

Phosphodiesterase 4D Gene, Ischemic Stroke, and Asymptomatic Carotid Atherosclerosis

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Background and Purpose—Phosphodiesterase 4D (*PDE4D*) was identified recently as the first novel stroke gene to predispose to ischemic stroke independently of conventional risk factors. An association was only found with large vessel and cardioembolic stroke, suggesting a mechanism of accelerated atherosclerosis. We sought to replicate this association in ischemic stroke as a whole, and individual stroke subtypes, in a non-Icelandic European population. To assess a role in early atherosclerosis, we also sought associations with underlying asymptomatic atherosclerosis itself, assessed by carotid ultrasound in a community population.

Methods—A total of 737 consecutive white patients with stroke and 933 white community controls free of symptomatic cerebrovascular disease were examined using a case control methodology. For association with atherosclerosis, intima-media thickness (IMT) in a community population (n=1000) was assessed using carotid ultrasound. Nineteen single nucleotide polymorphisms (SNPs) and 1 minisatellite in the *PDE4D* gene were determined, with haplotyping undertaken using Phase 2.0.

Results—No association with ischemic stroke overall was identified. Six of the 19 SNPs were associated with cardioembolic stroke and 2 different SNPs with large vessel disease. There was no association with carotid artery IMT or carotid plaque in the asymptomatic community subjects.

Conclusions—The *PDE4D* gene is not a major risk factor for ischemic stroke, or early atherosclerosis, within the 2 European population samples studied. On analysis of individual stroke subtypes, there is a possible association with cardioembolic stroke, but the lack of association with carotid IMT and plaque would suggest that this is via a mechanism other than accelerated atherosclerosis. (*Stroke*. 2005;36:949-953.)

Key Words: carotid stenosis ■ intima-media thickness ■ genetics ■ stroke

About half of the risk of stroke is unexplained by conventional cardiovascular risk factors. Both twin^{1,2} and family history studies^{3,4} suggest that genetic predisposition accounts for a proportion of this unexplained risk.

Recently, the first novel stroke gene, apparently acting independently of conventional risk factors, was described. By using a linkage-based approach, using its extensive population genealogical database, the DeCode group in Iceland identified a locus associated with stroke (*STRK1*) and mapped it to chromosome 5q12 using a genome-wide linkage methodology.⁵ Using a positional cloning, fine mapping, and subsequent case control association methodology, they suggested that this susceptibility was explained by variation in *phosphodiesterase 4D* (*PDE4D*).⁶ The *PDE4D* genotype was most strongly associated with the combination of cardioembolic and carotid stroke, whereas no association was found with small vessel disease stroke or cerebral hemorrhage. This would be consistent with a disease mechanism associated with atherosclerosis.

The action of *PDE4D* is poorly understood, but this class of enzymes is involved in the selective degradation of second

messenger cAMP, which has a central role in signal transduction and regulation of physiological responses.⁷ In vascular smooth muscle cells, low cAMP levels lead to cell proliferation and migration that is mediated in part by *PDE4D*.^{8,9} Studies in animal models have shown that elevation of cAMP reduces neointimal lesion formation and inhibits proliferation of smooth muscle cells after arterial injury. These mechanisms could mediate increased atherosclerosis.

Initially, the gene itself was sequenced and no deleterious mutations were found. A detailed analysis of 260 single nucleotide polymorphisms (SNPs) in the flanking and intronic regions was undertaken encompassing the known promoter regions of the gene together with the early coding exons.⁶ An at-risk haplotype present in 8.8% of controls was found, which conferred a relative risk (RR) of stroke of 1.98, whereas a second protective haplotype was associated with a reduced risk of 0.68, relative to the wild-type haplotype. Functional studies demonstrated that *PDE4D* high-risk isoforms present in affected individuals were associated with lower gene expression consistent with a functional role.

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This finding is of major potential importance and may reveal new mechanisms about stroke. However, these results now need to be replicated in independent populations for the following reasons. First, the Icelandic population may not be typical of other populations, being a relative genetic isolate, which may have strong founder effects. Second, although the results of this study demonstrated associations to a high level of statistical significance, it is important that they are confirmed in independent populations to exclude chance associations. Third, stroke subtyping in the DeCode study was fairly simple, and there was limited description of the clinical and imaging features used for subtyping. This may obscure or confound true relationships between the *PDE4D* gene and specific stroke subtypes. Fourth, it is unclear whether this gene predisposes to atherosclerosis itself rather than to stroke.

