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Association between the *PDE4D* gene and ischaemic stroke in the Chinese Han population

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A B S T R A C T

Recent findings suggests that *PDE4D* (gene encoding phosphodiesterase 4D) is a stroke-related gene in the Icelandic population, but it is still very controversial as to whether it is a susceptible gene for stroke in other populations. In the present study, we attempted to explore the role of the gene in the pathogenesis of stroke in the Chinese Han population of eastern China. A total of 649 ischaemic stroke patients and 761 unrelated control individuals with no history of stroke or transient ischaemic attack were examined in a case-control study. Four SNPs (single nucleotide polymorphisms) rs152312 (C/T), SNP56 (A/T), SNP83 (C/T) and SNP87 (C/T) with a minor allele frequency over 5% were genotyped and the corresponding haplotypes were constructed. In an analysis of the combined cardiogenic and carotid stroke group, both the allele ($P = 0.0060$) and genotype ($P = 0.0160$) frequencies between cases and controls at SNP83 showed significant differences. However, no difference in haplotype frequencies was observed between cases and controls at rs152312 and SNP56. In the analysis of the small-artery-occlusive stroke group, no difference in allele or genotype frequencies was observed at any marker between cases and controls; the global haplotype frequency in rs152312 and SNP56 had a significant difference between cases and controls ($P = 0.0162$); the frequency of haplotype C-A was higher in cases than in controls ($P = 0.0122$). In conclusion, our present findings show that polymorphisms in the *PDE4D* gene are associated with an increased risk of ischaemic stroke in the Chinese Han population. The present study adds further support to the role of *PDE4D* in stroke.

INTRODUCTION

Stroke is a leading cause of death and disability in the world. In China, the incidence and mortality rate resulting from stroke events are higher when compared with the world averages [1]. Stroke is considered to be a highly complex disease consisting of a group of heterogeneous disorders with multiple risk factors, both genetic and environmental [2,3]. To this end, the genetic determinants for the common forms of stroke still remain largely unknown.

The most intensively investigated candidate gene for common forms of stroke thus far is *PDE4D*, the gene encoding phosphodiesterase 4D, a member of the large

Key words: haplotype, ischaemic stroke, phosphodiesterase 4D gene (*PDE4D*), risk factor, single nucleotide polymorphism (SNP).
Abbreviations: BMI, body mass index; CI, confidence interval; CT, computed tomography; FAM, 6-carboxyfluorescein; HWE, Hardy–Weinberg equilibrium; LD, linkage disequilibrium; MRI, magnetic resonance imaging; OR, odd ratio; *PDE4D*, gene encoding phosphodiesterase 4D; SNP, single nucleotide polymorphism; UNG, uracil-N-glycosylase.

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superfamily of cyclic nucleotide phosphodiesterases. Gretarsdottir et al. [4] first mapped the main locus, *STRK1*, to 5q12 in an Icelandic population using a genome-wide linkage search for the common forms of stroke. They subsequently fine-mapped the region and found that *PDE4D* was highly associated with the combination of two forms of stroke: cardiogenic and carotid stroke ([5], but see [5a]). SNP41 (rs12153798; where SNP is single nucleotide polymorphism), SNP45 (rs12188950), one microsatellite marker (AC008818-1) and the haplotypes constructed by SNP45 and AC008818-1 show the most significant association with stroke. Furthermore, they observed a significant dysregulation of multiple PDE4D isoforms in affected individuals. They propose that this gene is involved in the pathogenesis of stroke through atherosclerosis.

Two other linkage studies on the *PDE4D* region at chromosome 5q12 have been performed. In a Swedish cohort, the linkage of stroke susceptibility has been replicated [6], but there is no evidence supporting the linkage in an American population [7]. Numerous follow-up association studies have examined genetic polymorphisms in *PDE4D* including the strongest genotype and haplotype associations from the Iceland study with all ischaemic stroke or its subtypes [6–19]. Some studies conclude that variants in the *PDE4D* gene are not a major risk factor for stroke [8,9], whereas other studies indicate a possible or modest association of *PDE4D* with ischaemic stroke or its subtypes [6,10–13]. Other studies support that *PDE4D* may contribute to the genetic risk of ischaemic stroke in multiple populations [7,14–19].

