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Association of the Phosphodiesterase 4D (PDE4D) Gene and Cardioembolic Stroke in an Australian Cohort

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None

Abstract

Background: Large-scale epidemiological studies support an important role for susceptibility genes in the pathogenesis of ischemic stroke, with PDE4D identified as the first gene predisposing to ischemic stroke. Several single nucleotide polymorphisms (SNPs) within the PDE4D gene have been implicated in the pathogenesis of stroke.

Aim: Undertake a multivariate analysis of six SNPs within the PDE4D gene in a previously defined Australian stroke cohort, to determine whether these SNPs have an association with ischemic stroke.

Methods: This case-control study was performed using an existing genetic database of 180 ischemic stroke patients and 301 community controls, previously evaluated for cerebrovascular risk factors (hypertension, hypercholesterolemia, diabetes, paroxysmal atrial fibrillation, smoking and history of stroke in a first degree relative). Based on previously reported associations with large vessel disease, ischemic stroke, cardioembolic stroke or a mixture of these, six SNPs in the PDE4D gene were selected for study, these being SNPs 13, 19, rs152312, 45, 83 and 87, based on previously utilised DeCODE nomenclature. SNPs were genotyped using a sequence-specific polymerase chain reaction (PCR) method and gel electrophoresis. Logistic regression was undertaken to determine the relevance of each polymorphism to stroke. Further analysis was undertaken to determine the risk of stroke following stratification for stroke subtype and etiology.

Results: Significant odds ratios were found to be associated with cardioembolic strokes in two SNPs: rs152312 and SNP 45 ($p<0.05$).

Conclusion: Our findings demonstrated an association between cardioembolic stroke and PDE4D SNPs rs152312 and 45. No significant association was found for the other 4 SNPs

investigated within the PDE4D gene. We propose that the results from this Australian population support the concept that a large prospective international study is required to investigate the role of PDE4D in the cardiogenic cause of ischemic stroke.

Introduction

Large-scale epidemiological studies support an important role for susceptibility genes in the pathogenesis of ischemic stroke (1-3). A unique Icelandic familial stroke study identified Phosphodiesterase 4D (PDE4D) as the first mapping of a gene to predispose to ischemic stroke, independent of conventional risk factors (4, 5). Vascular endothelial cells act as gatekeepers to control the infiltration of inflammatory cells via adhesion molecules that play a central role in the recruitment of inflammatory cells (6). PDE4D degrades 3',5'-cyclic AMP (cAMP), which is a key signalling molecule involved in the inflammatory responses of vascular cells (6, 7). Inhibitors of PDE4 have been reported to increase cAMP levels and adhesion in vascular endothelial cells (8, 9) and decrease migration of vascular smooth muscle cells (10), as well as inhibiting the expression of the cell-adhesion molecule E-selectin on human lung microvascular endothelial cells (11). Because of these effects, PDE4D has been postulated to contribute to vascular disease through its role in processes important in the pathogenesis of atherosclerosis, for example, through inflammation and plaque instability (5, 12).

However, the findings of many subsequent individual studies have not substantiated the original Icelandic association, with meta-analysis of PDE4D SNPs casting doubt on the conclusion of individual studies (13). In this meta-analysis, based on all PDE4D studies prior to 2008, Bevan et al. found that although there were several significant associations between

PDE4D and ischemic stroke, all became non-significant once the original Gretarsdottir et al. study (5) was excluded. When they pooled studies on white populations only, there were significant associations between several SNPs and ischemic stroke subtypes, particularly SNP 45 and combined large vessel disease and cardioembolic stroke subgroups. Nonetheless, when the original study by Gretarsdottir et al. was excluded, the results again became non-significant. Bevan et al. concluded that meta-analysis did not support studies replicating the original Gretarsdottir et al. study for any association of PDE4D with ischemic stroke (13). Since the data they presented could not totally exclude a link between PDE4D and ischemic stroke, they proposed that any association that may exist was likely to be weak and possibly restricted to specific populations: the studies analysed had been undertaken on populations in Iceland, USA, Greece, Japan, Sweden, Pakistan, Australia, Netherlands, United Kingdom and Germany.

