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Promoter Polymorphisms in the Nitric Oxide Synthase 3 Gene Are Associated With Ischemic Stroke Susceptibility in Young Black Women

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Abstract

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Background and Purpose—Endothelial nitric oxide exerts a variety of protective effects on endothelial cells and blood vessels, and therefore the nitric oxide synthase 3 gene (*NOS3*) is a logical candidate gene for stroke susceptibility.

Methods—We used the population-based Stroke Prevention in Young Women case-control study to assess the association of five *NOS3* polymorphisms in 110 cases (46% black) with ischemic stroke and 206 controls (38% black), 15 to 44 years of age. Polymorphisms included 3 single nucleotide polymorphisms (SNPs) in the promoter region (−1468 T>A, −922 G>A, −786 T>C), 1 SNP in exon 7 (G894T), and 1 insertion/deletion polymorphism within intron 4.

Results—Significant associations with both the −922 G>A and −786 T>C SNPs with ischemic stroke were observed in the black, but not the white, population. This association was attributable to an increased prevalence of the −922 A allele (OR=3.0, 95% CI=1.3 to 6.8; *P*=0.005) and the −786 T allele (OR=2.9, 95% CI=1.3 to 6.4; *P*=0.005) in cases versus controls. These 2 SNPs were in strong linkage disequilibrium (D′=1.0), making it impossible to determine, within the confines of this genetic study, whether 1 or both of these polymorphisms are functionally related to *NOS3* expression. Two sets of haplotypes were also identified, 1 of which may confer an increased susceptibility to stroke in blacks, whereas the other appears to be protective.

Conclusion—Promoter variants in *NOS3* may be associated with ischemic stroke susceptibility among young black women.

Keywords

genetics; nitric oxide; women and minorities; young, stroke in

Familial aggregation and twin studies provide evidence for a strong genetic component to ischemic stroke risk, particularly for early-onset cases.[1-6] Endothelial nitric oxide synthase (*NOS3*) is a logical candidate gene because of its ability to generate NO, which plays a central role in the maintenance of vascular homeostasis, including regulation of the cerebral circulation. NO is also a potent vasodilator[7] and inhibits platelet aggregation.[8] Impaired endothelial-mediated vasodilation is a common feature of many vascular risk factors, and experimental evidence strongly supports a role for impaired NO-dependent vasomotor reactivity in the pathophysiology of stroke.[9,10]

Association Studies with *NOS3* and ischemic stroke have been conducted exclusively in older populations and typically included only 1 or a few SNPs genotyped in various ethnic groups.[11-13] These studies have yielded conflicting results. To further characterize the role of *NOS3* in ischemic stroke, we evaluated 5 polymorphisms among black and white cases and controls in the Stroke Prevention in Young Women Study (SPYW).

Materials and Methods

Population

The SPYW study is a population-based, case-control study initiated to examine risk factors for ischemic stroke. Both cases and their comparison group (controls) were identified from a study area that included all of Maryland except the far Western panhandle, Washington DC, and southern portions of both Pennsylvania and Delaware. Cases and controls were recruited between February 26, 1992 and January 1, 1996. Institutional review committees at collaborating institutions approved the study and participants gave informed consent.

The baseline characteristics of the SPYW population are shown in Table 1. Cases (51 black, 59 white) were women 15 to 44 years of age with a first cerebral infarction, who were identified by discharge surveillance at all 59 hospitals in the study area or through direct referral by

regional neurologists. All stroke cases were adjudicated by 2 neurologists. The methods for discharge surveillance, chart abstraction, case adjudication, and assignment of probable and possible underlying causes have been described previously.[14,15] Recruitment within 1 year of stroke was required for participation. Controls (78 black, 128 white) were identified by random-digit dialing and included women without a history of stroke, frequency matched by age and geographic region of residence to the cases.

Hypertension, diabetes mellitus, and angina or myocardial infarction were determined by asking the participant (or her proxy when the participant was unable to answer) whether she had ever been told by a physician that she had the condition. Similarly, age, race, and current smoking status were determined by participant or proxy report. Total cholesterol was measured according to standard practice.[16]

Genotyping of *NOS3* Polymorphisms

The −1468 T>A, −922 G>A, and −786 T>C single nucleotide polymorphisms (SNPs) were genotyped using the MassARRAY genotyping system (Sequenom, Inc, San Diego, Calif). For the intron 4 tandem repeat, polymerase chain reaction was performed followed by separation on 2% agarose gels. The exon 7 SNP was genotyped by polymerase chain reaction followed by restriction fragment length polymorphism analysis using the enzyme *Ban*II.

