Genetic polymorphisms of methylenetetrahydrofolate reductase C677T and risk of ischemic stroke in a southern Chinese Hakka population

Jingyuan Hou, PhDb,c,d,e,f,g, Xing Zeng, BSbc,d,e,g, Yunquan Xie, BSbc,d,e,g, Hesen Wu, BSc,b,c,d,e,g, Pingsen Zhao, Phda,b,c,d,e,f,g,*

Abstract
Previous studies have shown that methylenetetrahydrofolate reductase (MTHFR) gene to be a genetic risk factor for the susceptibility to ischemic stroke. The aim of this case-control study was to investigate whether the polymorphisms of MTHFR C677T were associated with the susceptibility to ischemic stroke in a southern Chinese Hakka population. In this study, a total of 1967 ischemic stroke patients and 2565 controls of Chinese Hakka ethnicity were recruited. The MTHFR C677T polymorphisms were genotyped by polymerase chain reaction (PCR) amplification and microarray method. The risk of ischemic stroke was estimated by logistic regression analysis. The frequencies of CC, CT, and TT genotypes were 52.67% versus 55.63%, 40.31% versus 38.52%, and 7.02% versus 5.85% in patients with ischemic stroke versus controls, respectively. The frequency of T allele was significantly higher in ischemic stroke patients (27.17%) than in controls (25.11%) (\(P = 0.026\), odds ratio [OR] 1.113, 95% confidence interval [CI] 1.013–1.223). The homoyzgous TT genotype in the ischemic stroke patients was associated with increased risk (\(P = 0.049\), OR 1.132, 95% CI 1.001–1.281) when compared with the controls after adjustment for age and sex. The positive association was only found in dominant model without adjustment for age and sex (\(P = 0.047\), OR 1.127, 95% CI 1.002–1.268). Also, the carrier status of the MTHFRT allele was identified as an independent risk factor for the development ischemic stroke even after the adjustment for conventional risk factors (\(P = 0.047\), OR 1.109, 95% CI 0.964–1.225). Our results provide evidence that variants of MTHFR C677T gene may influence the risk of developing ischemic stroke in a southern Chinese Hakka population. Further studies are needed to confirm this association, which will promote the development of strategies for prevention and treatment of ischemic stroke in our study population.

Abbreviations: BP = blood pressure, CI = confidence interval, CT = computed tomography, DBP = diastolic blood pressure, EDTA = ethylene diamine tetra acetic acid, HDL = high-density lipoprotein, HWE = Hardy-Weinberg equilibrium, LDL = low-density lipoprotein, MR = magnetic resonance, MTHFR = methylenetetrahydrofolate reductase, OR = odds ratio, PCR = polymerase chain reaction, SBP = systolic blood pressure, SPSS = Statistical Package for the Social Sciences.

Keywords: C677T, Hakka population, ischemic stroke, methylenetetrahydrofolate reductase gene, polymorphisms

1. Introduction
Stroke is the second leading cause of death globally and a prominent cause of reduced disability-adjusted life-years worldwide.\(^{[1,2]}\) Epidemiological studies have reported that there are 2.5 million new cases of stroke, and more than one million people suffered from stroke-related causes every year in China.\(^{[3]}\) Ischemic stroke is the most common type of stroke that accounts for more than 80% of all stroke events, and it occurs when an
artery that supplies blood to the brain is interrupted. The etiology of ischemic stroke is heterogeneous and widely accepted as a complex multifactorial disease influenced by interactions between genetic and environmental factors.[6–8] Proper management of traditional risk factors for ischemic stroke, such as hypertension, diabetes mellitus, hypercholesterolemia, cigarette smoking, excessive drinking, and heart diseases, may reduce the incidence of ischemic stroke only to a certain degree.[7–9] To date, extensive research has been done focusing on the relationship between genetic variants and susceptibility to ischemic stroke.

Methylenetetrahydrofolate reductase gene (MTHFR) maps to chromosomal location 1p36.3, which is involved in the amino acid and purine biosynthesis pathway.[10,11] MTHFR is a critical regulatory enzyme that catalyzes the transformation of 5,10-
methylenetetrahydrofolate to 5-methylenetetrahydrofolate, which serves as the methyl group donor needed for the conversion of homocysteine to methionine.[12,13] A common mutation for the C to T substitution at nucleotide 677 of MTHFR gene (rs1801133, C677T), which is associated with decreased enzyme activity and eventually leads to elevation of plasma homocysteine levels.[14,15] Hyperhomocysteinemia has been reported to be associated with a variety of metabolic disorders and increased risk for complex diseases, including stroke.[16–19] Despite the MTHFR C677T polymorphisms is recognized as a risk factor for ischemic stroke, there are no consistent results among the populations studies.[16,20–22] These conflicting results may be due to small sample size, various ethnic groups, and lack of consideration of lifestyle. Additionally, to our knowledge, no previous report has examined the effect of MTHFR C677T polymorphisms on the risk of ischemic stroke in a Hakka population. Therefore, the aim of this case-control study was to investigate whether the polymorphisms in MTHFR C677T gene were associated with the susceptibility to ischemic stroke in a southern Chinese Hakka population.

