Phosphodiesterase 4D (PDE4D) Gene Variants and Risk of Ischemic Stroke in the Taiwanese Population

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Abstract

Objectives: It has been suggested that genetic variants in the phosphodiesterase 4D (PDE4D) gene confer risk of ischemic stroke. However, the cerebral infarction/cerebral hemorrhage ratio is lower in Asian populations compared with those in Caucasian and black populations. Thus, the association between variations in the PDE4D gene and ischemic stroke in Taiwan needs to be replicated. In the present study, we evaluated whether the PDE4D gene polymorphism confers a risk of ischemic stoke in Taiwanese patients.

Methods: Two single-nucleotide polymorphisms (SNPs) covering the PDE4D gene were genotyped using genomic DNA sequencing and a high-throughput TaqMan PCR assay in 100 patients who had suffered an ischemic stroke and 270 healthy individuals.

Results: No significant associations with ischemic stroke were observed with the SNP87 (rs2910829) or SNP41 (rs152312) SNPs from PDE4D, which were a part of the Icelandic at-risk haplotypes.

Conclusions: The present data does not support a significant role for PDE4D polymorphisms in genetic susceptibility to ischemic stroke in the Taiwanese population.

Strokes are a major cause of adult disability and mortality in the elderly human population worldwide.[1,2] Strokes can be divided into 2 major categories: ischemic and hemorrhagic.[3] The ischemic stroke is characterized by a sudden decrease in blood flow to 1 or more central nervous system areas, and it is the major type of stroke in Caucasians (80% to 90%). Compared with the Caucasian and black populations, the ratio of ischemic stroke/hemorrhagic stroke in Asian populations, including Chinese, Japanese, and Koreans, was much lower.[4] Epidemiological studies revealed that strokes can be attributed to a variety of genetic and environmental risk factors.[5] An epidemiologic study estimated the risk attributable for stroke in two-thirds of the population was due to genetic factors.[6]

Recently, a linkage and association study in Iceland showed that the chromosome 5q12 region was linked to ischemic stroke.[7] The same group then reported that the phosphodiesterase 4D (PDE4D) gene variants were associated with ischemic stroke as results of a genome-wide screening for stroke-susceptibility genes in Iceland.[8] PDE4D is involved in inflammation, cell proliferation, and migration—processes implicated in stroke occurrence.[9-11] PDE4D is a large gene that spans 1.5 Mb and has 22 exons and 8 splice variants.[12] Six PDE4D single-nucleotide polymorphisms (SNPs) were significantly associated with strokes after adjusting for multiple comparisons.[13] However, several replicated studies from different populations have provided apparently conflicting evidence as to the association between the PDE4D gene and stroke.[13-21] For example, PDE4D is a risk factor for ischemic stroke and, in particular, for cardio-embolic (CE) stroke among whites and blacks.[18] However, variants in the PDE4D gene are not a major risk factor for stroke in individuals from central Europe.[14] Population differences in allelic and haplotype frequencies as well as linkage disequilibrium (LD) structure may contribute to the observed differences among populations.

Although of great potential importance, the association of PDE4D with strokes has not been investigated in the Taiwanese population. The aim of the present study was to evaluate the potential of the PDE4D genes to increase the risk of stroke in Taiwan.

Materials and Methods

Patients and Control Individuals

Our study subjects consisted of 100 patients with a mean age of 70 ± 11 years who were recruited from the Department of Neurology, Taipei Municipal Wanfang Hospital, Taipei Medical University, Taipei, Taiwan. The control group consisted of 270 individuals with a mean age of 63 ± 23 years who had no history of vascular disorders. **Table 1** lists the clinical characteristics of all subjects who were recruited. Appropriate informed consent was obtained from all patients and control subjects in this study. The ethics committee of Taipei Medical University approved the study.

Genomic DNA

Genomic DNA from nucleated cells of patients and controls was extracted from EDTA peripheral blood using a DNA blood kit according to the manufacturer's instructions (Qiagen FlexiGene, USA).

Table 1_Clinical Data of Individuals Recruited in This Study

	Control Group	Ischemic Stroke Group
Number of subjects	270 (m 136; f 134)	100 (m 59; f 41)
Age (year)	63 ± 23 (m 68 ± 19; f 57 ± 24)	70 ± 11 (m 70 ± 9; f 71 ± 13)
Glucose (mg/dL)	109.81 ± 27.84	119.43 ± 39.65
Triglyceride (mg/dL)	105.39 ± 66.38	141.32 ± 73.69
Cholesterol (mg/dL)	183.28 ± 34.32	187.14 ± 39.66
HDL-CHOL	42.63 ± 8.39	41.06 ± 13.23
LDL-CHOL	124.55 ± 33.44	117.9 ± 31.67

HDL-CHOL, high density lipoprotein cholesterol; LDL-CHOL, low density lipoprotein cholesterol; m, male; f, female.