Genetic Analysis

Each of the biallelic polymorphisms was analyzed by comparing differences in genotype frequencies between cases and controls, stratified by race. χ^2 tests assuming a recessive model were performed in instances where only a small number of homozygotes existed for the rare allele. Hardy–Weinberg equilibrium and inter-marker linkage disequilibrium (LD) was calculated using SnpAnalyzer, an enhanced, Web-based version of the SNP-Analysis package (Kruglyak available at http://www.fhcrc.org/labs/kruglyak/Downloads). Haplotypes were determined using the PHASE computer program.[17]

Results

Five polymorphisms in *NOS3* were examined (Figure). All SNPs were in Hardy–Weinberg equilibrium. Using multiple pair-wise tests, significant LD was observed between four genotyped polymorphisms in the white population (D′=0.79 to 1.00). Lower D′ values were observed between all SNPs and G894T (D′=0.41 to 0.76). However, in the black population, only the promoter SNPs were in LD with each other (D′=0.82 to 1.00).

Two promoter SNPs, −922 G>A and −786 T>C, were significantly associated with stroke susceptibility in blacks when compared by genotype and allele (*P*=0.005 to 0.017; Table 2). The odds ratio for blacks was 3.0 (95% CI, 1.3 to 6.8) with the −922 AA genotype and 2.9 (95% CI, 1.3 to 6.4) with the −786 TT genotype. This association was independent of hypertension status, because stroke susceptibility was still significantly associated after adjusting for this dichotomous variable (*P*=0.030). No significant associations were observed with interaction analysis with “current smoking” status in either population.

Inspection of haplotypes revealed that the same haplotype was most common in both blacks and whites (TATbG; Table 3). One haplotype was significantly different between black cases and controls. This haplotype, AGCaG, was not observed in any cases but was observed in 8% (n=13) of control participants (*P*=0.001). Further inspection of haplotypes revealed potential “risk” and “protective” haplotypes, if 1 or 2 SNPs were ignored. For example, the TATxG haplotype (where x is any allele) occurred in 59% of the black cases versus 44% of controls (*P*=0.02). Conversely, the AGCxx haplotype occurred in 10% of black cases versus 24% of controls (*P*=0.004).

Discussion

NO is an important and well-characterized vasodilator, with homeostasis of NO essential in maintaining vascular tone in the systemic and cerebral circulation. We observed significant associations with the −922 and −786 promoter SNPs of the *NOS3* gene and susceptibility to stroke in black women. black women with the risk genotype for either SNP (−922 AA or −786 TT) had ≈3× the risk of stroke compared with women with the alternative genotypes. This effect was not observed in the white women.

There is a paucity of comparable data by which to assess our main finding that black women with the risk genotypes were at an increased risk of stroke. Two British studies have examined the association of *NOS3* polymorphisms with stroke in elderly white populations. In an earlier study, the exon 7 G894T SNP was not found to be associated with a combined case population of ischemic stroke and transient ischemic attacks, nor with the degree of carotid stenosis.[11] A subsequent study by the same group found an association between −786 T>C and intron 4 insertion/deletion haplotypes and lacunar infarction in the absence of associated ischemic leukoaraiosis and a protective effect of the intron 4 “a” allele.[12] We did not observe this protective effect in our sample. Although our allele frequencies for both the single SNPs and haplotypes are very similar for the overall white case and control groups, unfortunately, our sample size is too small to examine stroke subtypes to directly compare studies.

There are several potential explanations for the differing associations in different populations. First, these differences could be attributable to chance, although this is unlikely, given the small probability values and the small number of tested polymorphisms. Second, population-stratification bias may be involved. Because our results indicate that blacks have a higher prevalence of the −922 AA and −786 TT genotypes and an increased risk of early-onset stroke, these genotypes might be a marker for African ancestry in general, rather than a marker for increased stroke susceptibility. Third, the association could be caused by a polymorphism in LD with a nearby functional mutation. Finally, *NOS3* may contribute causally to the excess early-onset stroke risk among blacks and not among whites because of different environmental exposures or different non-*NOS3* genetic backgrounds.

The 2 SNPs associated with stroke in the SPYW population are both located in the 5′ promoter region of *NOS3* and were in strong LD in both the white and black populations. Accordingly, we cannot determine whether 1 of these is a true functional polymorphism. Promoter analysis performed by others suggests that the −786 SNP may be responsible for a functional change in *NOS3* gene expression.[18] The −786 T allele conveyed a lower level of *NOS3* expression than the C allele in the presence of the intron 4 polymorphic sequence. Lower levels of *NOS3* expression may lead to reduced protective effects in endothelial cells and thus predispose to stroke.

Haplotype inspection further identified potential risk and protective haplotypes in blacks. The putative risk haplotype, TATxG, suggests that the intron 4 polymorphism is not involved in the effects of *NOS3* on stroke susceptibility. However, it is difficult to determine whether the findings are attributable to a true haplotype effect or to the strong LD between the promoter SNPs. The same is true for the protective haplotype, AGCxx, which is independent of the intron 4 and exon 7 polymorphisms.

The present study is limited by its relatively small sample size and by the fact that the cases are heterogeneous in clinically determined etiology. Importantly, this limitation could lead to a reduced ability to identify genetic risk factors but would not account for significant positive associations. The small sample size precluded assessing the association of *NOS3* variants stratified by stroke subtypes and limited the power for detecting interactions with other risk factors.