A recent review (14) also commented on the relevance of differing findings in different populations, noting that many questions with respect to the role of PDE4D in stroke development remain unresolved. Genetic stroke studies from North India (15) and South India (16) showed that different populations demonstrated different linkages between SNP 83 and ischemic stroke, and SNP 83 with Large Artery Atherosclerosis and Small Artery Occlusion, respectively. Two separate recent medium-sized studies of Shanghai Chinese (17) and Beijing Chinese (18), found an association between SNP 83 and the combined cardiogenic and carotid stroke subgroups and of SNP 83 with atherothrombotic stroke, respectively. The only study of an Australian population in Western Australia has not been replicated independently (19). We initiated an independent South Australian study seeking an association between six PDE4D SNPs, ischemic stroke and stroke risk factors in a previously defined South Australian stroke cohort (21) and sought to determine the relevance of these polymorphisms to either

cardioembolic stroke or other stroke subtypes. We chose the six SNPs based on positive associations in previous studies, a SNP frequency in the population greater than 10% and underlying biological plausibility. Three of these SNPs (45, 83 and 87) were studied independently by the Western Australian researchers (19).

Methods

We genotyped DNA from 180 patients with acute ischemic stroke and 301 age- and gender-matched controls, sourced from our earlier prospective case-control study and using the same method (21), with research ethics approval. Patients for this study were admitted with acute ischemic stroke to one of five major hospitals within metropolitan Adelaide, South Australia and were invited to participate in the study. All participants were evaluated for known cerebrovascular risk factors, including age, gender, ethnicity, hypertension, hypercholesterolemia, diabetes mellitus, past history of stroke, smoking history, family history of stroke and atrial fibrillation (AF). The diagnosis of ischemic stroke was made by a neurologist (J.J.) in accordance with the World Health Organization definition (20). All patients underwent brain computerized tomography in order to allow exclusion of intracerebral and subarachnoid hemorrhage.

Stroke subtype was determined using the Oxford Community Stroke Project criteria (22). Subjects were further classified into lacunar syndrome versus non-lacunar syndrome (i.e. total anterior circular syndrome, partial anterior circulation syndrome and posterior circulation syndrome) and also cardioembolic versus non-cardioembolic causes, based on the presence of AF, determined by review of the twelve lead electrocardiograms (ECG) performed on admission for all patients. Atrial fibrillation was also deemed present if there was a reported

history of paroxysmal AF, regardless of the cardiac rhythm on ECG. Unless other investigations (e.g. high grade internal carotid artery stenosis or stroke affecting multiple cerebrovascular territories in the absence of an identifiable cause) showed otherwise, all patients with AF were recorded as cardioembolic in origin. Patients with cardiac valve prostheses were also coded as cardioembolic in origin if there was no clinical or radiological evidence of an alternate thrombotic source. Full study demographics and risk factor characteristics have been described previously (21) and are summarized in Table 1.

The control group consisted of non-hospitalized subjects from metropolitan Adelaide with no personal history of cerebrovascular disease. Controls, selected by random sampling of the South Australian electronic telephone directory, were group-matched with patients for age (within 5-year strata) and gender.

In this study, 201 consecutive patients with acute ischemic stroke were asked to participate. 182 (90.5%) agreed to participate, and 137 (75%) of these were first presentation ischemic stroke. No significant differences were observed between the two groups in terms of demographic variables (Table 1). Amongst the known risk factors examined, atrial fibrillation was found to be associated with the highest risk of ischemic stroke, with 23% of cases versus 3% of controls affected (OR: 8.5; 95% CI: 4.1 to 17.4). A history of smoking within the past 5 years (OR: 3.1; 95% CI: 1.9 to 5.2) and diabetes mellitus (OR: 2.7; 95% CI: 1.6 to 4.4) were also observed to be significantly associated with ischemic stroke. No association was found between ischemic stroke and a history of stroke in a first-degree relative, hypertension, hypercholesterolemia (Table1), or for the use of antihypertensive, antiplatelet, and lipid-lowering medication (results not shown). Two patients died after enrolment in the study and

before venous blood sampling, so that genetic analysis could not be performed; hence 180 patients remained for the full study.

Six SNPs were chosen based on positive associations in previous studies, a SNP frequency greater than 10% and underlying biological plausibility. We kept the DeCODE nomenclature from the original Icelandic study mapping the PDE4D gene: SNPs 13, 19, rs152312, 45, 83 and 87 (5). These SNPs are located in a region near the alternative forms of exon 1 in the gene.