Genotyping by Genomic DNA Sequencing

To characterize and confirm the polymorphisms, the polymerase chain reaction (PCR) products were directly sequenced using the following primers (**Table 2**): the reaction mixtures contained 10 μL of template DNA, 2 μL of dNTP (10 nM), 5 μL of 10 × PCR buffer (100 mM Tris-HCL [pH 9.0], 500 mM KCl, 15 mM $MgCl_2$, and 1% Triton X-100), 2 μL of each primer (10 μM), 0.5 μL of Taq DNA polymerase (5 U/μL), and 28.5 μL of double-distilled water (ddH_2O) in a volume of 50 μL. Thermal cycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s, with a final extension of 10 min at 72 °C. The PCR products were sequenced by Mission Biotech (Taipei, Taiwan).

Genotyping by TaqMan PCR Assay

Sequences of allele-specific TaqMan MGB probes and primers were used in the TaqMan PCR assay. These assays are designed for the allelic discrimination of specific SNPs. Primer sequences for the polymorphisms are given in **Table 2**. No-template controls with no DNA in the reaction were used as the criteria for the fluorescence level due to an uncleaved probe. TaqMan PCR and the genotyping analysis were performed on an Applied Biosystems 7300 Real-Time PCR System according to the manufacturer's instructions. The reaction mixtures were amplified in 1 μL of genomic DNA (10 ng/μL) or 1 μL of 100-fold-diluted PCR products, 10 μL of 2× TaqMan Universal Master Mix (Applied Biosystems), 0.5 μL of 40× primer/probe mix, and 8.5 μL of ddH_2O in a volume of 20 μL. PCR cycling conditions were as follows: 1 cycle at 95°C for 10 min, and 40 cycles at 92°C for 15 s and 60°C for 1 min. The results were analyzed on an Applied Biosystems 7300 Real-Time PCR System using an allelic discrimination assay program.

Table 2_Primer and Probe Sequences for the Polymorphisms

Name	Primers	Sequence
rs2910829 (SNP87)	Forward	5′-GTGCTTGCTGGACATTCATAACATC-3′
	Reverse	5′-TGTTTAAAGATGAGGAAGAATAATGGATGCA-3′
	VIC-labeled probe	5′-TCTAGTTTGGGAAATGTTGTGT-3′
	FAM-labeled probe	5′-CTAGTTTGGGAAATATTGTGT-3′
rs152312 (SNP41)	Forward	5′-CCACTAACGCGTCATCTAGCATTA-3′
	Reverse	5′-GAGCCTATTATATGGTGACTGCTCATT-3′
	VIC-labeled probe	5′-CCCTCCCGACAATT-3′
	FAM-labeled probe	5′-TCCCTCCTGACAATT-3′

Statistical Methods

Statistical analyses were carried out using Stat View version 5 (SAS Institute, Cary, NC) software, and $P < 0.05$ was considered significant. Allelic frequencies were calculated for each polymorphic site by the allele counting method.

Results

Genotyping by Genomic DNA Sequencing

We first compared gender and glucose and lipid profiles of all subjects in our study. **Table 1** shows there were no significant differences in these parameters between the patients and healthy controls. To evaluate whether the PDE4D genotype confers a risk for stroke, we performed single-marker association tests with the 2 SNPs of the PDE4D gene constituting SNP87 (rs2910829) and SNP41 (rs152312) using TaqMan PCR. Correlation studies using DNA sequencing as the gold standard revealed that this technique was highly reproducible with good sensitivity and specificity (data not shown).

Analysis of the PDE4D Gene

We compared the gene allelic frequencies of rs2910829 and rs152312 of the PDE4D. **Table 3** shows that the G and A allelic frequencies of rs2910829 in the control were 78.33% and 21.67%, respectively. Frequencies of the G/G, G/A, and A/A genotypes were 61.67%, 33.33%, and 5.0%, respectively. The G and A allelic frequencies of rs2910829 in stroke patients were 83.11% and 16.89%, respectively. Frequencies of the G/G, G/A, and A/A genotypes were 72.97%, 20.27%, and 6.76%, respectively.

The C and T allelic frequencies of rs152312 in the controls were 58.33% and 41.67%, respectively. Frequencies of the C/C, C/T, and T/T genotypes were 35.55%, 45.55%, and 18.90%, respectively. The C and T allelic frequencies of rs152312 in stroke patients were 66.21% and 33.79%, respectively. Frequencies of the C/C, C/T, and T/T genotypes were 43.24%, 45.95%, and 10.81%, respectively.

As shown in **Table 4**, 2 SNPs (rs2910829 and rs152312) in the PDE4D gene were tested for an association with ischemic stroke in the Taiwanese sample of stroke patients and control subjects. The rs2910829 (G/A) risk previously observed in the Icelandic population was significantly associated with a decreased risk odds ratio (OR)=0.949, confidence limits=0.588–1.53, $P>0.05$) in our population. We also examined the rs152312 risk previously observed in the Icelandic population and found no significant association with an increased risk (OR=0.917, confidence limits=0.577–1.455, $P>0.05$) in our population. We also analyzed macrovessel ischemia and microvessel ischemia and found no significant association with the control group.

