Effect of a pre-microRNA-149 (miR-149) genetic variation on the risk of ischemic stroke in a Chinese Han population

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ABSTRACT. Clinical and experimental data have demonstrated that genetic factors play an important role in determining the susceptibility to ischemic stroke (IS). The present study was performed to clarify the association between the pre-microRNA-149 (miR-149) single nucleotide polymorphism rs71428439 and the risk of IS in the Jiangsu Han population. Polymerase chain reaction and restriction fragment length polymorphism were performed to identify the genotypes of the miR-149 single-nucleotide polymorphism rs71428439 in 730 unrelated subjects (IS, 348; healthy controls, 382). Plasma levels of homocysteine were determined using a radioassay kit. Compared to healthy controls, IS patients had a lower frequency of GG genotype distribution of the hsa-mir-149 polymorphism (11.5 *vs* 16.0%) and a higher frequency of TT (46.6 *vs* 39.0%). The risk of IS was significantly

lower among subjects carrying the GG genotype than subjects carrying the AA genotype (odds ratio (95% confidence interval): 0.603 (0.382-0.952), P = 0.030) or at least carrying the G allele than patients carrying the A allele (odds ratio (95% confidence interval): 0.769 (0.620-0.954), P = 0.019). Levels of folate were statistically higher in patients with the TT genotype (8.59 ± 7.75 ng/mL) than in those with the CC genotype (6.32 ± 5.97 ng/mL) in IS patients. Our results suggest that the miR-149 single nucleotide polymorphism rs71428439 influences plasma levels of homocysteine and is associated with IS risk in the Jiangsu Han population.

Key words: Ischemic stroke; miR-149; Polymorphism

INTRODUCTION

Stroke is a multifactorial disorder with a high morbidity, mortality, disability, and recurrence rate in many developed countries (Hankey, 2006; Dichgans, 2007). There are 2 main types of stroke: ischemic strokes (IS) and hemorrhagic strokes. Hemorrhagic strokes account for 10-15% of all strokes. The remaining are IS, caused by a disruption of blood flow, oxygen, or glucose to the brain (Bendixen and Ocava, 2002). IS is the most commonly observed type of stroke in Chinese and accounts for the third major cause of morbidity and mortality in adults, behind only heart disease and cancer (Armstead et al., 2010). In China, IS is a leading cause of death and accounts for approximately two-thirds of all stroke-related deaths (Zhang et al., 2003). However, the pathophysiology of IS is complex and not completely understood. Data from family-based studies, twin studies, and animal experiments indicate that genetic factors play an important role in stroke (Jeffs et al., 1997; Liao et al., 1997; de Lange et al., 2001).

MicroRNAs (miRNAs) are a class of naturally occurring, small non-protein-coding RNAs of 21-24 nucleotides in length that regulate gene expression by base-pairing with target mRNAs at the 3'-untranslated region, leading to mRNA cleavage or translational repression (Lagos-Quintana et al., 2001; Lau et al., 2001). It has been suggested that miRNAs are important post-transcriptional regulators of gene expression and play a role in multiple cellular pathways. including cell proliferation, differentiation, cell cycle progression, and apoptosis (McDaneld, 2009). Accumulating evidence indicates that aberrant miRNA expression is involved in various diseases, including tumors, Down syndrome, schizophrenia, Alzheimer disease, Parkinson disease, and stroke (Jeon et al., 2013).

Recently, a well-known miRNA polymorphism in pre-miRNA sequences [miR-149 T>C (rs2292832; chromosome 2, 241395503)] has been studied in a variety of diseases and was found to contribute to pathogenesis of these diseases (Chen et al., 2013; Zhang et al., 2013). Ding et al. (2013) found that a pre-miRNA-149 (pre-miR-149) genetic variation affects miR-149 maturation. Bioinformatic analysis revealed that 5,10-methylenetetrahydrofolate reductase (MTHFR) may be a target gene of miR-149. A common polymorphism (C677T) in the gene encoding MTHFR, a critical enzyme in homocysteine metabolism, has been reported to be associated with both elevated plasma homocysteine levels and increased stroke risk in several studies (Frosst et al., 1995). However, there are currently no data regarding the role of miR-149 polymorphisms on homocysteine levels and stroke pathogenesis in a Chinese Han population. Therefore, we investigated the genetic association between the pre-miR-149 variant and IS.