It should be noted that the SNPs which are positively associated with stroke vary in different studies. These SNPs may be in LD (linkage disequilibrium) with a disease-related SNP, and the level of LD may vary by population. There also remains the possibility that a single disease phenotype can arise from multiple variants in the same gene, as is often the case in monogenic or Mendelian disorders. In an effort to clarify these contradictory findings and to identify the possible pathogenic role of *PDE4D* in relation to stroke, we attempted to replicate the Icelandic study in a Chinese Han population from eastern China.

MATERIALS AND METHODS

Subjects

All subjects in our present study were identified and recruited from Shanghai City in southeastern China. There is a high incidence of stroke in this large metropolitan city and, with the booming economy, the stroke subtypes of the Chinese population may rapidly be adopting a Western pattern. However, it is possible that genetic factors interact with environmental factors to determine the overall risk of stroke in this region.

All patients underwent CT (computed tomography) or MRI (magnetic resonance imaging) of the brain for initial imaging, and an ECG immediately after hospital admission. Detailed clinical information on each case, including clinical signs and symptoms (headache, vertigo, convulsions, aphasia and paralysis), previous medical history, medication use and life style, was obtained. Examinations such as TCD (transcranial Doppler) imaging, DCCU (double carotid chromatic ultrasonic) imaging, DSA (digital subtraction angiography) and electroencephalogram were performed where necessary. A total of 649 patients were recruited consecutively for the study from 2003 to 2006, all of whom had been diagnosed as suffering from ischaemic stroke according to WHO (World Health Organization) criteria [20]. The diagnosis was carried out by two neurologists with expertise in neurovascular diseases, who were blinded to the study hypotheses. The subtypes of ischaemic stroke were defined according to TOAST (Trial of Org 10172 in Acute Stroke Treatment) classification [21]. A total of 239 patients had large-vessel occlusive disease (or 'carotid stroke'), 11 patients had cardioembolic stroke (or 'cardiogenic stroke'), 249 patients had small-artery occlusion ('lacunar stroke') and 150 patients were diagnosed with an undetermined aetiology. The remaining 51 patients had a haemorrhagic stroke. A control group of 761 unrelated individuals, who came to the hospital to have a health check-up, was recruited during the same period. Controls were sampled to match stroke patients for age and gender. As they were in their seventies, a high risk for cerebrovascular diseases, controls were also underwent CT or MRI to exclude intracranial lesions to ensure that none of the controls had a history of stroke or transient ischaemic attack. Both the patients and the controls were native-born residents in Shanghai, speaking the local dialect and migration history was rare for them. Therefore the possibility of any systematic allele frequency difference between cases and controls that appeared as a disease association was small. A standardized risk factor assessment was completed for all subjects. Basic clinical characteristics of ischaemic patients and controls are shown in Table 1.

All subjects were Chinese Han in origin and were unrelated. The study protocol was approved by local research ethics committees, and written informed consent was obtained from either the participants or the participants' relatives, after the procedure had been fully explained. Genomic DNA was extracted from peripheral blood using a modified phenol/chloroform method.

Markers and genotyping

The human *PDE4D* gene spans approx. a 1.5 Mb region on chromosome 5q12 and contains at least 22 exons [OMIM® (Online Mendelian Inheritance in Man®) 600129]. In order to test the most significant disease-associated genetic markers reported previously, we selected eight SNPs: SNP32 (rs456009), SNP41 (rs12153798),

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Table 1 Demographics and clinical characteristics of subjects in the present study
Values are means ± S.D., or percentages.

Characteristic	Ischaemic stroke patients ($n = 649$)	Controls ($n = 761$)	P value
Age (years)	73.20 ± 9.41	73.27 ± 7.30	0.116
Male gender (%)	56%	55%	0.871
Risk factors (%)			
Hypertension	71.34	48.21	< 0.001
Diabetes mellitus	29.74	14.73	< 0.001
Heart disease	36.52	24.40	< 0.001
BMI (kg/m^2)	24.49 ± 11.72	23.95 ± 3.00	0.470

SNP45, SNP48 (rs37760), SNP56 (rs702553), SNP83 (rs966221), SNP87 (rs2910829) and SNP89 (rs1396476), and one microsatellite marker (AC008818-1). The nomenclature of markers used in the Icelandic study [5] was adopted and maintained throughout the present study.

