Original Article

# Interaction Between Methylenetetrahydrofolate Reductase (MTHFR) Gene Polymorphisms and Environment with Susceptibility to Ischemic Stroke in Chinese Population

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## Abstract

**Aims:** To investigate the association of several single-nucleotide polymorphisms (SNPs) within methylenetetrahydrofolate reductase (MTHFR) gene, and additional gene–environment interaction with ischemic stroke (IS) risk. **Methods:** Testing for Hardy–Weinberg equilibrium in controls was conducted using SNPstats (online software: http://bioinfo.iconcologia.net/SNPstats). Generalized multifactor dimensionality reduction (GMDR) was used to screen the best interaction combination among four SNPs within MTHFR gene and smoking or alcohol drinking. **Results:** The frequency of the rs4846049-A allele was 28.6% in IS patients and 19.1% in normal controls, in addition, the frequency of the rs3737967-T allele was 27.9% in IS patients and 20.3% in normal controls, which was also indicating a statistically significant difference. The rs4846049-A and rs3737967-T were associated with an increased risk of IS risk; adjusted odds ratios (ORs) (95% confidence interval [CI]) were 1.76 (1.28–2.13) and 1.51 (1.13–1.97), respectively. GMDR model found significant gene–alcohol drinking interaction combination, but no significant gene–tobacco smoking interaction combinations. In order to obtain the odds ratios and 95% CI for the joint effects of gene–alcohol drinking on IS, we conducted stratified analysis for interaction effect using logistic regression. We found that alcohol drinkers with rs4846049-CA/AA genotype also have the highest IS risk, compared with never drinkers with rs4846049-CC genotype, OR (95% CI) = 3.12 (1.83–4.45), after adjustment for age, smoke, and smoking status. **Conclusions:** The rs4846049-A and rs3737967-T, gene–environment interaction between rs1764391 and rs918592, gene–environment interaction between rs4846049 and alcohol drinking were all associated with increased IS risk.

**Keywords:** Alcohol drinking, interaction, ischemic stroke, methylenetetrahydrofolate reductase, single-nucleotide polymorphisms

## Introduction

Ischemic stroke (IS) is a complex disorder and the second leading cause of death and a major cause of serious disability for adults in the world.[1] In China, with the development of the economy, nearly 1.6 million stroke deaths per year, or 157 stroke deaths per 100,000 deaths.[2,3] To date, several risk factors have been reported,[4,5] including age, hypertension, diabetes mellitus, smoking, hyperlipidemia, and hyperhomocysteinemia. Some studies including twin studies,[6] family history studies,[7] and animal stroke model studies[8] have suggested that stroke is partly due to genetic influences. It has been reported that the elevation of homocysteine, which could be obtained by conversion from 5,10-methylenetetra-hydrofolate into 5-methyltetrahydrofolate by 5,10-methylenetetrahydrofolate reductase (MTHFR), is associated with an increased risk for some diseases, such as arteriosclerosis and stroke.[9]

The MTHFR gene locus is located at chromosome 1p36.3 in humans.[10] The 5,10-methylenetetrahydrofolate reductase was the product encoded by the MTHFR gene; this product could catalyze the rate-limiting step in the remethylation of homocysteine to methionine.[11] Some previous studies have suggested that MTHFR gene was a genetic risk factor for cerebrovascular diseases, including IS.[12] Some single-nucleotide polymorphisms (SNPs) within MTHFR gene have been reported associations with IS risk; in these SNPs, C677T has been more studied previously.[13-15] However, to date, the association between IS risk and the others SNPs within MTHFR gene have not been extensively examined; especially for variants in the MTHFR 3′-UTR, just one study[16] for Korean population investigated whether MTHFR 3′-UTR polymorphisms correlate with IS susceptibility. In addition, it was also considered that IS susceptibility was resulting from the interaction between genetic and environmental factors.[17] However, till now, no study investigated the interaction between MTHFR gene and environment. Therefore, this study aimed to evaluate the impact of four SNPs

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in the MTHFR 3′-UTR region and its interaction with environmental risk factors on susceptibility to IS.