MATERIAL AND METHODS

Study subjects

A total of 348 patients with IS were enrolled from the Department of Neurology at The Affiliated People's Hospital, Jiangsu University. All study protocols were approved by The Institutional Review Board of The Affiliated People's Hospital, Jiangsu University, and written informed consent was obtained from each participant. The diagnosis of all stroke patients was confirmed to be IS using brain computed tomography or magnetic resonance imaging (Liao et al., 2013). Patients with history of myocardial infarction, cerebral vein thrombosis, transient ischemic attack, or hemorrhagic stroke were excluded. We selected 382 gender- and age (± 5 years)-matched control subjects from patients presenting at our hospitals during the same period for health examinations. Control subjects had no recent history of cerebrovascular disease or myocardial infarction. Exclusion criteria were the same as those used for the patient group. All study participants were Chinese Han residing in Jiangsu. Subjects were deemed to have hypertension if the systolic blood pressure was ≥140 mmHg or diastolic pressure was ≥90 mmHg, or if they were taking antihypertensive medications. Diabetes mellitus was defined as a fasting plasma glucose level >126 mg/dL (7.0 mM) and included patients taking diabetic medications. Smoking refers to patients who currently smoke. Hyperlipidemia was defined by total cholesterol serum levels ≥200 mg/dL or an antihyperlipidemic agent treatment history.

Genotyping

DNA was extracted from leukocytes using the Qiagen DNA Blood Mini kit (Qiagen, Hilden, Germany) according to manufacturer instructions. Genotypes of rs71428439 were analyzed by polymerase chain reaction (PCR)-based direct DNA sequencing. DNA specimens were amplified by using standard PCR protocols. The primers were designed by Tu et al. (2012). The PCR profile included an initial melting step at 94°C for 5 min, 35 cycles at 94°C for 60 s, 55°C for 45 s, and 72°C for 1 min, followed by a final extension step at 72°C for 10 min. The PCR product was a 493-bp fragment covering the A-G polymorphism site (rs71428439) in the miR-149 precursor.

Laboratory analyses

We collected plasma samples to measure total homocysteine levels within 48 h of stroke onset in patients. Additionally, 12-14 h after the subject's previous meal, 5 mL whole peripheral blood was collected in a tube containing anticoagulant. We centrifuged the tube for 10 min at 1000 g to separate the plasma. Plasma levels of homocysteine were determined using a radio-assay kit (Bio-Rad, Hercules, CA, USA), according to manufacturer instructions.

Statistical analysis

The SPSS 12.0 statistical software was applied for statistical analysis (SPSS, Inc., Chicago, IL, USA). Hardy-Weinberg equilibrium analysis was evaluated for the miR-149 alleles in the patient and control groups. The significance of differences between allelic and phe-

notype frequencies in study groups was determined using the Pearson χ^2 test. The relationship between genetic polymorphisms and IS was analyzed by the odds ratio (OR) and its 95% confidence interval (95%CI), which was obtained by logistic regression. Statistical significance was accepted at the $P < 0.05$ level.

RESULTS

Study population

A total of 348 IS patients and 382 controls were included in the study. All patients belonged mainly to a Southeast Chinese Han population from Jiangsu. The clinical and demographic data for the study subjects, including age, gender, hypertension, smoking status, diabetes mellitus, hyperlipidemia, smoking, and homocysteine, are shown in Table 1. The mean age was 66.3 ± 10.2 years in the control group and 65.7 ± 11.5 years in the patient group. No statistical difference was observed in age or gender distribution between the control and patient groups. However, a significantly higher proportion of IS patients were smokers compared to the control group ($P < 0.05$). IS patients were significantly more likely to have high-density lipoprotein cholesterol, diabetes mellitus, hypertension, hyperlipidemia, and increased homocysteine levels ($P < 0.05$).

