

## Original Article

# Tagging functional SNPs rs191876689 in MTHFR decrease risk of ischemic stroke

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**Abstract:** *Objective:* Functional polymorphisms located in MTHFR have been identified to be highly associated with the risk of ischemic stroke (IS). In this study, we mainly focused on the miRNA associated MTHFR polymorphisms and function of which in the development of IS. *Methods:* The hospital based case-control study was conducted with 350 patients diagnosed with IS and 350 healthy volunteers as control enrolled. The genotype was conducted by using Taqman probe. The binding site between miR-22 and MTHFR was predicted by bioinformatical analysis. The relative expression of MTHFR was detected by qRT-PCR. Further confirmation was determined by dual-luciferase assay. *Results:* The potential function polymorphism rs191876689 located in the 3' untranslated region of MTHFR was predicted by bioinformatical analysis with highly interaction with miR-22. The genotype assay further indicated polymorphism rs191876689 were significantly associated with decreased risk of ischemic stroke compared with the wild genotype. According to the bioinformatical analysis and further confirmed by luciferase assay, the polymorphism rs191876689 could attenuate the binding of miR-22, resulting in the accumulation of MTHFR. *Conclusion:* This study demonstrated that the MTHFR rs191876689 is associated with decreased risk of IS and might be a biomarker for early intervention for IS patients.

**Keywords:** MTHFR, polymorphism, Taqman, miR-22

## Introduction

As the major cause of mortality in elder population and a common cause of long-term disability in the world, stroke burden continues to increase, especially in developing countries like China [1, 2]. Accordingly, stroke can be divided into ischemic and hemorrhagic stroke [3, 4]. Ischemic stroke is usually caused by blockage of a blood vessel, whereas hemorrhagic stroke is caused by rupture of a vessel leading to bleeding in the brain. Increasing evidence revealed that the environmental factor and genetic factor might be responsible for the occurrence of IS [5-7].

Methylene tetrahydrofolate reductase (MTHFR) is the key enzyme for the metabolism of circulating homocysteine (tHcy) [8, 9]. tHcy is a crucial intermediate in methionine metabolism and causes excessive production of reactive oxygen species (ROS) [10, 11]. The accumulation of tHcy could evaluate the ROC levels by

activating the MAPK signaling, resulting in the cell apoptosis [11, 12]. As reported, the functional polymorphism located in the exon region of MTHFR has been proved highly associated with the pathogenesis of IS. For example, the polymorphism rs1801133 could cause approximately 70% and 35% reductions of normal MTHFR enzymatic activity in TT and CT genotype carriers, respectively, and finally causing the increasing level of tHcy which predicting the poor prognosis of IS [13, 14].

Currently, miRNA has been documented as function factor involved in the SNP associated post-transcriptional regulation. The miRNA are small, non-coding RNA molecules. Through binding to the 3' untranslated region (3'-UTR) of messenger RNA (mRNA), miRNA can cause degradation of mRNA, thus regulating the expression of mRNA at post-transcription level [15, 16]. As reported, miR-22 directly targeted the 3'UTR sequence of the MTHFR gene [17]. Researchers also demonstrated a critical con-

**Table 1.** Clinical characteristics of ischemic stroke patients and healthy controls enrolled in this study

Characteristics	Cases (n = 350)	Control (n = 350)	p value <sup>a</sup>
Age			>0.05
>60	299	301	
<60	51	49	
Gender			>0.05
Male	256	239	
Female	94	111	
Smoking exposure			<0.001
Yes	279	202	
No	71	148	
Drinking exposure			<0.001
Yes	244	122	
No	106	228	
Diabetes			0.007
Yes	228	109	
No	122	241	
Hypertension			<0.001
Yes	281	111	
No	69	239	

a: Student t test for continuous variable, Chi-squared test for categorical variable.

tribution of miR-22 in the inhibition of MTHFR gene expression during 2-AAF-induced rat hepatocarcinogenesis [18].

In this study, according to the direct binding between miR-22 and MTHFR, we aimed to screen the potential polymorphisms which may associate with miR-22 by bioinformatical analysis.

## Materials and methods

### Study subjects

Total 350 patients diagnosed with ischemic stroke and 350 healthy controls were enrolled. Diagnosis of patients with ischemic stroke was established clinically and confirmed by using X-ray computed tomography and/or magnetic resonance imaging scans of the brain. All control individuals visited our hospital for annual health check-ups and were free of ischemic stroke. Diabetes mellitus was defined as a fasting glucose level  $\geq 7.0$  mmol/L and/or  $\geq 11.1$  mmol/L 2 h after oral glucose challenge, or receiving anti-diabetic medication. Subjects

who had smoked more than 10 cigarettes daily for 5 years were considered as smokers. Subjects who drank more than 50 ml of alcoholic beverages daily for 5 years were considered as alcohol drinkers. This study was approved by the Ethics Committee of Qinghai University and the study was conducted in accordance with the Helsinki Declaration.