2. Methods

2.1. Study population

Meizhou is a small town located in the northeast of Guangdong Province, with a total area of 15,876 m² and a population of 5.43 million, and approximately 95% inhabitants in Meizhou are Hakka. In this retrospective case-control study, 1967 consecutive unrelated subjects (1293 males and 674 females) were recruited from the patients who visited the neurology department of Meizhou People’s Hospital (Huangtang Hospital), Meizhou Academy of Medical Sciences, Meizhou Hospital Affiliated to Sun Yat-sen University, Guangdong Province, China. All participants in this study provided signed informed consent before the study. Specially trained interviewers administered a standardized questionnaire. A careful anamnestic evaluation and physical examination were performed in all participants. All patients underwent a comprehensive medical history, physical examination, and clinical biochemistry analysis at the time of ischemic stroke. Risk factors for ischemic stroke were recorded, including age, sex, history of hypertension, diabetes mellitus, smoking, drinking alcohol, and dyslipidemia.

2.2. MTHFR genotyping

A 5-ml peripheral venous blood sample was drawn and collected in vacuum tubes containing ethylene diamine tetra acetic acid (EDTA). Genomic DNA was isolated from the blood by using a commercial DNA isolation kit (Quiagen, Hilden, Germany) according to the manufacturer’s protocol. All DNA samples were stored at −80°C until use. Genotyping of MTHFR C677T (rs1801133) polymorphisms was performed by polymerase chain reaction (PCR) amplification and microarray method using a commercially available kit (BaiO Technology Co, Ltd, Shanghai, China). The 25-μl PCR reaction mixture contained 20 ng DNA template and the recommended amounts of primers, dNTPs, and Taq DNA polymerase. The PCR condition was as follows: pre-denaturating at 94°C for 5 minutes, then 35 cycles (94°C for 25 seconds, 56°C for 25 seconds, and 72°C for 25 seconds), and final extension at 72°C for 5 minutes. The PCR products were then dispensed into a hybridization reaction chamber for hybridize reactions. Genotypes of MTHFR C677T were visualized by using the BAI Array Doctor Version.2.0 software and BaiOBE-2.0 software according to the instructions of the manufacturer (BaiO Technology Co, Ltd, Shanghai, China). The genotyping results were confirmed by direct sequencing of the PCR product with an ABI 3500xL DNA sequence analyzer by using a commercially available kit (SinoMDgene Technology Co., Ltd., Beijing, China).

2.3. Statistical analysis

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) version 20.0 statistics software (SPSS Inc., Chicago, IL). Continuous data are expressed as means ± standard deviation and categorical data are expressed as percentages. The significance of differences between continuous variables was determined by Student t test or 1-way analysis of variance (ANOVA). Differences between categorical variables were evaluated with a chi-square test. Allele frequency was determined via direct counting, and the standard goodness-of-fit test was used for the testing of Hardy–Weinberg equilibrium (HWE) by chi-square test. Univariate and multivariate logistic regressions were used to estimate odds ratio (OR) and 95% confidence interval (CI) for the association between ischemic stroke and any of the confounds. Differences were significant at P < .05.

3. Results

3.1. Baseline characteristics

A total of 1967 ischemic stroke patients and 2565 controls who met our inclusion criteria were enrolled in this study. The general baseline clinical characteristics of the 2 groups showed that there
were significant differences in the mean age for which $68.6 \pm 12.1$ years for the patients and $65.1 \pm 13.2$ years for the controls. Both systolic blood pressure (SBP) and diastolic blood pressure (DBP) were significantly higher in the ischemic stroke patients compared with the controls. As expected, the ischemic stroke patients had a significantly higher prevalence of smoking, hypertension, diabetes, dyslipidemia, and hyperhomocysteinemia ($P < .05$). There were no significant differences in the rate of drinking between the groups ($P = .511$). Moreover, the high-density lipoprotein (HDL) cholesterol level was lower ($P < .001$) and total plasma homocysteine level was significantly higher in the patient group than in the control group ($P < .001$). However, there was no significant difference in total cholesterol, triglycerides, and low-density lipoprotein (LDL) cholesterol levels between the 2 groups ($P > .05$).

Table 2 shows the baseline clinical characteristics of ischemic stroke patients according to MTHFR C677T genotypes. The prevalence of hyperhomocysteinemia ($P = .004$) and the level of homocysteine ($P < .001$) were significantly higher in the TT genotype group than in the CC and CT genotype groups.