## Materials and Methods

### Subjects

There were total of 1,722 participants including 860 patients with IS and 862 normal controls. All IS cases were diagnosed by brain imaging using computed tomography (CT) scanning and/or magnetic resonance imaging (MRI). A diagnosis of IS was diagnosed by at least two related senior neurologists. Age-matched (±2 years) control subjects, in nearly 1:1 ratio to IS patients, did not have IS and any history or symptoms of cerebrovascular disease. Those patients with family history of hemorrhagic stroke, tumor, trauma, myocardial infarction, or atrial arrhythmia were excluded. All of the recruited subjects were Han Chinese with no genetic relationships to one other. At recruitment, all subjects signed the written informed consent.

### Genomic DNA extraction and genotyping

In total, 3-mL EDTA-treated blood samples were obtained from all of the participants for genomic DNA extraction according to the instructions of DNA Blood Mini Kit (Qiagen, Hilden, Germany) and the DNA was kept at −20°C until use. The genotyping for the selected four SNPs was conducted using polymerase chain reaction-based restriction fragment length polymorphism. All SNP sequences were obtained from the HapMap database (http://www.hapmap.org). The primers used in this study were shown in Table 1. In addition, a randomly chosen 15% of the samples were genotyped again by different individuals; the results of these assays were 100% concordant.

### Statistical analysis

In our study, the percentages were calculated for categorical variables and a Chi-squared test was performed for difference analysis between groups, and the Student's *t*-tests was used to check difference for the continuous variables with normal distribution (means ± standard deviations). Hardy–Weinberg equilibrium (HWE) were tested for controls using SNPstats (http://bioinfo.iconcologia.net/SNPstats). Logistic regression calculated the odds ratios (ORs) (95% confidence interval [CI]) for associations between the four SNPs and IS risk. The ORs were adjusted for age, gender, body mass index (BMI), smoking and alcohol drinking. The best combination of SNP–smoking and SNP–alcohol drinking interactions was assessed by generalized multifactor dimensionality reduction (GMDR). A sign or permutation test (providing empirical *P* values) was used for predicting accuracy and measuring the significance of an identified model.

## Results

A description for IS patients and normal controls regarding demographic and general or clinical characteristics is shown in Table 2. There were total of 1,722 participants including 860 patients with IS and 862 normal controls. The average age for all participants was 64.1 ± 12.2 years. No significant different existed in the parameters of interest, including gender, age, BMI, duration of smoking and alcohol drinking, and smoking quantity between the two groups (all *P* values were >0.05). In contrast, the percentages of participants who smoked cigarettes, consumed alcohol, hypertension, T2DM, and hypertension were higher in the IS patients than that in controls.

The genotype frequencies in the control of this study were all distributed according to HWE. The frequency of the rs4846049-A allele was 28.6% in IS patients and 19.1% in normal controls, in addition, the frequency of the rs3737967-T allele was 27.9% in IS patients and 20.3% in normal controls, which was also indicating a statistically significant difference. The rs4846049-A and rs3737967-T were associated with an increased risk of IS, adjusted ORs (95% CI) were 1.76 (1.28–2.13) and 1.51 (1.13–1.97), respectively [Table 3]. We also found that the others SNPs - rs1537514 and rs4846048 - were not significantly associated with susceptibility to IS.

The comparison of cross-validation consistency and the test accuracy of gene–environment interaction model was determined by GMDR model [Table 4]. We found significant gene–alcohol drinking interaction combinations, but no significant gene–gene and gene–tobacco smoking interaction combinations, after adjusting for sex, age, hypertension, and T2DM covariates. A two-locus including rs4846049 and alcohol drinking was significant in the GMDR model. In order to obtain the ORs and 95%CI for the joint effects of gene–alcohol drinking on IS, we conducted stratified analysis for interaction effect using logistic regression.