We tested these allele frequencies of SNPs in 32 healthy Chinese individuals using DNA sequencing and found that none of the subjects were heterogeneous for SNP32, SNP41, SNP45 and SNP48, and the minor allele frequency of SNP89 was under 5 %. When we genotyped SNP41 (rs12153798), rs152312 was detected upstream of SNP41 separated by 175 bases. SNP41 was incorrectly labelled as rs152312 in the original paper [5], and has since been corrected to rs12153798 [5a]. Finally, four SNPs with minor allele frequencies over 5 % [rs152312 (C/T), SNP56 (A/T), SNP83 (C/T) and SNP87 (C/T)] were selected for further study. The genotyping of SNP83 and SNP87 was conducted by direct sequencing according to our standard protocol [23]. Two other SNPs (rs152312 and SNP56) were tested by combined kinetic (real-time quantitative) PCR with allele-specific amplification. As described previously [24], this method takes less time than direct sequencing, and the sequences around these two SNPs are suitable for this method. We used two separate real-time quantitative PCRs, each of which contained an allele-specific primer of the SNP and a common primer. Heterozygous samples have an equal amount of the two alleles, which should reach a detectable level of fluorescence at the same cycle number in the two separate PCRs, but for heterogeneous samples the cycle number has to be different for the two amplification reactions. PCRs for each sample were carried out in a total volume of 5 μl containing 10 ng of genomic DNA, 2.5 μl of TaqMan universal PCR master mix (Applied Biosystems), 0.2 μmol/l allele-specific primer, 0.2 μmol/l common primer and 0.2 × SYBR Green I (Molecular Probes). Kinetic PCRs were performed with an ABIPRISM 7900 Sequence Detection System (Applied Biosystems). After an initial 2-min incubation step at 50 °C to activate the AmpErase UNG (uracil-N-glycosylase) and a step of 12 min at 95 °C to deactivate UNG and activate the AmpliTaq Gold enzyme, 50 cycles consisting of 15 s at 95 °C and 30 s at an annealing temperature were performed, followed by a final dissociation stage to check the PCR product. An automated dispenser (Hydra microdispenser; Robbins Scientific) and digital multi-channel pipettes (Thermo LabSystems) were used to reduce well-to-well variability in PCR conditions. To check for genotyping errors, eight DNA samples were randomly selected from each 96-well plate and re-genotyped. All genotypes were identical with those obtained from the first round of genotyping.

The genotyping of microsatellite marker AC008818-1 was performed with one side of a fluorescence-marked [FAM (6-carboxyfluorescein)] primer by Megabace 1000 (Amersham Biosciences), which is a fluorescence-based DNA genotyping system that uses capillary electrophoresis. PCR amplification was done in a final volume of 5 μl, consisting of 10 ng of genomic DNA, 0.2 μmol/l primer, 2.5 μmol/l dNTP, 0.25 unit of HotStar Taq DNA polymerase (Qiagen), 0.5 μl of 10 × buffer and 2.5 mmol/l $MgCl_2$. Themocycling was performed according to a modified touchdown protocol, with an initial denaturation at 95 °C for 10 min to activate the polymerase, followed by 45 cycles of denaturation at 95 °C for 30 s and extension at 72 °C for 1 min. The annealing temperature was decreased from 64 °C by 0.5 °C per cycle for a total of 15 cycles, followed by 30 cycles at the final annealing temperature of 57 °C. PCR products were then subject to electrophoresis on Megabace. The primer sequences for SNPs and the microsatellite are listed in Table 2.

