polymorphism of NOS3 is associated with ICA stenosis, especially in patients with ulceration, the latter being a major indicator of plaque instability. Multiple logistic regression analysis of CC mutant homozygosity and other major risk factors for atherosclerosis showed

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<th>Table 1. Clinical characteristics of the study participants.</th>
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<th>Table 2. Distribution of Glu298Asp polymorphism in patients and controls.</th>
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* OR for genotype was calculated with TT vs GT + GG.

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<th>Table 3. Distribution of T786C polymorphism on promoter region of NOS3 gene in patients and controls.</th>
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* OR for genotype was calculated with CC vs CT + TT.
that the mutant CC genotype of the NOS3 gene seems to be a reliable predictor of ICA stenosis (OR, 2.61).

Recently, Nakayama et al. (14) showed a relationship between C mutant allele in the NOS3 promoter region and coronary spasm. They also demonstrated, as assessed by luciferase reporter gene assay, that the T786C mutation produced a significant reduction in NOS3 gene promoter activity. Their study thus strongly suggested that the presence of the NOS3 gene mutant allele reduces endothelial production of NO in vessels and predisposes the patients carrying the mutant allele to coronary spasm.

We are not aware of previous studies assessing this polymorphism in a Caucasian population. The wide racial differences in polymorphism distribution are well known. The results of the present study indicate that the prevalence of the T786C polymorphism appears different in Italian compared with Japanese populations; the same is true for the more well-known polymorphism in exon 7 of the NOS3 gene that encodes the Glu298Asp polymorphism in the protein. In our study, controls and patients were all Italian and the genotype distribution in the controls was 54 TT (41%), 61 CT (46%), and 18 CC (13%), which is quite different from the distribution in the Japanese population (89% TT and 0% CC) studied by Nakayama et al. (14). The distribution of the mutant allele in our study was compatible with the Hardy–Weinberg equilibrium in both patients and controls, indicating that the screening method was appropriate.

In the present study, the Glu298Asp polymorphism was not linked to ICA stenosis, demonstrating that the T786C polymorphism is not in linkage disequilibrium with other genetic variants within the same gene. Lembo et al. (20) recently found an excess of homozygotes for the Glu298Asp variant of eNOS among asymptomatic Italians who had atherosclerotic plaques in their carotid arteries compared with individuals without carotid plaques. This finding is apparently in contrast to the observations by Markus et al. (21), showing no linkage between the Glu298Asp polymorphism and carotid atheroma. In contrast to Lembo et al. (20), Markus et al. (21) considered patients with severe carotid stenosis; the difference in population selection may explain the apparently conflicting results.

This Glu298Asp polymorphism is not located in any known functional consensus sequences. The functionality of this polymorphism should thus be investigated (22). On the other hand, the T786C polymorphism has been demonstrated to suppress NOS3 gene transcription (23). Thus, our results might suggest that the presence of the NOS3 mutant allele could predispose the patients carrying the mutant allele to moderate to severe ICA stenosis, possibly reducing endothelial NO production in the carotid arteries.

Although it is likely that some strokes associated with ICA stenosis result from uncompensated hypoperfusion (24), the majority of such strokes appear to occur as a result of embolization of an atherosclerotic carotid plaque or from acute occlusion of the carotid artery and cephalic propagation of thrombus. Histologic studies comparing carotid plaques removed from symptomatic and asymptomatic patients showed the characteristic features of unstable plaques: surface ulceration and plaque rupture, thinning of the fibrous cap, and infiltration of the cap by greater numbers of macrophages and T cells (25). In our study, the CC genotype was present in 44% of patients with ulcerative stenosis, with an OR of 3.82 for CC vs patients with nonulcerative stenosis. It is well known that NO produced by eNOS has a protective physiologic role and orchestrates the paracrine homeostatic functions of the endothelium, which include inhibition of leukocyte and platelet adhesion, control of vascular tone, and maintenance of the thromboresistant interface between the bloodstream and the vessel wall.

In conclusion, our study has identified an association between moderate to severe ICA stenosis and the T786C polymorphism in the NOS3 gene. As shown by multiple logistic regression analysis, C allele homozygosity may be considered an independent risk factor for carotid atherosclerotic plaque formation, with an OR of 2.61. C allele frequency was significantly higher among patients compared with controls (P = 0.003), suggesting a possible facilitating role of the T786C mutation in plaque formation at the carotid bifurcation. In particular, the CC genotype is associated with the more unstable ulcerative plaques.

References