**Table 1: Description and primer sequences designed for sequencing four SNPs within MTHFR gene**

| SNPs | Chromosome | Major/minor alleles | Primer (5′→3′) |
|---|---|---|---|
| 2572 C >A rs4846049 | 1:11790308 | C/A | Forward: 5′-TTGCCA ACTAAGCCCTCG AAACAA-3′<br>Reverse: 5′-TGCCACATCTCTTCTACGATGCCA-3′ |
| 4869 C >G rs1537514 | 1:11788011 | C/G | Forward: 5′-AGGCAAGCCCCTCAGCCCTT-3′<br>Reverse: 5′-TCCAGCCCTGAGCCCAGAGTC T-3′ |
| 5488 C >T rs3737967 | 1:11787392 | C/T | Forward: 5′-GAGGCACCAGCTCTGTGG-3′<br>Reverse: 5′-CCCCAGGAAGTCCAAGC-3′ |
| 6685 T >C rs4846048 | 1:11786195 | T/C | Forward: 5′-CCAGACCAGAAGCAGTTA-3′<br>Reverse: 5′-GCTGTGCAGTGTCATTT-3′ |

SNP=Single-nucleotide polymorphism; MTHFR=Methylenetetrahydrofolate reductase

**Table 2: General characteristics of 1,722 study participants in case and control group**

| Variables | IS patients (*n*=860) | Normal controls (*n*=862) | *P* |
|---|---|---|---|
| Age (year), means±SD | 63.8±13.2 | 64.4±13.8 | 0.357 |
| Gender, *n* (%) | | | 0.813 |
| Males | 505 (58.7) | 511 (59.3) | |
| Females | 355 (41.3) | 351 (40.7) | |
| Hypertension, *n* (%) | 354 (41.2) | 270 (31.3) | 0.000022 |
| T2DM, *n* (%) | 132 (15.4) | 97 (11.3) | 0.012322 |
| BMI (kg/m$^2$), means±SD | 24.5±8.1 | 23.9±8.5 | 0.134 |
| Smoking, *n* (%) | | | 0.044 |
| Never smoking | 575 (66.9) | 615 (71.4) | |
| Ever or current smoking | 285 (33.1) | 247 (28.6) | |
| Smoking-quantity (per day), means±SD | 13.4±8.8 | 12.7±8.2 | 0.088 |
| Duration of smoking (years), means±SD | 18.2±10.2 | 17.3±10.7 | 0.074 |
| Alcohol drinking, *n* (%) | | | 0.000668 |
| Never drinking | 538 (62.6) | 606 (70.3) | |
| Ever or current drinking | 322 (37.4) | 256 (29.7) | |
| Duration of alcohol drinking (years), means±SD | 15.3±7.2 | 14.6±7.0 | 0.041 |

BMI=Body mass index; T2DM=Type 2 diabetes mellitus, SD=Standard deviation

**Table 3: Association analysis for four SNPs within MTHFR gene and IS susceptibility**

| SNPs | Genotypes or Alleles | Frequencies, *n* (%) | | OR (95%CI)* | HWE test for controls |
|---|---|---|---|---|---|
| | | Normal controls (*n*=862) | IS patients (*n*=860) | | |
| 2572 C >A; rs4846049 | | | | | |
| | CC genotype | 566 (65.7) | 441 (51.3) | 1.00 (ref) | 0.595 |
| | CA genotype | 262 (30.4) | 346 (40.2) | 1.59 (1.19-1.97) | |
| | AA genotype | 34 (3.9) | 73 (8.5) | 2.15 (1.41-2.79) | |
| | C allele | 1,394 (80.9) | 1,228 (71.4) | 1.00 (ref) | |
| | A allele | 330 (19.1) | 492 (28.6) | 1.76 (1.28-2.13) | |
| 4869 C >G; rs1537514 | | | | | |
| | CC genotype | 539 (62.5) | 498 (57.9) | 1.00 (ref) | 0.246 |
| | CG genotype | 278 (32.3) | 307 (35.7) | 1.22 (0.81-1.83) | |
| | GG genotype | 45 (5.2) | 55 (6.4) | 1.53 (0.71-2.34) | |
| | C allele | 1,356 (78.6) | 1,303 (75.8) | 1.00 (ref) | |
| | G allele | 368 (21.4) | 417 (24.2) | 1.26 (0.79-1.97) | |
| 5488 C >T; rs3737967 | | | | | |
| | CC genotype | 553 (64.2) | 462 (53.7) | 1.00 (ref) | 0.249 |
| | CT genotype | 268 (31.1) | 316 (36.7) | 1.30 (0.90-1.79) | |
| | TT genotype | 41 (4.8) | 82 (9.5) | 1.93 (1.32-2.61) | |
| | C allele | 1,374 (79.7) | 1,240 (72.1) | 1.00 (ref) | |
| | T allele | 350 (20.3) | 480 (27.9) | 1.51 (1.13-1.97) | |
| 6685 T >C; rs4846048 | | | | | |
| | TT genotype | 530 (61.5) | 488 (56.7) | 1.00 (ref) | 0.506 |
| | TC genotype | 296 (34.3) | 316 (36.7) | 1.22 (0.86-1.74) | |
| | CC genotype | 36 (4.2) | 56 (6.5) | 1.31 (0.78-1.99) | |
| | T allele | 1,356 (78.6) | 1,292 (75.1) | 1.00 (ref) | |
| | C allele | 368 (21.4) | 428 (24.9) | 1.25 (0.84-1.78) | |