Statistical analysis

Means ± S.D. of continuous data and percentages of categorical data for the basic clinical characteristics of each group as shown. Statistical analysis was determined using a two-sample Student's t test, a χ^2 test and an ANCOVA (analysis of covariance) with risk factors, and these were calculated using SPSS (version 13.0). In the genetic association study, allele and genotype frequencies of each SNP marker were calculated and compared between cases and controls using SHEsis [25] (a robust and user-friendly software platform with a series of tools designed specifically for association study analysis). The genotype frequencies of each SNP marker were tested for HWE (Hardy–Weinberg equilibrium) also using SHEsis [25]. The CLUMP

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Table 2 Primers sequences used for genotyping

SNP	Primer sequence for PCR or direct sequencing		Primer sequence for combined kinetic PCR with allele-specific amplification	
AC008818-1	Left	5′-GGAGGGTGTGCAGTCAGAGG-3′		
	Right	5′-FAM-CTAAGAAGCAGCAGAAGAAAGAAC-3′		
SNP83	Left	5′-GTTTCTAGTGTTAGCCTTGCTCTCT-3′		
	Right	5′-ATTTCACAATGTGGAAGTTTGGT-3′		
SNP87	Left	5′- ATTGCTATTTCCTTCTGAGACTGC -3′		
	Right	5′- AGATGTTGGGAGGGATGATGA -3′		
rs152312	Left	5′- TGGTCTCAAACTCCTGGATTCAA-3′	Left	5′-GTTACTGTGTGTGGCACCCTTTT-3′
	Right	5′- TTTCCTCCTATATAAAAGGGTGCC -3′	Right 1	5′-CACATCCCAGCTCTGACGCT-3′
			Right 2	5′-CACATCCCAGCTCTGACGCC-3′
SNP56	Left	5′- TCTTTACATCGCAACCAGAA -3′	Left 1	5′-GATAATGGTTAATTTCCTACACGT-3′
	Right	5′- AAAGCCCACAAATAGCAGAC -3′	Left 2	5′-GATAATGGTTAATTTCCTACACGA-3′
			Right	5′-AGGAGTTGAATAATTTTTCCAAG-3′

program (version 2.3) implementing a Monte Carlo simulation strategy [26] was used to compare the discrepancies of allele frequencies of the microsatellite marker between cases and controls. Pair-wise LD of all possible pairs of the four polymorphisms was estimated using 2LD software, where the extent of LD was measured by standardized D′ [27], and EMLD software, where the extent of LD was measured by r^2 (https://epi.mdanderson.org/~qhuang/Software/pub.htm). The individual and global haplotype frequency differences between cases and controls were compared using SHEsis [25], with an FPI (Full-Precise-Iteration) algorithm in haplotype reconstruction and frequency estimation in randomly chosen samples, and those haplotypes with a frequency under 5 % were excluded from the analysis to avoid the statistical warp caused by low-frequency haplotypes. ORs (odd ratios) with 95 % CIs (confidence intervals) were estimated for the effects of high-risk haplotypes. The haplotype frequencies were also checked using UNPHASED3.0.3 with an expectation-maximization method of constructing haplotypes [28]. In the present study, the P values were two tailed and significance was accepted when $P < 0.05$. All of the P values were adjusted for multiple comparisons using the QVALUE software, which measures the minimum false discovery rate and offers a sensible balance between the number of true and false positives [29]. The statistical power of our sample size were estimated using the G*Power program [30].

RESULTS

No association was found between any allele of the microsatellite AC008818-1 and stroke or any of its subtypes ($P = 0.6329$ for the ischaemic stroke group; $P = 0.0913$ for the combined cardiogenic and carotid stroke group; and $P = 0.4816$ for the small-artery-occlusive stroke group). We analysed rs152312 (C/T), SNP56 (A/T), SNP83 (C/T) and SNP87 (C/T) in the 5′ region of *PDE4D* in all ischaemic stroke patients (649 subjects) and controls. Genotype frequencies of all four markers showed no deviations from HWE in both cases and controls. Genotype and allele frequencies and single locus association analysis on SHEsis are shown in Table 3. There was no significant difference in allele or genotype frequencies between cases and controls at any SNP, except SNP83. The C allele frequency of SNP83 was higher in cases (23.4 %) than in controls [19.7 %; $P = 0.018$; OR, 1.2429 (95 % CI, 1.0379–1.4484)].