Allele-specific and consensus primers were designed using sequences from the Entrez Human Genome public database. Specific sequences were synthesised by GeneWorks Pty Ltd, Adelaide, South Australia and the resultant PCR products were confirmed by sequencing (Department of Molecular Pathology, Institute of Medical and Veterinary Science, Adelaide, South Australia). Genotyping and quality control methods have been described previously (21, 23). An internal positive control was run for every PCR well, consisting of a pair of primers specific to an invariant section of the HLA DRB1 gene, to ensure that negative alleles were not due to failed polymerase reactions.

Power was estimated (with Win Episcope [ver.2, http://www.clive.ed.ac.uk/winepiscope/]) using frequencies reported for two representative SNPs (45 and 87) with lower and higher at-risk frequencies, respectively (5). Given a control population allele frequency of 78% (SNP 45, and hence an at-risk frequency of 22%) with 95% confidence levels and 80% beta, 472 subjects were required to detect a minimum odds ratio (OR) of 2.0 (minimum detectable OR <0.81 or >1.52). Using the control population allele frequency of 48.4% (SNP 87) with 95%

confidence intervals (C.I.) and 80% beta, 270 subjects were required to detect a minimum OR of 2.0 (minimum OR <0.79 or >1.36).

All statistics were analyzed with the R statistical language (24). The relevance of each polymorphism was assessed by logistic regression of allelotypes and genotypes, for all strokes and stroke subtypes, adjusting for important vascular risk factors. Hardy-Weinberg equilibrium was also assessed for each SNP. Confounders, defined as extraneous factors or variables that partially (or wholly) account for the observed effect of a risk factor on the occurrence of stroke, included age, gender, family history of stroke, hypertension, diabetes, smoking, cholesterol and atrial fibrillation. Confounders causing ±10% or more change in the odds ratio in the regressions of stroke incidence on SNPs were considered 'important' (25). Confidence intervals were also calculated to assess the significance of the odds ratio.

	Controls **n (%)**	**Cases** **n (%)**	**Odds Ratio** **(95% CI)**	**p** **value**
Age (years, mean +/- 1 SD)	73.4 +/- 11.6	73.6 +/- 12		0.8
Females	134 (45)	80 (44)	1.0 (0.7 – 1.5)	0.9
Males	167 (55)	102 (56)		
White	299 (99.7)	178 (98)	6.7 (0.8 – 60.6)	0.09
Non-white	1 (0.3)	4 (2)		
Family history of stroke	100 (33)	60 (33)	1.0 (0.7 – 1.5)	0.95
No family history of stroke	201 (67)	122 (67)		
Hypertension	137 (46)	85 (52)	1.3 (0.9 – 1.9)	0.2
No hypertensive	164 (54)	87 (48)		
Hypercholesterolemia	119 (40)	65 (36)	0.9 (0.6 – 1.2)	0.4
No hypercholesterolemia	182 (60)	117 (64)		
Atrial fibrillation	10 (3)	41 (23)	8.5 (4.1 – 17.4)	<0.0001
No atrial fibrillation	291 (97)	141 (77)		
Smoking in last 5 years	30 (10)	47 (26)	3.1 (1.9 – 5.2)	<0.0001
No smoking in last 5 years	271 (90)	135 (74)		
Diabetes mellitus	33 (11)	45 (25)	2.7 (1.6 – 4.4)	<0.0001
No diabetes mellitus	268 (89)	137 (75)		

Totals	301	182		

TABLE 1. Demographic Characteristics and Prevalence of Risk Factors for Patients with Ischemic Stroke and Controls.

PDE4D genotype:	n	SNP 13	SNP 19	rs152312	SNP 45	SNP 83	SNP 87
Minor Allele:		*G*	*G*	*T*	*A*	*T*	*C*
Frequency in Controls:	301	45.3%	26.7%	10.3%	17.6%	42.5%	48.3%
Ischemic Stroke:	180	43.1%	28.6%	12.2%	15.3%	43.9%	50.0%
Stroke Subtypes							
Lacunar Stroke:	44	47.7%	25.0%	9.1%	14.8%	47.7%	52.3%
Non-Lacunar Stroke:	136	41.5%	29.8%	13.2%	15.4%	42.6%	49.3%
Total:	180						
Cardioembolic Stroke:	24	22.9%	31.3%	27.1%	2.1%	47.9%	58.3%
Non-Cardioembolic Stroke:	156	45.9%	28.3%	9.9%	17.2%	43.6%	49.0%
Total:	180						

TABLE 2. Minor allele frequency of PDE4D SNPs.