This study also has several strengths. First, we examined multiple polymorphisms, as well as estimated haplotypes, in our population, allowing for a more comprehensive evaluation of the gene. Second, our study is the first to examine the association of *NOS3* variants with stroke in blacks. Third, this study involved the use of a young stroke population, which may have enhanced our ability to detect a genetic contribution to risk.

In conclusion, our study extends prior work showing the association of *NOS3* promoter variants with isolated lacunar stroke in an elderly white population[12] to early onset ischemic stroke of diverse subtypes among blacks. More comprehensive studies of the association between *NOS3* genetic variation and both functional correlates and stroke risk in larger, ethnically diverse populations are needed.

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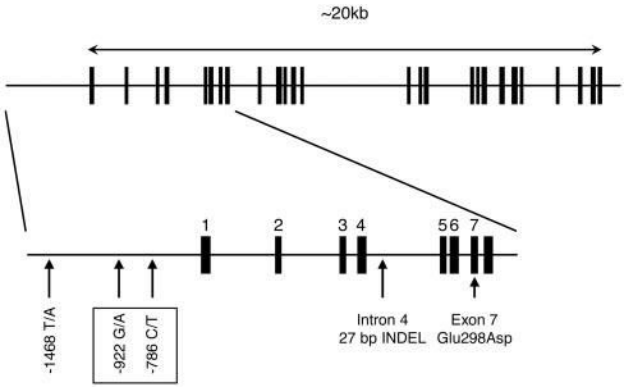

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Genomic structure of the *NOS3* gene and polymorphisms genotyped. The 26 exons (vertical lines) of *NOS3* span 20 kb on chromosome 7. The locations of the five polymorphisms genotyped are shown. The SNPs associated with stroke are boxed.

TABLE 1

Clinical Characteristics

Characteristics	Cases	Controls	P
Sample size	110	206	
Blacks, no. (%)	51 (46)	79 (38)	
Whites, no. (%)	59 (54)	127 (62)	
Median age, y	39	38	
High blood pressure, %	27	14	0.004
Diabetes, %	15	4	0.0003
High blood cholesterol, %	17	18	NS
Current smoking, %	43	29	0.007

NS indicates not significant.

TABLE 2

Analysis of Stroke Susceptibility and *NOS3* Genotypes

	−1468 T/A		−922 G/A		−786 T/C		Intron 4		G894T (T=Asp, G=Glu)	
	Cases	**Controls**	**Cases**	**Controls**	**Cases**	**Controls**	**Cases**	**Controls**	**Cases**	**Controls**
Whites	n=50	n=116	n=52	n=110	n=50	n=117	n=55	n=121	n=59	n=120
	TT: 0.42	TT:0.33	AA: 0.40	AA:0.36	TT: 0.40	TT:0.34	bb: 0.75	bb:0.73	GG: 0.49	GG:0.47
	AT: 0.42	AT:0.52	AG: 0.42	AG:0.49	CT: 0.40	CT:0.50	ab: 0.22	ab:0.25	TG: 0.39	TG:.042
	AA: 0.16	AA:0.16	GG: 0.17	GG:0.15	CC: 0.20	CC:0.15	aa: 0.04	aa:0.02	TT: 0.12	TT:0.12
	0.95		0.66		0.54		0.74		0.76	
Genotype, *P* value										
Allele, *P* value	0.54		0.88		0.84		0.91		0.86	
Blacks	n=50	n=76	n=49	n=75	n=50	n=75	n=46	n=67	n=50	n=78
	TT: 0.46	TT:0.30	AA: 0.80	AA:0.57	TT: 0.78	TT:0.56	bb: 0.41	bb:0.45	GG: 0.08	GG:0.68
	AT: 0.36	AT:0.46	AG: 0.20	AG:0.35	CT: 0.22	CT:0.36	ab: 0.37	ab:0.45	TG: 0.02	TG:0.32
	AA: 0.18	AA:0.24	GG: 0.00	GG:0.08	CC: 0.00	CC:0.08	bc: 0.07	bc:0.03	TT: 0.00	TT:0.00
							aa: 0.11	aa:0.08		
							ac: 0.02	ac:0.00		
							cc: 0.02	cc:0.00		
Genotype, *P* value	0.36		0.0093		0.017		0.12		0.14	
Allele, *P* value	0.078		0.005		0.005		0.79		0.14	

TABLE 3

Haplotype No.	Haplotype	White Cases (n=118)	White Controls (n=254)	Black Cases (n=102)	Black Controls (n=158)
1	AACaG			0.01	
2	AATaG		0.02	0.19	0.18
3	AATbG			0.04	0.04
4	AATcG			0.02	
5	AGCaG	0.14	0.13		0.08*
6	AGCaT	0.01			
7	AGCbG	0.03	0.03	0.06	0.08
8	AGCbT	0.20	0.23	0.04	0.08
9	TACbG	0.01	0.01		
10	TATaG		0.01	0.09	0.04
11	TATbG	0.51	0.48	0.46	0.39
12	TATbT	0.10	0.09	0.06	0.08
13	TATcG			0.04	0.01
14	TGCbG				0.02

n indicates number of haplotypes.

*P=0.001.