LD between each pair of all of the markers is shown in Table 4. The LD results indicated that rs152312 and SNP56 were in strong LD [31] and a haplotype analysis was therefore performed. Haplotypes can represent a combined effect of several sites along the same chromosome that cannot be detected when these sites are tested one by one. There were significant differences between cases and controls in the haplotype C-A in rs152312(C/T) and SNP56 (A/T). The frequency of haplotype C-A was higher in cases (25.8 %) than in controls [22.0 %; $P = 0.012$ after multiple comparisons correction; OR,1.326 (95 % CI, 1.112–1.582)]. The global haplotype frequency also had a significant difference between cases and controls ($P = 0.019$, after multiple comparisons correction).

In the original Icelandic study, *PDE4D* was strongly associated with a combination of cardiogenic and carotid stroke [5]. Subsequent reports have since found *PDE4D* to also be associated with different subtypes of stroke [7,10,11,17]. To this end, we conducted a breakdown comparison between cases and controls within the combined cardiogenic and carotid stroke group and the small-artery-occlusive stroke group respectively, to explore whether the role of *PDE4D* with ischaemic stroke was subtype related. Genotype and allele frequencies and single locus association analysis results are shown in Table 3. In the combined cardiogenic and carotid stroke group, both the allele ($P = 0.0060$) and

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Table 3 Genotype and allele frequencies and single locus association analysis results
C&C, combined cardiogenic and carotid stroke group; small, the small-artery-occlusive stroke group; df, degree(s) of freedom.

SNP		Allele (*n*)		*P* value (df = 1)		Genotype (*n*)			*P* value (df = 2)
rs152312		C	T		OR (95 % CI)	CC	CT	TT	
Ischaemic	Cases	798 (62.1 %)	486 (37.9 %)	0.5641	0.9958 (0.8198–1.1144)	250 (38.9 %)	298 (46.4 %)	94 (14.6 %)	0.7192
	Controls	962 (63.2 %)	560 (36.8 %)			301 (39.6 %)	360 (47.3 %)	100 (13.1 %)	
C&C	Cases	304 (61.8 %)	188 (38.2 %)	0.5716	0.9413 (0.7633–1.1608)	94 (38.2 %)	116 (47.2 %)	36 (14.6 %)	0.8215
	Controls	962 (63.2 %)	560 (36.8 %)			301 (39.6 %)	360 (47.3 %)	100 (13.1 %)	
Small	Cases	304 (61.5 %)	190 (38.5 %)	0.5052	0.9314 (0.7557–1.1480)	94 (38.1 %)	116 (47.0 %)	37 (15.0 %)	0.7500
	Controls	962 (63.2 %)	560 (36.8 %)			301 (39.6 %)	360 (47.3 %)	100 (13.1 %)	
SNP56		A	T			AA	AT	TT	
Ischaemic	Cases	733 (57.4 %)	543 (42.6 %)	0.6995	1.0301 (0.8862–1.1973)	212 (33.2 %)	309 (48.4 %)	117 (18.3 %)	0.8221
	Controls	861 (56.7 %)	657 (43.3 %)			241 (31.8 %)	379 (49.9 %)	139 (18.3 %)	
C&C	Cases	281 (57.3 %)	209 (42.7 %)	0.8073	1.0259 (0.8351–1.2604)	81 (33.1 %)	119 (48.6 %)	45 (18.4 %)	0.9192
	Controls	861 (56.7 %)	657 (43.3 %)			241 (31.8 %)	379 (49.9 %)	139 (18.3 %)	
Small	Cases	283 (58.2 %)	203 (41.8 %)	0.5581	1.0638 (0.8649–1.3083)	81 (33.3 %)	121 (49.8 %)	41 (16.9 %)	0.8367
	Controls	861 (56.7 %)	657 (43.3 %)			241 (31.8 %)	379 (49.9 %)	139 (18.3 %)	
SNP83		C	T			CC	CT	TT	
Ischaemic	Cases	303 (23.4 %)	993 (76.6 %)	0.0180	1.2429 (1.0379–1.4484)	40 (6.2 %)	223 (34.4 %)	385 (59.4 %)	0.0673
	Controls	300 (19.7 %)	1222 (80.3 %)			35 (4.6 %)	230 (30.2 %)	496 (65.2 %)	
C&C	Cases	127 (25.5 %)	371 (74.5 %)	0.0060	1.3944 (1.0993–1.7687)	15 (6.0 %)	97 (39.0 %)	137 (55.0 %)	0.0160
	Controls	300 (19.7 %)	1222 (80.3 %)			35 (4.6 %)	230 (30.2 %)	496 (65.2 %)	
Small	Cases	105 (21.1 %)	393 (78.9 %)	0.5064	1.0883 (0.8479–1.3969)	13 (5.2 %)	79 (31.7 %)	157 (63.1 %)	0.8097
	Controls	300 (19.7 %)	1222 (80.3 %)			35 (4.6 %)	230 (30.2 %)	496 (65.2 %)	
SNP87		C	T			CC	CT	TT	
Ischaemic	Cases	1060 (82.0 %)	232 (18.0 %)	0.1464	0.8638 (0.7089–1.0526)	439 (68.0 %)	182 (28.2 %)	25 (3.9 %)	0.2955
	Controls	1280 (84.1 %)	242 (15.9 %)			539 (70.8 %)	202 (26.5 %)	20 (2.6 %)	
C&C	Cases	401 (80.8 %)	95 (19.2 %)	0.0917	0.7980 (0.6138–1.0376)	161 (64.9 %)	79 (31.9 %)	8 (3.2 %)	0.2149
	Controls	1280 (84.1 %)	242 (15.9 %)			539 (70.8 %)	202 (26.5 %)	20 (2.6 %)	
Small	Cases	402 (81.0 %)	94 (19.0 %)	0.1132	0.8085 (0.6214–1.0520)	166 (66.9 %)	70 (28.2 %)	12 (4.8 %)	0.1753
	Controls	1280 (84.1 %)	242 (15.9 %)			539 (70.8 %)	202 (26.5 %)	20 (2.6 %)	