Table 1. Baseline characteristics of ischemic stroke patients.

Characteristics	Control	Ischemic stroke	P value
Total (N)	348	382	
Male [N (%)]	193	205	0.399[#]
Age, years (mean ± SD)	66.3 ± 10.2	65.7 ± 11.5	0.455[#]
Hypertension [N (%)]	136 (39.1)	190 (49.7)	0.005*
Diabetes mellitus [N (%)]	45 (12.9)	77 (17.5)	0.010*
Hyperlipidemia [N (%)]	69 (19.8)	101 (26.4)	0.036*
Smoking [N (%)]	92 (26.4)	135 (35.3)	0.010*
Homocysteine (ng/mL)	10.12 ± 5.19	12.43 ± 6.09	0.000*

Compared to controls *$P < 0.05$, [#]$P > 0.05$.

Association between hsa-mir-149 polymorphism and IS

The genotype at rs71428439 was identified in patients and healthy volunteers. The allele and genotype distributions are shown in Table 2. The distribution of genotypes for the pre-miR-149 SNP rs71428439 in the patient and healthy volunteer groups complied with Hardy-Weinberg equilibrium (patient group: $P = 0.419$; healthy volunteers: $P = 0.338$). The AG genotype of the hsa-mir-149 gene was more common in the control group, while the AA genotype of pre-miR-149 gene was more common in the patient group. Compared to healthy controls, IS patients had a lower frequency of the GG genotype of the hsa-mir-149 polymorphism (11.5 *vs* 16.0%) and a higher frequency of TT (46.6 *vs* 39.0%). The risk of IS was significantly lower among subjects carrying the GG genotype than in subjects carrying the AA genotype (OR (95%CI): 0.603 (0.382-0.952), $P = 0.030$) or at least carrying the G allele than patients carrying the A allele (OR (95%CI): 0.769 (0.620-0.954), $P = 0.019$).

Table 2. Distribution of miR-149 genotypes in patients and the control group.

		Control [N (%)]	Ischemic stroke [N (%)]	OR (95%CI)	P
rs71428439	AA	149 (39.0)	162 (46.6)		
	AG	172 (45.0)	146 (42.0)	0.781 (0.571-1.068)	0.130
	GG	61 (16.0)	40 (11.5)	0.603 (0.382-0.952)	0.030
	A allele	470	470		
	G allele	294	226	0.769 (0.620-0.954)	0.019

Effect of hsa-mir-149 polymorphism on plasma levels of homocysteine

Bioinformatic analysis revealed that MTHFR is a target gene of miR-149 (Figure 1A). We assessed the plasma levels of homocysteine in patients with different genotypes. As shown in Figure 1, homocysteine levels were statistically lower in patients with the GG or AG genotype (11.53 ± 5.02 or 12.31 ± 5.29 ng/mL) than in those with the AG or AA genotype (15.37 ± 7.31 ng/mL) in IS patients (Figure 1B). No statistical difference was observed in the plasma levels of homocysteine between the AG and GG genotypes ($P = 0.3895$).
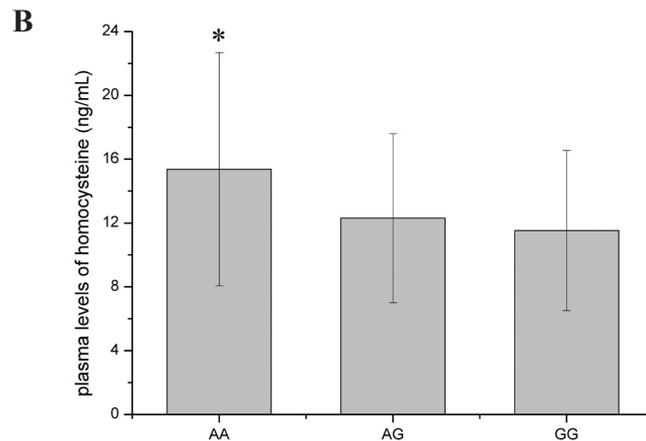

Figure 1. Effect of pre-miR-149 polymorphism rs71428439 on plasma levels of homocysteine. **A.** Targetscan predicted that MTHFR was a target gene of miR-149. **B.** Plasma levels of homocysteine were statistically lower in patients with the GG genotype compared to the AA genotype group, *$P < 0.05$.