Therefore, we attempted to replicate this association in 2 non-Icelandic European populations. First, to investigate associations with ischemic stroke as a whole and with individual stroke subtypes, we studied a case-control series of well-phenotyped ischemic strokes and community controls. Second, to determine whether the association was with atherosclerosis itself, we studied associations with carotid intima-media thickness (IMT) and carotid plaque in a community population.

Subjects and Methods

Ischemic Stroke Case-Control Study

A total of 737 consecutive white patients with ischemic stroke attending a cerebrovascular service were recruited in the United Kingdom. All patients had a standard stroke investigation including brain imaging with computed tomography or MRI and imaging of the carotid arteries with duplex or magnetic resonance angiography. Echocardiography was performed when clinically indicated. A standardized risk factor assessment was completed. Hypertension was defined as taking antihypertensive therapy for high blood pressure or having a systolic blood pressure >140 mm Hg or a diastolic blood pressure >90 mm Hg. Raised cholesterol was defined as statin therapy for raised cholesterol or a serum cholesterol >5.3 mmol/L. Cases were classified into stroke subtypes using the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification¹⁰ by the same stroke neurologist with review of original imaging.

A total of 933 white community controls free of symptomatic cerebrovascular disease were also recruited by sampling family doctor lists from the same geographical regions as the patients. Sampling was stratified to provide a similar distribution of age and sex as in the patient group. A similar risk factor assessment was performed for all controls, including blood pressure and serum cholesterol measurement. The study protocol was approved by local research ethics committees, and informed consent was obtained from all participants.

Carotid IMT and Plaque Study

To investigate associations with subclinical atherosclerosis, we used samples from a community study performing duplex carotid ultrasound, the Carotid Atherosclerosis Progression Study (CAPS). Full details have been published previously,¹¹ but in brief, 5460 community individuals ≥ 40 years of age who lived within a radius of 50 km from 5 study sites in western Germany were recruited without exclusion criteria. For the current study, we used 2 subsets of this cohort. First, to look at associations with IMT, the first consecutive 1000 subjects in the 50- to 65-year-old group (mean [SD] age 57.9 [4.4] years; 51.5% women) were included. Second, to look at associations with more advanced atherosclerosis, we performed a case-control study, selecting those individuals with carotid plaque. From among the 5460 individuals, we selected the first 250 individ-

uals with carotid plaque, defined as an IMT >1.8 mm, matched 1:2 for age, sex, and conventional cardiovascular risk factors, with 500 individuals from the 5460 who had no carotid plaque. The study was approved by the ethical review committees of Düsseldorf and Frankfurt University hospitals. The method used to determine IMT and inter-reproducibilities and intrareproducibilities has been described in detail previously.¹²

Choice of Polymorphisms

The previous study by Gretarsdottir et al identified 260 novel SNPs and numbered them numerically.⁶ To avoid confusion and to aid replication, we maintained this nomenclature throughout. Gretarsdottir et al also identified 3 haplotypes defining a wild-type, an at-risk, and a protective haplotype from SNP45, minisatellite AC008818-1, and a common linkage haplotype designated Lc comprising 25 SNPs. We used these as our starting point and assessed SNP45, minisatellite AC008818-1, and 17 SNPs from Lc. The remaining 7 SNPs from Lc showed complete redundancy in haplotypes, with a frequency $>2\%$, so they were not analyzed. Gretarsdottir⁶ also identified 2 coding SNPs at low frequency in their population. We were unable to identify any incidence of either SNP in 100 alleles of our population, so further testing of these was not performed.

Laboratory Methods

All genotyping assays were performed blind to patient details. DNA was extracted from whole blood with the use of Nucleon kits (Tepnel Life Sciences). Genotyping of SNPs was performed by automated polymerase chain reaction and fluorescent calling by a commercial company (K-Bioscience) using a patent-protected process. All ambiguous genotypes were excluded from the analysis, and genotyping success rates are shown in Table I (available online at <http://www.strokeaha.org>). Duplicate testing of a 96-well plate of samples revealed 100% agreement between runs. Minisatellite AC008818-1 was typed on an ABI3100 genotyper (Applied Biosystems). The Centre d'Etude du Polymorphisme Humain control 1347-02 was used on all runs as a reference sample, with the smaller allele of this individual being called a 0 by Gretarsdottir et al.⁶

Statistical Methods

Data were analyzed with the use of SPSS (version 11). Univariate analysis was performed followed by either logistic regression for case-control analyses or multiple regression for IMT as a continuous variable. SNPs were tested for dominant and additive effects individually. For dominant effects, the presence of ≥ 1 risk allele was compared with no copies of the risk allele using dummy variables for the number of risk alleles in a logistic regression. For additive effects, a trend test using the number of SNP risk alleles as a continuous variable in a logistic regression analysis was performed. Haplotype reconstruction was performed using Phase version 2.0¹³ and analyzed in SPSS for dominant and additive effects in the same manner as for an individual SNP. Assuming a frequency of 35% for the at-risk haplotype as identified by Gretarsdottir et al, we have $>99\%$ power to detect the same size effect in our case-control study and a $>98\%$ power to detect the same size effect in our IMT population. Even at 10% frequency of the at-risk haplotype, both sample sets retain $>92\%$ power to detect the RR of 1.98 identified by the DeCode group.

