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Investigation of single nucleotide polymorphisms in phosphodiesterase 4D gene in Mongol and Han patients with ischemic stroke in Inner Mongolia

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ABSTRACT. We investigated single nucleotide polymorphisms (SNP) at 87 sites of the phosphodiesterase 4D (*PDE4D*) gene in Mongol and Han patients with ischemic stroke in Inner Mongolia. SNPs in 226 patients with ischemic stroke (case group, 110 Mongol patients, 116 Han patients) and 220 patients without neurological disease (control group, 102 Mongol patients, 118 Han patients) were detected by polymerase chain reaction-restriction fragment length polymorphism and gene sequencing. The genotype and allele frequencies of all groups were compared. There were no statistically significant differences in genotypes in the *PDE4D* gene at 87 sites between the case and control groups ($P > 0.05$). The C allele frequency

in the case group was significantly higher than that in the control group ($P < 0.05$). The CC genotype and C allele frequencies in the Mongol case subgroup were higher than those in the Mongol control subgroup ($P < 0.05$). The CC genotype and C allele frequencies in the Han case subgroup were higher than those in the Han control subgroup ($P < 0.05$). In the case group, there were no significant differences at 87 sites for genotypes and allele frequencies between the Mongol and Han subgroups. In the control group, there were no significant differences at 87 site genotypes and allele frequencies between the Mongol and Han subgroups. The increase in the C allele frequency at 87 SNP sites in *PDE4D* may increase ischemic stroke risk. We found no differences in the risk between Mongol and Han populations in Inner Mongolia.

Key words: Han people; Ischemic stroke; Phosphodiesterase 4D gene; Mongol; Single nucleotide polymorphism

INTRODUCTION

Stroke has emerged as the second most common cause of mortality worldwide and is a major public health problem. It accounted for nearly 5.7 million deaths worldwide in 2005 (Strong et al., 2007). More than two-thirds of these deaths occurred in under-developed countries (Feigin, 2007). In China, with a population of 1.4 billion, the annual stroke mortality rate is approximately 1.6 million, exceeding heart disease to become the leading cause of death. In addition, 2.5 million new stroke cases are documented in China each year, and there are 7.5 million stroke survivors. Ischemic stroke is the most common type of stroke, and approximately 43-79% of all strokes are ischemic in China (Liu et al., 2011). Traditional factors such as hypertension and smoking account for a significant proportion of ischemic stroke risk, but many risks remain unexplained (Thrift et al., 2011). Genetic risk factors, suggested by evidence from twin, case-control, and cohort studies of familial aggregation, may contribute to a predisposition to ischemic stroke (Meschia et al., 2011).

The phosphodiesterase 4D (*PDE4D*) gene is at the locus on chromosome 5q12 and encodes cAMP-specific 30, 50-cyclic phosphodiesterase 4D (Flossmann et al., 2011), belonging to a superfamily of phosphodiesterases (PDE4 family) (Munshi and Kaul, 2008). In 2003, in a genome-wide association, Gretarsdottir et al. (2003) identified the association between 87 single nucleotide polymorphisms (SNPs) in *PDE4D* and carotid stroke in an Icelandic population. Since then, studies of different populations have been performed to determine whether 87 SNPs in *PDE4D* gene could participate in ischemic stroke in non-Icelandic populations. However, based on previous studies, the relationship between the 87 SNPs in the *PDE4D* gene and ischemic stroke remains unknown. There have been no reports regarding the correlation association between the 87 SNPs and ischemic stroke in Mongol and Han populations. To clarify these relationships, we conducted a case-control study to examine SNPs at 87 sites of the *PDE4D* genes in Mongol and Han patients with ischemic stroke in Inner Mongolia.

MATERIAL AND METHODS

Patients

Ischemic stroke patients (*case group*)

A total of 256 non-relative in-patients with ischemic stroke, diagnosed according to the 4th National Cerebrovascular Disease Conference Standards and treated by the Department of Neurology, Affiliated Hospital of Inner Mongolia Medical University from October 2010 to November 2013, were selected. All patients were confirmed once by head computed tomography or magnetic resonance imaging, excluding patients with subarachnoid hemorrhage and cerebral hemorrhage. The patients included 130 Mongol patients (76 men, 54 women), with ages and courses of disease ranging from 41-81 years (60.87 ± 8.1) and from 1-8 days (3.4 ± 1.2), respectively. A total of 126 Han patients (75 men, 51 women), aged from 40-82 years (60.9 ± 9.7) and with their courses of disease ranging from 1-8 days (3.5 ± 1.9) were also included.