Table 4 Estimates of LD between the four markers
*Extent of LD between two SNPs measured by standardized D′; †extent of LD between two SNPs measured by r^2.

Marker	rs152312	SNP56	SNP83	SNP87
rs152312	–	0.747*	0.188*	0.027*
SNP56	0.252†	–	0.049*	0.012*
SNP83	0.006†	0.000†	–	0.192*
SNP87	0.000†	0.000†	0.027†	–

genotype ($P = 0.0160$) frequencies between cases and controls at SNP83 showed significant differences. The C allele frequency of SNP83 was higher in cases (25.5 %) than in controls [19.7 %; OR, 1.3944 (95 % CI, 1.0993–1.7687)]. After adjusting for age, gender, hypertension, diabetes, heart diseases and BMI (body mass index), the SNP83 risk allele remained associated with cardiogenic and carotid stroke ($P = 0.011$). No difference in haplotype frequencies was observed between cases and controls at rs152312 and SNP56 in the cardiogenic and carotid stroke group. In the analysis of the small-artery-occlusive stroke group, no difference in allele or genotype frequencies was observed with any marker between cases and controls. The global haplotype frequency showed a significant difference between cases and controls ($P = 0.0162$, after multiple comparisons correction). The frequency of haplotype C-A in rs152312 (C/T) and SNP56 (A/T) was higher in cases (27.5 %) than in controls [22.0 %; $P = 0.0122$, after multiple comparisons correction; OR, 1.490 (95 % CI, 1.176–1.888)].

We also performed a power calculation on the G*Power program [30]. When an effect size index of 0.15 (corresponding to a 'weak-to-moderate' gene effect) was used, the present sample size revealed a

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power $>99\,\%$ for detection of a significant association ($\alpha < 0.05$).