Results

Stroke Case-Control Study

The demographics of the stroke and control populations are shown in Table 1. Cases and controls show no difference for age and sex but do show significant differences for conventional risk factors predisposing to stroke.

TABLE 1. Demographics and Cardiovascular Risk Factors Profiles in Cases and Controls

Risk Factor	Cases n=737	Controls n=933	P Value
Age	65±12.5	65±8.9	0.781
Male gender	59%	57%	0.594
Hypertension	76%	59%	<0.001
Diabetes	15%	5%	<0.001
Serum cholesterol (mmol/L)	5.8±1.4	5.6±1.1	0.018
Hypercholesterolemia	71%	65%	0.022
Smoking current/former/never	34%, 38%, 28%	17%, 40%, 43%	<0.001

Significance calculated using χ^2 analysis.

Associations With All Ischemic Stroke

All SNPs were confirmed to be in Hardy–Weinberg equilibrium in the control population before associations were performed. There was no significant association between any individual SNP or any allele of minisatellite AC008818-1 and all ischemic strokes. Genotype frequencies and *P* values are shown in Table I.

Associations With Individual Stroke Subtypes

Association of SNPs with individual stroke subtypes are shown in Table I. No association was found between any allele of minisatellite AC008818-1 and any stroke subtype (data not shown). No association was found between any of the 19 SNPs and small vessel disease stroke. Two SNPs showed a significant difference between large vessel disease cases and controls, and 6 SNPs showed a significant difference between cardioembolic cases and controls (Table 2). The SNPs showing associations with large vessel and cardioembolic stroke were different, and no SNPs were associated when the 2 subtypes combined were compared with the control population. Following multivariate analysis and adjustment for age, gender, smoking, hypercholesterolemia, hypertension, and diabetes, 7 of the SNPs showing associations on univariate analysis still showed a significant association when present at 2 copies of the risk allele (Table 2).

Stroke Subtype Haplotype Analysis

SNPs 19 and 87 were determined to be necessary as 2 copies of the at-risk allele to be an independent risk factor for large

vessel disease. Thus, presence of the SNP19 G and SNP87 T haplotype would also be expected to be a risk factor for large vessel disease. Using the computational program phase 2.0¹³ for haplotype reconstruction, at least 1 copy of the GT haplotype was identified in 9.3% of controls (n=933) and 11.7% of large vessel disease cases (n=223), whereas the frequency of individuals displaying 2 copies of the GT haplotype was 0.5% and 0.9% in controls and large vessel disease patients, respectively. Analysis shows that the RR of ≥ 1 copies of the GT haplotype is 1.158 (0.822 to 1.632; *P*=0.292), whereas the low frequency means the risk of possessing 2 copies of the GT haplotype cannot be assessed reliably from our data set. Thus, from our data set, no haplotype gave a greater risk of large vessel disease stroke than possessing 2 copies of SNP19 G.

In cardioembolic stroke, 2 copies of the at risk allele of SNPs 2, 13, 14, 20, and 26 were found to show a significant risk of cardioembolic stroke. Thus, the presence of 2 copies of the TGATC haplotype should also confer an increased risk of cardioembolic stroke. This haplotype was identified in 60.8% of controls (n=933) and 67.5% of cardioembolic patients (n=80), with the presence of 2 copies being identified in 13.7% of controls and 21.3% of cases, respectively. There was no increased risk associated with either ≥ 1 copies of the haplotype (RR, 1.340 [0.824 to 2.179]; *P*=0.237) or the presence of 2 copies (RR, 1.729 [0.946 to 3.160]; *P*=0.075) after adjustment for risk factors. This may be because of the small sample size of the cardioembolic group or a true effect. No other haplotype from these 5 SNPs had a greater risk than the presence of 2 copies of the SNP13 risk allele alone (RR 2.26 [1.36 to 3.75]; *P*=0.003).