Control group

A total of 250 non-relative in-patients without ischemic stroke, cerebral vascular, or nervous system disease diagnosed through the Department of Neurology, Affiliated Hospital of Inner Mongolia Medical University from October 2010 to November 2013 were selected. Among these were 122 Mongol patients (69 men, 53 women) aged 42-81 years (59.13 ± 8.9), with the course of disease ranging from 1-9 days (3.9 ± 1.8). A total of 128 Han patients (70 men, 58 women) aged 44-85 years (62.1 ± 9.4), with the courses of disease ranging from 1-8 days (3.8 ± 1.6) were also included. All patients were residents of Inner Mongolia and did not have serious heart, liver, kidney, and blood system diseases, or autoimmune diseases and tumor. The indicators for patients in the 2 groups showed no statistically significant differences, as ascertained using the SPSS 13.0 statistical software (SPSS, Inc., Chicago, IL, USA), and were found to be similar.

All patients signed informed consent before enrolling in the study, and the study was approved by the Ethics Committee of Inner Mongolia Medical University.

Methods

Extraction of genomic DNA

In accordance with the kit instructions, we extracted the whole blood genomic DNA from 2 mL peripheral venous blood in all groups using the Axygen whole blood genomic DNA ion kit (AP-96-BL-GDNA-1; Union City, CA, USA).

PCR and product identification

All primers were synthesized by Shanghai Sangon Biotechnology Company (Shanghai, China). The following primers were used: 5'-AGG TAT GAA GAC ACCTGA AAG ATC-

3'; post-primers: 5'-GCA GTA TGT TTA AAG ATG AGG AAG-3'. The amplified PCR product length was 211 bp. The total PCR volume was 25 μL, including 3 μL genomic DNA template, 0.5 μL of each primer, 10 μL 2X Taq PCR MasterMix, and 10 μL ddH_2O. The PCR conditions were as follows: 95°C for 5 min; 95°C for 30 s, 57.5°C for 30 s, and 72°C for 30 s for a total of 35 cycles; then 72°C for 7 min. Amplification was tested by 2% agarose gel electrophoresis.

Enzyme digestion reaction and product identification

The total enzyme digestion reaction volume was 20 μL, including 10 μL PCR product, 20 U *Ssp*I enzyme, 2 μL buffer G, and ddH_2O to 20 μL. The enzyme digestion reaction was tested by 3.5% agarose gel electrophoresis.

Gene sequencing

We selected 3 patients with different genotype samples that were determined by enzyme digestion from each group for PCR. The PCR-amplified products were sequenced by Shanghai Sangon Biotechnology Company. To verify the correct amplification of the 87 sites in the *PDE4D* gene, we used NCBI BLAST searching for homology analysis of the sequencing results.

Statistical analysis

All experimental data were analyzed using the statistical package of SPSS 13.0 statistics software. Alleles were calculated by direct counting, and genotype frequencies were computed. Measurement data were compared using the *t*-test, and the distribution frequency of various genotypes and alleles was compared using the chi-square test. Polymorphism distribution in genes was tested by determining whether the population was in Hardy-Weinberg.

RESULTS

PCR amplification results

The PCR amplification of 87 sites of the *PDE4D* gene showed 1 band (211 bp) on gel electrophoresis. After restriction enzyme digestion with *Ssp*I, the PCR amplification of the 87 sites of the *PDE4D* gene showed 3 bands: homozygote (CC genotype), 211 bp; heterozygote (CT genotype), 211, 159, and 52 bp; homozygote (TT genotype), 159 and 52 bp. Gene sequencing results were consistent with the 3 different genotypes (CC, CT, TT), which were determined by enzyme digestion (Figures 1-3). Each genotype polymorphism distribution was in accordance with Hardy-Weinberg equilibrium.

Genotype distribution of the 87 sites on the *PDE4D* gene and allele frequency comparison

There was no statistically significant difference ($P > 0.05$) in genotype distribution of the 87 sites of *PDE4D* between the case group and control group. The C allele frequency at the 87 sites of the *PDE4D* gene in the case group was higher than that in the control group (P

< 0.05). The CC genotype and C allele frequencies in the Mongol case subgroup were clearly higher than those in the Mongol control subgroup (P < 0.05). The CC genotype and C allele frequencies in Han case subgroup were clearly higher than those in the Han control subgroup (P < 0.05). In the case group, there were no significant differences in the 87 sites of the *PDE4D* gene genotypes and allele frequency between the Mongol subgroup and the Han subgroup. In the control group, there were no significant differences at the 87 sites of the *PDE4D* gene and allele frequencies between the Mongol subgroup and Han subgroup (Table 1).

DISCUSSION

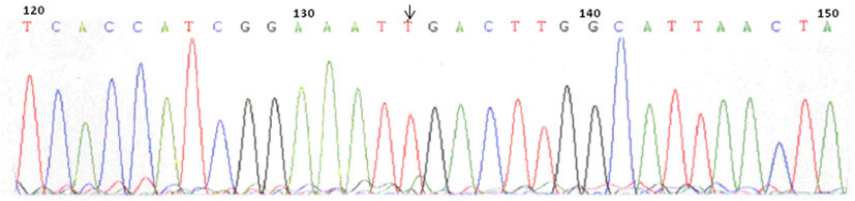

Figure 1. Genotype CC of SNP 87.