SNP=Single-nucleotide polymorphism; MTHFR=Methylenetetrahydrofolate reductase; IS=Ischemic stroke; OR=Odds ratio; CI=Confidence interval; HWE=Hardy-Weinberg equilibrium. *Adjusted for age, gender, body mass index, smoking and alcohol drinking

We found that alcohol drinkers with rs4846049-CA/AA genotype also have the highest IS risk, compared with never drinkers with rs4846049 - CC genotype, OR (95%CI) = 3.12 (1.83–4.45), after adjustment for age, smoke, and smoking status [Figure 1].

## Discussion

In this study, we found that rs4846049-A and rs3737967-T were associated with an increased risk of IS risk. We also found that SNPs - rs1537514 and rs4846048 - were not significantly

**Table 4: GMDR analysis for the best interaction combination models**

| Locus no. | Best combination | Cross-validation consistency | Testing balanced accuracy | *P** |
|---|---|---|---|---|
| Gene-tobacco smoking interactions* | | | | |
| 2 | 1, tobacco smoking | 7/10 | 0.476 | 0.624 |
| 3 | 1, 2, tobacco smoking | 8/10 | 0.521 | 0.171 |
| 4 | 1, 2, 3, tobacco smoking | 5/10 | 0.562 | 0.857 |
| 5 | 1, 2, 3, 4, tobacco smoking | 7/10 | 0.545 | 0.377 |
| Gene-alcohol drinking interactions ** | | | | |
| 2 | 1, alcohol drinking | **10/10** | **0.632** | **0.0010** |
| 3 | 1, 2, alcohol drinking | 8/10 | 0.557 | 0.172 |
| 4 | 1, 2, 3, alcohol drinking | 7/10 | 0.476 | 0.377 |
| 5 | 1, 2, 3, 4, alcohol drinking | 7/10 | 0.512 | 0.532 |

GMDR=Generalized multifactor dimensionality reduction. *Adjusted for age, gender, body mass index, hypertension, T2DM, and alcohol drinking. **Adjusted for age, gender, body mass index, hypertension, T2DM, and smoking; SNPs named with 1-4 were rs4846049, rs1537514, rs3737967, and rs4846048 respectively