Results

All SNPs showed accordance with expected Hardy-Weinberg equilibrium ratios. Data in Table 1 show that atrial fibrillation, smoking and diabetes were significant confounders affecting the risk of stroke in this population group, as reported previously (21).

The minor allele frequencies we found for each SNP in the control population for all patients with ischemic stroke and for distinctive subtypes are presented in Table 2. For all stroke and for the subtypes classified as lacunar stroke, non-lacunar stroke and non-cardioembolic stroke, no significant associations with these six PDE4D SNPs were found. However, for the cardioembolic stroke subtype, an association was found with two of the SNPs, rs152312 and SNP 45, as shown in Table 3. Significant confounding factors for cardioembolic stroke and rs152312 were atrial fibrillation, hypertension, male gender and diabetes, whilst those for SNP 45 were atrial fibrillation and smoking. However, the data for both of these SNPs remained significant, even after adjustment for these confounding factors.

SNP & *allele*	**Stroke Subtype**	**Odds Ratio**	**95% C.I.**	**p value**
rs152312 *T v C*	cardioembolic stroke (n=24)	3.25	(1.57, 6.36)	0.002
	adjusted for significant variables *	3.85	(1.43, 10.13)	0.007
45 *A v G*	cardioembolic stroke (n=24)	0.11	(0.005, 0.52)	0.002
	adjusted for significant variables ^	0.08	(0.004, 0.50)	0.03

TABLE 3. Association of PDE4D rs152312 and SNP 45 alleles with cardioembolic stroke.

***** Significant confounding effects for rs152312 were atrial fibrillation, hypertension, diabetes and male gender. ^ Significant confounding effects for SNP 45 were atrial fibrillation and smoking.

Table 4 shows the genotypes for SNP rs152312 and SNP 45 and their association with the cardioembolic stroke subtype (CE Stroke column) after important confounding factors were taken into account. It demonstrates that the SNP rs152312 shows significant effects on cardioembolic stroke. The odds ratios for the SNP (TT) versus the wild genotype (CC) (OR=16.37, CI 1.69 to 127.8, p=0.01) and the combined non-wild genotypes (CT+TT) versus CC (OR=3.54, CI 1.05 to 12.33, p=0.04) for cardioembolic strokes are both significant. There are also significant effects with SNP 45 and cardioembolic stroke after the confounding effects are taken into account for the combined non-wild (GA+AA) versus the wild (GG) genotype, although not significant for the single gene copy GA versus the wild genotype (p=0.09). There is obviously no association with the non-cardioembolic stroke subgroup (labelled Non-CE Stroke in Table 4), nor was any association with the other four SNPs' genotypes found (data not shown).

In the analysis of the other four SNPs, what appeared to be a third significant OR with the SNP 13 'G' allele and cardioembolic stroke (OR=0.360; C.I. 0.17 to 0.70, p=0.002) became non-significant when adjusted for the confounding factors of atrial fibrillation, diabetes and hypertension (OR=0.45; C.I. 0.18 to 1.03, p=0.07) (data not shown).

Genotype	Controls n (%)	CE Stroke n (%)	Odds Ratio (95% CI)	p	Non-CE Stroke n (%)	Odds Ratio (95% CI)	p
rs 152312 *							
CC	243 (81)	14 (58)	1.00		125 (80)	1.00	
CT	54 (18)	7 (29)	2.50 (0.65, 9.43)	0.17	31 (20)	0.98 (0.58, 1.64)	0.94
TT	4 (1)	3 (13)	16.37 (1.69, 127.8)	0.01	0		
CT + TT	58 (19)	10 (42)	3.54 (1.05, 12.33)	0.04	31 (20)	0.91 (0.53, 1.51)	0.71
SNP 45 ^							
GG	206 (68)	23 (96)	1.00		105 (67)	1.00	
GA	84 (28)	1 (4)	0.114 (0.005, 0.89)	0.09	48 (31)	1.34 (0.86, 2.10)	0.20
AA	11 (4)	0 (0)	0		3 (2)	0.48 (0.10, 1.68)	0.29
GA + AA	95 (32)	1 (4)	0.085 (0.004, 0.62)	0.04	51 (33)	1.23 (0.79, 1.89)	0.36

TABLE 4. Analysis of SNPs between controls and stroke subtypes, after adjustment for significant confounders. * Significant confounding effects for rs152312 were atrial fibrillation, hypertension, diabetes and male gender. ^ Significant confounding effects for SNP 45 were atrial fibrillation and smoking.