Evaluation of Previously Identified At-Risk, Protective, and Wild-Type Haplotypes

Gretarsdottir et al previously identified an at-risk haplotype designated G0 between SNP45 and allele 0 of minisatellite AC008818-1 and a protective haplotype of AX from the same 2 markers. Therefore, we have addressed whether these same haplotypes confer similar risks in our population. There were no significant differences between all cases and controls or any ischemic subtype and controls (Table 3). Our observed

TABLE 2. SNPs Found to be Significantly Associated With Large Vessel Disease and Cardioembolic Stroke

Large Vessel Disease						
SNP	Minor Allele	% Cases	% Controls	1 Copy OR (95% CI)	2 Copy OR (95% CI)	Trend Significance
SNP19	G	28.4%	23.3%	1.118 (0.820–1.526)	2.256 (1.288–3.953)	0.024
SNP87	C	44.5%	48.3%	0.655 (0.465–0.921)	0.779 (0.522–1.162)	0.157
Cardioembolic Stroke						
SNP2	A	36.6%	45.0%	0.548 (0.361–0.952)	0.590 (0.295–1.182)	0.067
SNP13	G	44.6%	43.2%	1.124 (0.616–2.050)	2.346 (1.269–4.335)	0.007
SNP14	A	50.7%	40.9%	1.260 (0.696–2.282)	2.105 (1.091–4.061)	0.031
SNP15	T	37.3%	46.6%	0.660 (0.398–1.094)	0.508 (0.261–0.988)	0.032
SNP20	C	17.5%	28.7%	0.601 (0.360–1.003)	0.657 (0.197–2.186)	0.067
SNP26	C	52.5%	42.9%	1.224 (0.695–2.156)	2.118 (1.136–3.946)	0.021

The minor allele is the least frequent allele, not the risk allele. Odds ratios (ORs) are shown for 1 and 2 copies of the minor allele, and the trend significance shows the significance of the additive effects model for increasing minor allele No.

TABLE 3. Frequency of the A0, AX (protective), G0 (at-risk), and GX (wild-type) Haplotypes Derived From SNP45 and Minisatellite AC008818-1 by Ischemic Stroke Subtype

	A0	AX	G0	GX	Significance
Controls (933)	0.9%	15.9%	23.2%	60.0%	...
All cases (737)	0.4%	14.9%	23.5%	61.2%	<i>P</i> =0.772
LVD (223)	0.5%	16.7%	23.8%	61.0%	<i>P</i> =0.977
SVD (134)	0.4%	13.4%	23.9%	62.3%	<i>P</i> =0.715
CE (80)	0%	13.3%	24.7%	62.0%	<i>P</i> =0.614
Unknown (203)	0.5%	14.8%	23.2%	61.5%	<i>P</i> =0.994
LVD+CE (303)	0.3%	15.8%	23.6%	60.3%	<i>P</i> =0.791

LVD indicates large vessel disease; SVD, small vessel disease; CE, cardioembolic.

Significance calculated using χ^2 analysis.

frequency of the at-risk haplotype was 23.5%, which equated to a power of >99.8% to detect an RR of 1.98 should such a risk exist in our case control and IMT populations.

Carotid Artery IMT and Plaque Study

Associations With Carotid IMT

There were no significant differences between left- and right-sided measurements at any IMT site. Therefore, a mean of values from the 2 sides was used for analysis. There was no association between the 19 SNP genotypes or any allele of minisatellite AC008818-1 and mean IMT at any of the 3 sites, except for 1 copy of the SNP87 risk allele with increased IMT in the carotid bulb (*P*=0.008). After adjusting for age, sex, body mass index, cholesterol, diabetes, smoking, and hypertension, presence of ≥ 1 copies of the SNP87 risk allele remained associated with carotid bulb IMT (*P*=0.041). Two copies of the SNP87 risk allele showed no significantly greater risk than a single copy. SNP87 showed no association with either increased common carotid artery nor internal carotid artery IMT values. No association with any allele of minisatellite AC008818-1 was found with IMT measurements at any of the 3 sites.

Associations With Carotid Plaque in Case-Control Study

There was no association between the presence of carotid plaque and any of the 19 SNPs or the minisatellite AC008818-1. An analysis of top-quartile IMT against bottom 3 quartiles for each of the SNPs and minisatellite alleles at each of the 3 sites also revealed no significant associations. Haplotype analysis also revealed no significant association between any haplotype and IMT (data not shown).

Discussion

Our results suggest that the genetic variants in the *PDE4D* gene, described by the DeCode group, are not major risk factors for ischemic stroke in our populations. We found no association between any individual SNP, or with the at-risk haplotype identified by the DeCode group, and ischemic stroke. Furthermore, we found no association between either individual SNPs, or the at-risk haplotype, and carotid IMT or plaque. This suggests that these variants in the *PDE4D* gene are not a major risk factor for early carotid atherosclerosis in the populations under investigation.