DISCUSSION

In the present study, based on previous reports, we selected a potential functional SNP rs71428439 in miR-149 and investigated its relationship with IS in a Chinese Han population. We found that rs71428439 may affect the susceptibility to IS. This is the first study to evaluate the association between rs71428439 polymorphisms and IS in a Chinese Han population. Our findings suggest that genetic variants of pre-miR-149 play a role in IS pathogenesis.

Stroke is a complex disease caused by a combination of various genetic and environmental factors and is the leading cause of long-term disability and mortality in China. Its incidence is more than 4-5 times higher than that in developed countries (Wu et al., 2012). miRNAs are known to play important roles in many physiological and pathological processes, including tumorigenesis (Mocellin et al., 2009), proliferation (Johnnidis et al., 2008), hematopoiesis (Merkerova et al., 2008), metabolism (Aumiller and Förstemann, 2008), immune function (Carissimi et al., 2009), epigenetics, and neurodegenerative diseases (Bushati and Cohen, 2008). miRNAs have also been found to be beneficial for identifying the etiology of lymphoma (Lawrie et al., 2007) and progression of certain neurological diseases (Nelson et al., 2008). Over the past decade, miRNA microarray has been performed to examine the differential expression profiles of miRNAs in stroke *in vivo* and *in vitro*. The studies indicated that miRNAs are key modulators and play important roles in both pathogenic and pathological aspects of IS biology.

Jeyaseelan et al. (2008) determined an miRNA profile under ischemic conditions from the ischemic brain and blood of rats suffering middle cerebral artery occlusion and found that some of the miRNAs that are overexpressed in the ischemic brain tissue can be detected in serum or plasma. An miRNA expression profile has been implicated in endothelial/vascular function, erythropoiesis, angiogenesis, and neural function, demonstrating the potential of using peripheral blood miRNA profiles as biomarkers of cerebral IS (Kong et al., 2011). Similarly to cardiac-specific miRNA, Kawamoto et al. (2005) found that miR-124 was a brain-specific microRNA and was increased in the plasma of a rat acute IS model, generating interest in the exploration of brain-specific miRNAs, including miR-124, miR-134, miR-153, miR-9, and miR-219, as potential molecular biomarkers of human acute IS (Laterza et al., 2009). However, few studies have examined the roles of genetic variation in IS. In this study, we investigated the association between the pre-miR-149 (rs71428439) polymorphism and IS.

MTHFR is a key enzyme involved in folate metabolism. It catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. This may be used as a predictive marker for IS in patients with associated type 2 diabetes (Kawamoto et al., 2005). The effects of MTHFR C677T polymorphism appear to be more common in regions with low dietary folate consumption than in those with high dietary folate consumption (Holmes et al., 2011). However, some recent studies observed no direct association between any single polymorphism of key genes of the homocysteine metabolic pathway (Low et al., 2011). In the present study, we found that the risk of IS was significantly lower among subjects carrying the GG genotypes than subjects carrying the AA genotype or at least carrying one G allele than among patients carrying the A allele. To investigate the gene-environment interaction, we explored the combined effects of pre-miR-149 polymorphisms and plasma tHcy levels. IS patients show increased tHcy levels compared to controls ($P < 0.05$). Bioinformatic analysis revealed that MTHFR is a target gene of miR-149. The polymorphism in pre-miR-149 may modulate folate levels by modulating MTHFR activity.

In summary, we found that the miR-149 polymorphism is correlated with susceptibility to IS in a Chinese Han population. These findings add to the body of evidence demonstrating a role for microRNA in disease susceptibility and pathology. Additional studies should be conducted to provide a detailed understanding of the molecular mechanisms linking the miR-149 polymorphism to IS.

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