Discussion

In our study, after allowing for important confounders such as atrial fibrillation, significant odds ratios were found to be associated with cardioembolic strokes in SNPs rs152312 and 45 ($p<0.05$). We were powered sufficiently to show no association for any of the six SNPs with total ischemic stroke in our study. Although our subgroup analysis was not powered sufficiently to detect significant association with subgroups of ischemic stroke, we consider our findings overall add to the body of evidence supporting the hypothesis that PDE4D may contribute to the risk of cardioembolic stroke.

The SNP 41 used in the Gretarsdottir et al. study is actually rs12153798 (26), which showed links with combined cardiogenic and carotid stroke (5). It was originally identified as rs152312, but this SNP, although studied by the Gretarsdottir group, was not reported in their supplementary tables as showing any association with stroke. Due to the potential for confusion, we decided to use the nomenclature rs152312 rather than SNP41 in this paper. Our SNP rs152312 data support the findings of Woo et al., who reported an association between rs152312 and cardioembolic stroke in their white US population (27). However, in contrast to their reported association of SNP 87 with cardioembolic stroke, we found no association of SNP 87 with either cardioembolic stroke or total ischemic stroke.

With respect to SNP 45, we found that when compared to the major 'G' allele, the non-wild 'A' confers a protective effect (OR < 1) against having a cardioembolic stroke, either before or after adjustment for confounding factors. A more recent Swedish study also found a protective association between SNP 45 and total ischemic stroke (28). In contrast to both these studies, in

the original Icelandic study an at-risk or positive association (greater risk of stroke) was reported between SNP 45 and the combined cardiogenic and carotid stroke subtype patient groups (5). Our finding is also in contrast with a study from USA that found the 'A' allele had a "non-significant at-risk effect" in non-hypertensive ischemic stroke patients (29). A study in a Swedish population found a "non-significant link" between the 'G' allele and stroke which increased in the Large Artery Atherosclerosis (LAA) subtype of stroke, and was a component of a SNP 45-SNP 41 "GA" haplotype that showed a significant increased risk of stroke in both the LAA group and for the combined LAA and cardioembolic group (30). It remains to be seen if the protective effect of the non-'G' allele is due to an allele exchange between the Icelandic population's 'G' allele and another nearby, undetected "at-risk" allele.

Our study is the second study of PDE4D in an Australian cohort with the first being in a population from the western state, whilst this study is from a southern state. In the Western Australian study, the authors reported a significant association between PDE4D and ischemic stroke (19). They also conducted a meta-analysis of nine case-controlled studies (including their own) that demonstrated a strong association with SNPs 41, 83 and 87. They noted that there was statistical heterogeneity in the direction of the association between individual SNPs and stroke that suggested that the SNPs tested were in linkage disequilibrium with the causal alleles (19). Some of the alleles we examined were also investigated by their study: SNPs 45, 83 and 87. For these SNPs, we report the same minor alleles, which the Western Australian authors noted were the converse of the at-risk alleles reported by Gretarsdottir et al. In contrast with the Western Australian study which found an association of SNPs 83 and 87 with ischemic stroke (19), we found no association of these two SNPs with either total ischemic stroke or with subgroups.

We assigned primers for rs152312 based on the original report detailing 'SNP 41' (5). In the Genebank sequence gb|AC016607.6|AC016607 (Homo sapiens chromosome 5 clone CTD-2165C), rs152312 is 175 base pairs distant from SNP 41. Our chosen SNP rs152312, the correct SNP 41 (rs12153798) and SNP 45 are all contained within the promoter 1a isoform of the gene, being at relative positions in exon 1a of 144510, 144685 and 149009 respectively (31, 32). Since SNP 41 and SNP 45 (4324 base pairs apart) are highly linked ($r^2 = 0.94$) (31), the linkage between rs152312 and SNP 41 (only 175 base pairs apart) would be expected by genetic theory to be even greater.