DISCUSSION

Using a cohort of Chinese Han subjects, we attempted in the present study to confirm the association studies implicating *PDE4D* as a susceptibility factor for stroke. We investigated ten genetic variants at the 5′-end of the gene, among which SNP41, SNP45 and AC008818-1 had the most significant association with stroke in the Icelandic study [5]. However, we did not find any risk allele of the microsatellite AC008818-1 in any group. Furthermore, none of the subjects we tested were heterogeneous for SNP32, SNP41, SNP45 and SNP48. Finally, four SNPs rs152312 (175 bases upstream of SNP41), SNP56, SNP83 and SNP87 with minor allele frequencies over 5 % were selected for the present association study. After adjustment for multiple comparisons with QVALUE, we still found a positive indication that *PDE4D* is associated with an increased risk of ischaemic stroke in the Chinese Han population. Although our findings did not fully replicate the results from the Icelandic study [5], the power of our sample size to detect the same associations should be $>99\,\%$. Population differences in allele and haplotype frequencies as well as LD structure may contribute to the observed differences between populations.

In the Icelandic study [5], the strongest association found in the two main subtypes of ischaemic stroke, cardiogenic and carotid stroke, suggests a role for *PDE4D* in the vascular biology of atherosclerosis. In our combined cardiogenic and carotid stroke group from southeastern China, SNP83 had significant differences both in the allele and genotype frequencies between cases and controls ($P = 0.0189$ after correction for the allele, and $P = 0.0368$ after correction for the genotype). Gretarsdottir et al. [5] found this polymorphism to be significantly associated with the carotid subtype of stroke. In a cohort of North American subjects, there was a significant association with SNP83 in the large-artery group [7]. In the Pakistani population, SNP83 is also found to be significantly associated with ischaemic stroke [14]. A recently published study has also suggested that the SNP83 is a genetic risk factor for atherothrombotic strokes in a northern Chinese population [19]. PDE4D is expressed in most cell types involved in the pathogenesis of atherosclerosis [32–35] and selectively degrades the second messenger cAMP [36], which is a key signalling molecule mediating cell proliferation, migration and secretion related to atherosclerosis [37,38]. At this point, our results from the combined cardiogenic and carotid stroke group support the hypothesis that the SNP83 of *PDE4D* may affect differential expression and activity of the PDE4D enzyme, which modifies the stroke risk on atherosclerotic effects.

In our analysis of the small-artery-occlusive stroke group, where the stroke is thought to be a non-atherosclerotic narrowing of small end-arteries in the brain, the global haplotype frequency in rs152312 (C/T) and SNP56 (A/T) showed a significant difference between cases and controls ($P = 0.0162$ after correction), and the frequency of single haplotype C-A was significantly higher in cases than in controls ($P = 0.0122$ after correction; OR, 1.490). In a U.K. study [10], none of the *PDE4D* polymorphisms associated with stroke correlated with carotid artery IMT (intima media thickness) or plaque. In an American population [18], an effect of *PDE4D* on stroke risk was demonstrated among young adults, a population with a very low prevalence of atherosclerotic disease. These findings suggest that a mechanism other than accelerated atherosclerosis exists. Expression of PDE4D is abundant in neurons as well as in vessel cells [39]. Rolipram, a selective PDE4 inhibitor, especially to PDE4D [39], has been reported to attenuate neuronal cell death in the CA1 sector after transient ischaemia [40,41]. It has also been reported that the substrate of PDE4D, cAMP, is responsible for neuron survival and neurogenesis [42–44]. Recently, it has been reported that rolipram significantly increases the survival of nascent neurons, accompanied by an enhancement of phospho-CREB (cAMP-response-element-binding protein) staining and decreased newborn cell death after ischaemia [45]. These observations point to the possibility that genetic variants of *PDE4D* might modulate the PDE4D/cAMP pathway and alter neuronal properties, possibly rendering neurons to undergo permanent damage from a pre-ischaemic insult.

In conclusion, the present study suggests that polymorphisms of *PDE4D* are a significant risk factor for stroke among the Chinese Han population, and possibly exerts its effects on arteriosclerosis and neuron-protection mechanisms. Our present findings emphasize differences in allele and haplotype frequencies between populations and add independent support to the role of *PDE4D* with stroke.

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