Discussion

In this report, we used the TaqMan PCR assay based on allele-specific probes to examine the association of the PDE4D gene variation with ischemic strokes. This method combines the amplification and detection steps and requires no post-PCR processing, which makes the TaqMan PCR assay easy to use and allows a high-throughput operation. The present method can be most effectively used with 1 to 20 ng of DNA. Despite its high accuracy, this method is effective only for mutations that are already

Table 3_Frequency and Number of rs2910829 and rs152312 (PDE4D) Alleles

SNP Site	Nucleic Acid Substitution/Gender	No.	Allelic Frequency % (2N)		Genotype % (No.)		
			Major	Minor	Major/Major	Major/Minor	Minor/Minor
Control							
rs2910829	G→A	280	81.25 (455)	18.75 (105)	66.79 (187)	28.93 (81)	4.28 (12)
	Male	146	81.51 (238)	18.49 (54)	67.12 (98)	28.77 (42)	4.11 (6)
	Female	134	80.97 (217)	19.03 (51)	66.42 (89)	29.10 (39)	4.48 (6)
rs152312	C→T	280	60.36 (338)	39.64 (222)	37.86 (106)	45 (126)	17.14 (48)
	Male	146	62.63 (182)	37.67 (110)	39.73 (58)	45.21 (66)	15.06 (22)
	Female	134	58.21 (156)	41.79(112)	35.82 (48)	44.78 (60)	19.40 (26)
Stroke							
rs2910829	G→A	108	80.09 (173)	19.91 (43)	65.74 (71)	28.70 (31)	5.56 (6)
	Male	67	78.36 (105)	21.64 (29)	62.69 (42)	31.34 (21)	5.97 (4)
	Female	41	82.93 (68)	17.07 (14)	70.73 (29)	24.39 (10)	4.88 (2)
rs152312	C→T	108	63.89(138)	36.11 (78)	39.81 (43)	48.15 (52)	12.04 (13)
	Male	67	67.91 (91)	32.09 (43)	46.27 (31)	43.28 (29)	10.45 (7)
	Female	41	57.32 (47)	42.68 (35)	29.27 (12)	56.10 (23)	14.63 (6)

SNP, single nucleotide polymorphism; G, guanosine; A, adenosine; C, cytidine; T, thymidine.

known. The TaqMan PCR assay presented in this paper can offer a complementary and confirmative test with sequencing.

The risk factors for ischemic stroke can be classified as environmental and genetic factors. Although several environmental risk factors have been implicated, little is known about the genetic factors of predisposition to ischemic stroke. The association of PDE4D gene variants with ischemic stroke as demonstrated by the decode group has caused great interest in the stroke genetic community. A number of replication studies performed outside the Iceland community have confirmed an independent association between PDE4D SNP87 and SNP41 and ischemic stroke.[15-21] Nevertheless, at least 2 groups failed to replicate the findings.[13,14]

Our study fails to replicate the association between PDE4D haplotypes and ischemic stroke. One possible consideration is that our analysis of the PDE4D gene was limited to relatively small sample sizes and may have missed true association of modest effect. However, we consider this possibility unlikely because Bevan and colleagues have used a meta-analysis on 5,200 cases and 6,600 controls, and the meta-analysis revealed that no genetic variants examined in PDE4D showed a robust and reproducible association to ischemic stroke.[13] Our results are in line with those published by Lohmussaar and colleagues, in which they used haplotype-tagging SNPs across the PDE4D region and examined them among cases and controls from a central European region.[14] They were unable to identify a positive association of PDE4D variants with ischemic stroke.

Differences in incidence, prevalence, and clinical patterns among different ethnic populations may produce different results in the genetic studies. One possible explanation for the lack of association of PDE4D with ischemic stroke in the Taiwanese population may be due to the different incidences of stroke subtypes in Taiwan as compared with those in Caucasian populations. Recently, Nakayama and colleagues identified another region within the 5q12 STRK1 locus that was associated with cerebral infarction in Japan.[22] Further studies are needed to identify the causative variants in other genetic markers in the Taiwanese population. LM

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Table 4_Analysis of the Association of the PDE4D (rs2910829 and rs152312) Gene with Ischemic Stroke

Total	Effect	Odds Ratio	95% Confidence Limits	*P*
rs2910829 (SNP87)	GG	1	Reference	
	GA	0.949	0.588–1.530	NS
	AA	1.793	0.686–4.689	NS
rs152312 (SNP41)	CC	1	Reference	
	CT	0.917	0.577–1.455	NS
	TT	0.712	0.362–1.402	NS
Macro-vessel ischemia				
rs2910829 (SNP87)	GG	1	Reference	
	GA	0.956	0.533–1.715	NS
	AA	0.815	0.177–3.743	NS
rs152312 (SNP41)	CC	1	Reference	
	CT	0.735	0.415–1.303	NS
	TT	0.696	0.309–1.569	NS
Micro-vessel Ischemia	**Effect**	**Odds Ratio**	**95% Confidence Limits**	***P***
rs2910829 (SNP87)	GG	1	Reference	
	GA	1.034	0.486–2.201	NS
	AA	2.687	0.696–10.366	NS
rs152312 (SNP41)	CC	1	Reference	
	CT	1.501	0.698–3.226	NS
	TT	1.021	0.337–3.094	NS

SNP, single nucleotide polymorphism.

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