With regard to our finding of a lack of association between the SNP 13 'G' allele and the cardioembolic stroke subtype ($p=0.07$), it is of interest to note that a previous association has been reported in the United Kingdom between homozygous 'G' SNP 13 alleles and cardioembolic stroke after controlling for risk factors (33).

As reported previously, the conventional risk factors of atrial fibrillation, smoking and diabetes are highly significant confounders affecting the risk of stroke ($p<0.001$) in all stroke patients (21). In our present analysis of stroke subgroups, hypertension and male gender were also found to be significant confounders in strokes of cardioembolic origin, although they had no significant impact in the non-cardioembolic or total ischemic stroke groups (non-cardioembolic data not shown).

Populations of varied ethnic origins report different SNP frequencies for PDE4D in their control population. For example, SNP 45 is one of two PDE4D SNPs that showed linkage in the original Icelandic population (5), but was found to be monomorphic in an Asian population

(34). Data from our control group for SNP 87 demonstrated that the 'T' major allele frequency is 51.8%, very close to the 51.7% found in a United Kingdom study (33), but at slight variance to the dataset from Western Australia that showed a control population frequency of 56.1% (19). Similarly, our control group had SNP 83 'C' and SNP 45 'G' frequencies of 57.7% and 82.3%, respectively; the Western Australian dataset had frequencies of 60.1% and 86.3% (19). These variances in frequency may represent different sampling methods or differences in the ethnic origins of the populations studied.

In relation to the cardiogenic causes of strokes, aortic arch atherosclerosis (AAA) is an important source of cerebral emboli which may increase the risk for ischemic stroke (35). Atherosclerotic plaques that are at least 4 mm in thickness in the aortic arch have been shown to be significant predictors of stroke and other vascular events (36). Patients with severe AAA have over four times the risk of stroke or peripheral embolism and recurrent stroke is also more common in this same patient group (37). Indeed, the prevalence of severe AAA in stroke patients is over 20% (38). One study of 100 consecutive patients with embolic cerebral ischemia of the internal carotid or vertebrobasilar arteries found a cardiac source of emboli in 28% of their patients and AAA was found to be the probable source for embolic stroke in 14% of patients (35).

In our assessment of cardioembolic stroke, some patients may have had aortic arch atherosclerosis (AAA). Cardioembolic stroke was defined as due to an intracardiac source, as determined by the neurologist, for example, atrial fibrillation, recent myocardial infarction or thrombus on cardiac imaging. One plausible explanation is that there is coexistent AAA, but this was not actively investigated or assessed and requires further research. We are unaware of any publications linking AAA with genetic risk.

Recent meta-analysis studies of different ethnic populations provide conflicting results with respect to the importance of the role of PDE4D in the polygenic risk of ischemic stroke (13, 28, 31, 34). However, the PDE4D gene has been postulated to contribute to large vessel disease through its role in inflammation and plaque stability, with changes involving processes that are important in the pathogenesis of atherosclerosis (5, 12). As such, there is biological plausibility for the role of PDE4D in the pathogenesis of atherosclerosis and ischemic stroke, including strokes of cardiogenic origin. Further prospective studies of PDE4D in different populations with larger sample numbers may help clarify the role of PDE4D in ischemic stroke and subtypes.

Conclusion

We report new findings that SNP rs152312 and SNP 45 are associated with the cardioembolic stroke subtype ($p<0.05$) in a mainly Caucasian population from South Australia. However, we found no association of SNP 13, SNP 19, SNP 83 or SNP 87 with ischemic stroke in our study. This is the second Australian study to investigate PDE4D and ischemic stroke (19). Our findings support the original Icelandic study suggesting SNP 45 in the PDE4D gene may be an important association in the cardioembolic subtype of ischemic stroke (5). These findings add to the body of evidence supporting the hypothesis that PDE4D may contribute to the risk of cardiogenic stroke. We propose that the results from our Australian study support the concept that a large, sufficiently powered, prospective international study is required to elucidate the role of PDE4D in cardioembolic ischemic stroke.

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