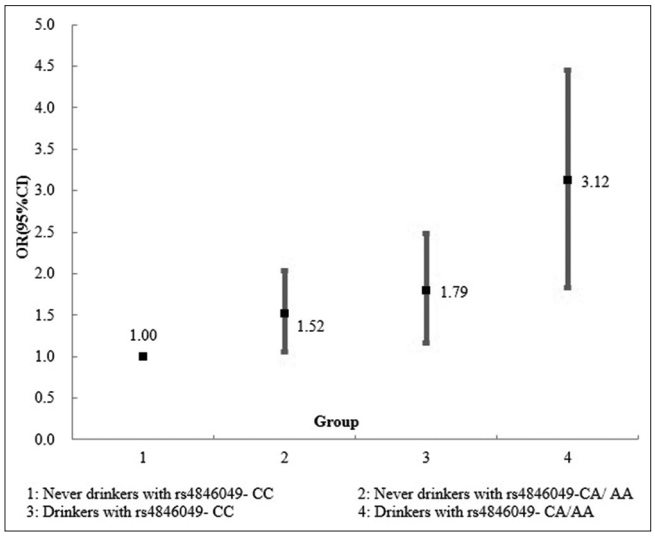


**Figure 1:** Stratified analysis for rs4846049–alcohol drinking interaction effect

associated with susceptibility to IS; previously the study on IS related genetic factors has been wildly performed in different populations. In terms of MTHFR gene, several studies also were performed on this gene, but most studies were involved in one SNP - C677T. Many studies including meta-analysis[13-15] have reported the relationship between C677T and IS risk. So, in this study, we did not investigate whether C677T was associated with IS risk, and some other SNPs were included in this study. As far as we know, just one study was performed on association between rs4846049 and IS risk for Korean subjects.[16] In this study, Kim *et al*.[16] concluded that MTHFR rs4846049 and rs4846048 are associated with ischemic stroke pathogenesis. The common ground between this study and current study was that rs4846049 was a genetic risk factor for IS susceptibility, but the difference was that we also found rs3737967-T was associated with increased IS risk, Kim *et al.* found that rs4846048 was a risk factor for IS. The inconsistent results may be caused by different races and different sample size, which was larger in our study. Previously, some studies also reported the correlation between rs4846049 and others diseases, including migraine,[18] colorectal cancer,[19] preeclampsia,[20] and coronary heart disease.[21,22] Yu *et al*.[22] demonstrated a contributory role of genetic defects in MTHFR gene individually, or interacted with XRCC1 genes, were associated with the development of CAD in Han Chinese. Another Chinese study also suggested that rs4846049 of MTHFR is associated with increased risk for CHD. The mechanism for genesis of ischemic events was not well studied. The MTHFR gene is critical for Hcy and folate metabolism, and polymorphic variants of the enzymes.[23] MTHFR gene could influence the MTHFR enzyme activity, and increase the total plasma homocysteine (tHcy) levels and decrease plasma folate levels, contributes to stroke development.[24,25]

Generally, the pathological mechanism underlying IS risk was a complex process and mainly caused by the interaction between genetic and environmental factors.[17] Some studies have showed that gene–environment[26,27] interaction could influence the IS risk in different populations and involved different gene. This study is the first report attempting to elucidate the possible impact of MTHFR gene–environment interaction on IS risk. We found a significant gene–environment interaction between rs4846049 and alcohol drinking. Previous study has been performed by Kim *et al*.[28] indicated a significant interaction between MTHFR gene and metabolic syndrome (MS) on IS prevalence, but this SNP was 677C >T. The association between alcohol consumption and IS occurrence have been reported in previous studies.[29,30] In this study, the alcohol drinking rate was higher in IS patients than that in controls, and the significant interaction effect indicated that genetic and environment factors could singly and jointly influence IS risk, to date, no experimental evidence at the molecular level were reported to explain the mechanisms for the interaction between alcohol consumption and MTHFR gene variants to affect IS risk. So, future studies *in vitro* and/or *in vivo* would be warranted to verify this finding.

The limitations of this study were: First, we only examined the Chinese Han patients, which may not present on behalf of the Chinese populations, because there were 56 races in China. Second, just four SNPs were studied; more SNPs should be

investigated in the future. Last, we did not examine the levels of serum homocystiene and levels of vitamin B12 in cases as well as controls, so we did not know whether the levels of serum homocysteine could influence the results obtained in current study.

In conclusion, we found that the rs4846049-A and rs3737967-T, gene–environment interaction between rs1764391 and rs918592, gene–environment interaction between rs4846049 and alcohol drinking were all associated with increased IS risk.

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Nil.

## Conflicts of interest

There are no conflicts of interest.

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