The DeCode group found an association with carotid and cardioembolic stroke but not with small vessel disease stroke. Therefore, we examined associations with individual stroke subtypes. We found associations between some SNPs and carotid and cardioembolic stroke. Two copies of the risk allele for SNPs 2, 13, 14, 20, and 26 were associated with cardioembolic stroke. These associations remained significant after controlling for other cardiovascular risk factors. All cases in our study had brain imaging and imaging of the carotid arteries, but echocardiography was only performed when clinically indicated. For this reason, the proportion of cases with cardioembolic stroke was low. Our results suggest a possible association in this stroke subtype, and further studies in larger numbers of patients with cardioembolic stroke are indicated.

We found 2 of 19 SNPs were associated with large vessel disease stroke. These were different from the SNPs associated with cardioembolic stroke, and this would argue against the same variants in the *PDE4D* gene predisposing to carotid and cardioembolic stroke via the same mechanism. However, this is not incompatible with *PDE4D* having a role in both subtypes independently. For example, increased *PDE4D* expression could lead to decreased cAMP activity and increased smooth muscle migration and plaque instability, a risk factor for large vessel disease;^{8,9} whereas decreased *PDE4D* activity could lead to an increase in cAMP, consequent activation of protein kinase C, and influx of Ca^{2+} leading to atrial fibrillation, a risk factor for cardiogenic stroke.¹⁴

The results of our carotid IMT study argue against a major role for genetic variation in the *PDE4D* gene as a risk factor for large artery stroke via accelerated atherosclerosis. IMT is increasingly used as an intermediate phenotype for large artery stroke to examine genetic associations with early atherosclerosis. Increased IMT associates strongly with evidence of established carotid and coronary atherosclerosis and is an independent predictor of future cardiovascular events.¹⁵ It offers a number of advantages, including the use of a continuous variable and overcoming the problem of subclinical disease, whereby a control in a case-control study will develop stroke shortly after being recruited.

We examined associations between the 19 SNPs and the minisatellite with IMT in the common carotid artery, at the bifurcation, and the proximal internal carotid artery. Only 1 of these 60 associations was significant, and this was likely to be attributable to chance. These results suggest that the *PDE4D* gene is not a risk factor for early carotid atherosclerosis. IMT increases may also represent remodeling rather than atherosclerosis. Therefore, we also looked for associations with more advanced atherosclerosis, as evidenced by carotid plaque, in the same population. No associations were found with any of the SNPs or the minisatellite.

In our case-control study, we studied consecutive patients of all ages presenting with ischemic stroke. This is the most appropriate population to determine whether a particular genetic variant is important on a population basis. However, genetic influences are more important in younger stroke patients, and it is possible that study of a younger population may reveal associations. It is also possible that our failure to

replicate the DeCode results may be attributable to differences between our western European populations and the Icelandic population, as evidenced by the different frequencies of the at-risk, wild-type, and protective haplotypes of SNP45 and minisatellite AC008818-1 in our control population and the Icelandic control populations.

Despite finding an association between *PDE4D* and ischemic stroke, the DeCode group was unable to find specific mutations conferring this increased risk. Therefore, they described a haplotype on the basis of a number of SNPs associated with increased risk. To attempt to replicate these results in our population required genotyping of 19 SNPs and 1 minisatellite. This involved performing $\approx 60\,000$ genotypes. In view of the multiple comparisons made, our results have to be interpreted cautiously. Our primary aim was to determine whether the same haplotype identified by the DeCode group was a risk factor for ischemic stroke as a whole in our case-control study or for increased IMT and the presence of carotid plaque in our IMT sample. Both results were negative. The optimal method for controlling for multiple comparisons when looking at associations with individual SNPs is uncertain. Applying a multiple comparisons statistic, such as the Bonferroni correction, is inappropriate because the individual SNPs are not independent. For this reason, we presented unadjusted *P* values but have interpreted the results cautiously.

In conclusion, our results suggest that the *PDE4D* gene is not a major risk factor for ischemic stroke or early atherosclerosis in the 2 western European populations studied, although *PDE4D* cannot be excluded entirely as a risk factor for ischemic stroke as a result of this study. On analysis of individual stroke subtypes, there is a suggestion of an association with cardioembolic stroke, but the lack of association with carotid IMT and plaque would suggest that this is via a mechanism other than accelerated atherosclerosis. Additional studies are required in well-phenotyped groups of patients with cardioembolic stroke to explore this association further.

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