### Genomic DNA extraction and genotyping

Blood samples were collected after a 12 h overnight fasting period and then separated into serum, red blood cells, and buffy coat. Genomic DNA from peripheral whole blood of every validation subject was extracted by using QIAamp DNA blood mini kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, genomic DNA samples of 10 ng each were amplified using PCR in a total volume of 5  $\mu$ L containing TaqMan Universal Master Mix, 80x SNP Genotyping Assay-Mix, DNase-free water and 10-ng DNA. The PCR conditions were 50°C for 2 min and then 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min in a 384-well ABI 7900HT Real Time PCR System.

### Bioinformatics analysis

Bioinformatics software (<http://bioinfo.life.hust.edu.cn/miRNASNP2/index.php>) was used to detect the candidate SNPs which was associated with miR-22.

### Cell transfection

The 293T cells were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and cultured in RPMI-1640 (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) in a humidified incubator with 5% CO<sub>2</sub> at 37°C. The miR-22 mimics and control were obtained from Genepharma (Shanghai, China) and transfected into 293T cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

### Luciferase reporter assay

MTHFR 3'-UTR fragments containing either G or C alleles were amplified using PCR from genomic DNA and cloned into pGL3-promoter-

**Table 2.** Genotype frequencies of the MTHFR rs191876689 in IS patients and healthy controls

Genotype	Cases (n = 350)		Controls (n = 350)		OR (95% CI) <sup>a</sup>	P Value <sup>a</sup>
	N	%	N	%		
	rs191876689					
GG	288	82.3	276	78.9	1.00	
GC	45	12.9	55	15.7	0.44 (0.22-0.57)	0.01
CC	17	4.8	19	5.4	0.32 (0.19-0.42)	0.006
C carrier	62	17.7	74	21.1	0.37 (0.31-0.54)	0.02

<sup>a</sup>The ORs, 95% CIs and P value were calculated after adjusting for age, gender, smoking, drinking and other characteristics listed in **Table 1**.

less luciferase-based plasmids (Promega, Madison, WI, USA) multiple cloning sites. MTHFR 3'-UTR fragments potentially binding to miRNAs, predicted by bioinformatical analysis or a mutated sequence with the predicted target sites, were cloned using PCR from genomic DNA and inserted into the pGL3 promoter vector (Genscript, Nanjing, China). The cloned plasmids were amplified and verified by DNA sequencing.

For the dual-luciferase reporter assay, 293T cells were plated onto 24-well plates and transfected with 100 ng of pGL3-MTHFR wild, pGL3-MTHFR mutant, and miRNA mimics, respectively, using Lipofectamine 2000 (Invitrogen). A Renilla luciferase vector pRL-SV40 (5 ng) was also co-transfected as a normalization and transfection efficiency control. Each experiment was performed in triplicate and luciferase activity was assessed 48 h after transfection using the dual-luciferase reporter assay system (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity.

#### Statistical analysis

The association between different genetic variants and ischemic stroke risk was evaluated by calculation of the odds ratios (ORs) and 95% confidence intervals (CIs) using univariate and multivariate logistic regression analysis. Student t test was applied to analyze the difference between the expression of MTHFR in IS patients and controls. All statistical analyses were two-sided and P<0.05 was considered statistically significant using SPSS 13.0 (SPSS, Chicago, IL, USA) or SAS software (version 9.1.3; SAS Institute, Cary, NC, USA).