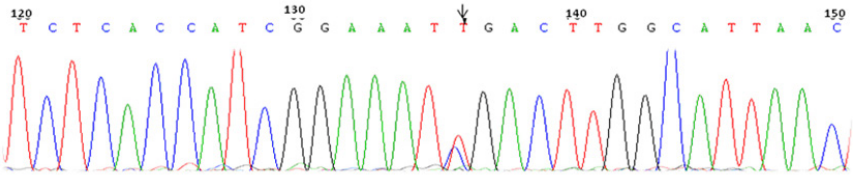

Figure 2. Genotype CT of SNP 87.

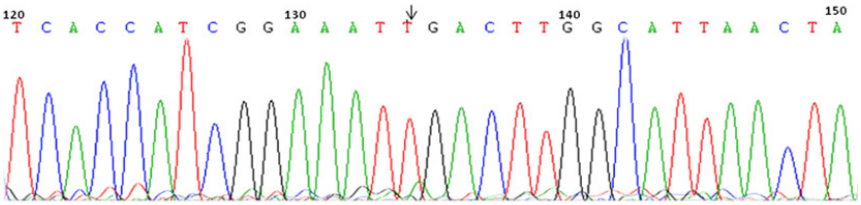

Figure 3. Genotype TT of SNP 87.

Table 1. Comparison of SNP 87 genotype and allele frequency between the case group and the control group in Mongol and Han people (%).

Group	Case	Genetype frequency			Allele frequency	
		TT	CT	CC	C	T
Case group	256	22 (8.6)	56 (21.9)	178 (69.5)	82.3[a]	17.7
Mongol	130	8 (6.4)	37 (28.2)	85 (65.4)[b]	79.5[b]	20.5
Han	126	4 (3.2)	30 (23.8)	92 (73.0)[c]	84.9[c]	15.1
Control group	250	15 (6)	89 (35.6)	146 (58.4)	76.4	23.6
Mongol	122	10 (7.8)	46 (38.1)	66 (54.1)	73	27
Han	128	5 (3.9)	43 (33.6)	80 (62.5)	79.2	20.8

[a]P = 0.04 < 0.05, compared to the control group; [b]P = 0.03 < 0.05, compared to the Mongol subgroup of the control group; [c]P = 0.01 < 0.05, compared to the Han subgroup of the control group.

The *PDE4D* gene is at the 5q12 locus on chromosome, has a total length of 1.6 Mb, contains 22 exons and 21 introns, and encodes cAMP-specific 30, 50-cyclic phosphodiesterase 4D, which belongs to a superfamily of phosphodiesterases (PDE4 family). There are several SNP loci in the *PDE4D* gene that affect its expression. Increased *PDE4D* expression can cause the loss of cAMP activity, promote vascular smooth muscle proliferation and migration, and aggravate local inflammation in impaired vessels, accelerate atherosclerosis formation, and then induce ischemic cerebral apoplexy (Stangherlin and Zaccolo, 2009). However, decreased *PDE4D* expression can cause increased cAMP activity, protein kinase C activation, and promote Ca^{2+} inflow, leading to atrial fibrillation, followed by induction of cardioembolism (Shao and Yi, 2009). Thus, gene polymorphism can affect *PDE4D* expression levels.

In the present study, we identified polymorphisms at 87 sites of the *PDE4D* gene. The results showed that the CC genotype was the most prevalent, and the C allele frequency was higher than the T allele frequency in the Mongol and Han populations. There were no statistically significant differences between the case group and control group in the distributions of the 87 sites in the *PDE4D* gene, but the C allele frequency in the case group was higher than that in the control group. In addition, the CC genotype and C allele frequencies in the Mongol case subgroup were clearly higher than those in the Mongol control subgroup ($P < 0.05$). The CC genotype and C allele frequencies in the Han case subgroup were clearly higher than those in Han control subgroup ($P < 0.05$). In the case group, there were no significant differences in the genotypes and allele frequencies of the 87 sites in the *PDE4D* between the Mongol subgroup and Han subgroup. In the control group, there were no significant differences in the SNPs at 87 sites of the *PDE4D* gene for genotype and allele frequency between the Mongol subgroup and Han subgroup. Therefore, there was no difference in the gene polymorphisms in *PDE4D* gene between the Mongol and Han populations in Inner Mongolia. However, the C allele at the 87 sites may be an important risk factor in the progression of ischemic cerebral infarction in the Mongol and Han populations in Inner Mongolia. The results of the present study were in accordance with those of Zhang et al. (2009). Gretarsdottir et al. (2003) showed that there was significant correlation between the 87 SNPs in the *PDE4D* gene and cardioembolism, but different results were observed in different region or nations.

In conclusion, we found that a high frequency of the C allele at the 87 sites of the *PDE4D* gene may increase the risk of ischemic stroke, but there was no difference between the Mongol and Han populations in Inner Mongolian. This may be because a small sample size was included. Additional studies should include a large number of subjects to verify the results.

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