## Results

### Clinical characteristics analysis

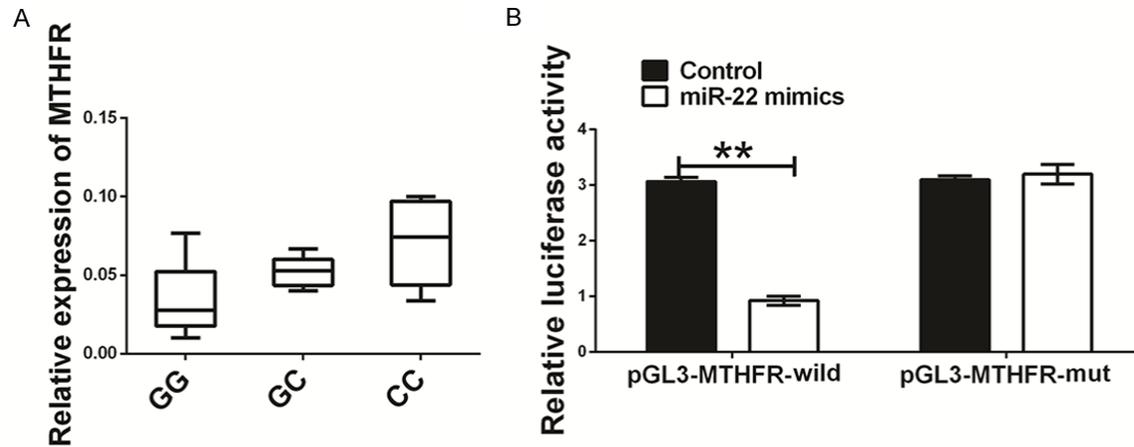
As presented in **Table 1**, no significant difference was observed in the distribution of age, gender of patients with IS and healthy controls. The common risk factors including smoking exposure, drinking exposure, patients with diabetes or hypertension were confirmed with the increased risk of IS comparing with the control group (p<0.001). In addition, the traditional biomarker for cardiovascular and cerebrovascular diseases including total cholesterol, HDL-C and LDL-C were also associated with the occurrence of IS.

### MTHFR polymorphism rs191876689 acting as a protective factor in IS

As predicted by bioinformatical analysis, the polymorphism rs191876689 located in the 3'UTR of MTHFR presented highly association with the binding of miR-22. As presented in **Table 2**, among the 350 IS patients, we found that polymorphism rs191876689 indicated a different distribution comparing with the control group. The logistic regression analysis revealed that MTHFR rs191876689 GC and CC genotypes significantly associated with decreased risk of developing of ischemic stroke compared with the GG genotype (OR = 0.44; 95% CI = 0.22-0.57 for GC genotype, while OR = 0.32; 95% CI = 0.19-0.42 for AA genotype; **Table 2**). A higher number of C-alleles were also associated with an increased risk of ischemic stroke (OR = 0.37; 95% CI = 0.31-0.54).

### The miR-22 binding was attenuated by function SNP

As confirmed, functional SNP could cause an abnormal binding of miRNA and target genes. The expression of MTHFR with different genotype was detected by RT-PCR in plasma mRNA. As presented in **Figure 1A**, the expression of MTHFR was increased with the patients harbored the C allele indicating the SNP located in the 3'UTR of MTHFR might causing an abnormal expression. We next clone the miR-22 mimics and transfected with either wild type or mutant type of MTHFR in cells. The luciferase assay to confirm such binding and found that



**Figure 1.** Binding of miR-22 to MTHFR rs868014 minor alleles. A: Relative expression of MTHFR mRNA with different genotype in ischemic stroke patients. B: Both firefly and renilla luciferase activities were measured in the same sample using a luciferase assay kit. Firefly luciferase signals were normalized with renilla luciferase signals. Data were presented as mean  $\pm$  SE. \* $P < 0.05$ .

luciferase activity of G-allele-specific pGL3 construct was significantly suppressed by miR-22 (Figure 1B).

### Discussion

ROS generation is enhanced and can lead to damage to various types of cells, including brain endothelial cells, leading to formation of thrombosis and loss of brain function. Among the ROS signaling, MTHFR is an important enzyme especially for the degradation of tHcy [19]. There is accumulating evidence demonstrating that SNPs localized at miRNA binding sites (miRSNPs) could affect the binding of miRNAs to the target genes and in turn result in reduction or increase in translation of the target mRNA and altered susceptibility to cancer [20]. For example, previous studies showed that the rs2910164 polymorphism harboring the sequence for miR-146a could influence susceptibility to gastric cancer in a Chinese population, while rs4143815 and rs4819388 SNPs in the 3'-UTR of B7-H1 and B7-H2 genes, respectively, associated with development of gastric cancer [21].

In this study, we focused on the functional SNP located in MTHFR which might associated with miR-22, a reported regulator of MTHFR. As predicted by bioinformatical analysis, we reveal that rs191876689 might decrease the risk of ischemic stroke. Besides, the binding ability of miR-22 in MTHFR could also attenuated by the

functional SNP causing a increasing level of MTHFR. Consistent with previous report, MTHFR rs868014 TC or CC genotypes were also associated with increased serum tHcy and a poor ischemic stroke outcome.

In summary, our current study demonstrated MTHFR rs191876689 is associated with decreased risk of IS and might be a short-term outcome biomarker for IS patients.

### Disclosure of conflict of interest

None.

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