The genotypes distribution and allele frequencies of the studied MTHFR C677T polymorphisms in the ischemic stroke patients and controls are depicted in Table 3. Distribution of MTHFR C677T genotypes in the ischemic stroke patients ($\chi^2 = 0.681$, $P = .409$) and in the controls ($\chi^2 = 1.507$, $P = .220$) were consistent with the HWE expectations. Frequencies of CC, CT, and TT genotypes were 52.67% versus 55.63%, 40.31%...
Genotypes and allele frequencies of the MTHFR C677T polymorphisms were associated with ischemic stroke in the Chinese population. In our study population, the homozygous TT genotype was associated with increased risk \((P=0.049, \text{OR} = 1.132, 95\% \text{CI 1.001–}1.281)\) when compared with the control group \((5.85\%)\) after adjustment for age and sex. Also, dominant \((TT + CT \text{ vs } CC)\) and recessive models \((TT \text{ vs } CT + CC)\) were also performed to assess the effect of MTHFR C677T on the risk of ischemic stroke. The positive association was only found in ischemic stroke \((P=0.047, \text{OR} = 1.127, 95\% \text{CI 1.002–}1.268)\) when compared with the controls \((25.11\%)\) \((P=0.026, \text{OR} = 1.113, 95\% \text{CI 1.013–}1.223)\).

Multivariate logistic regressions were used to examine the risk factors for ischemic stroke. As shown in Table 4, age, hypertension, diabetes mellitus, dyslipidemia, and hyperhomocysteinemia, but not sex, was found to be significant risk factors for ischemic stroke \((P<.01)\). Moreover, the carrier status of the MTHFR T allele was identified as an independent risk factor for ischemic stroke even after the adjustment for conventional risk factors \((P=0.047, \text{OR} = 1.109, 95\% \text{CI 0.964–}1.225)\).

### Table 2
Baseline clinical characteristics of ischemic stroke patients according to MTHFR genotypes.

| Variables                      | Genotype          |
|--------------------------------|-------------------|
|                                | CC (n=1036)       | CT (n=793)       | TT (n=138)       | \(P\)          |
| Age, y                         | 68.3±12.3         | 69.0±11.7        | 68.4±12.1        | .456         |
| Male, n (%)                    | 678 (65.4)        | 532 (67.1)       | 83 (60.1)        | .273         |
| BP, mm Hg                      | 145.7±31.4        | 147.5±29.2       | 150.1±24.8       | .168         |
| SBP                            | 83.8±18.3         | 85.2±17.1        | 84.4±14.2        | .251         |
| Cerebrovascular risk factors   |                  |                  |                 |             |
| Smoking, n (%)                 | 204 (19.7)        | 141 (17.8)       | 31 (22.5)        | .344         |
| Drinking, n (%)                | 39 (3.8)          | 25 (3.2)         | 9 (6.5)          | .153         |
| Hypertension, n (%)            | 732 (70.7)        | 582 (73.4)       | 100 (72.5)       | .430         |
| Diabetes, n (%)                | 318 (30.7)        | 260 (32.8)       | 42 (30.4)        | .609         |
| Dyslipidemia, n (%)            | 306 (29.5)        | 251 (31.7)       | 38 (27.5)        | .479         |
| Hyperhomocysteinemia, n (%)   | 325 (31.4)        | 298 (37.6)       | 58 (42.0)        | .004         |
| Laboratory results             |                  |                  |                 |             |
| Total cholesterol, mmol/L     | 4.02±1.38         | 4.78±1.37        | 4.83±1.31        | .111         |
| Triglycerides, mmol/L         | 1.73±1.75         | 1.73±1.33        | 1.68±1.08        | .906         |
| LDL cholesterol, mmol/L       | 2.81±0.98         | 2.75±0.88        | 2.80±0.96        | .431         |
| HDL cholesterol, mmol/L       | 1.26±0.39         | 1.24±0.36        | 1.23±0.35        | .523         |
| Homocysteine, μmol/L          | 17.04±7.26        | 17.88±7.23       | 22.22±12.39      | <.001        |

Table 3
Genotypes and allele frequencies of the MTHFR C677T polymorphisms in ischemic stroke patients and controls.

| MTHFR            | Ischemic stroke (n=2061) | Controls (n=2965) | Unadjusted OR \((95\% \text{CI})\) | \(P\)       | Adjusted OR \(^\dagger\) \((95\% \text{CI})\) | \(P\)       |
|------------------|--------------------------|-------------------|-----------------------------------|-------------|-----------------------------------|-------------|
| Genotype         |                          |                   |                                   |             |                                    |             |
| CC               | 1036 (62.67)             | 1427 (55.63)      | 1.000 (reference)                 | 1.000       | 1.000 (reference)                 | 1.000       |
| CT               | 793 (40.31)              | 988 (38.52)       | 1.106 (0.978–1.250)               | .110       | 1.091 (0.964–1.236)               | .169       |
| TT               | 138 (7.02)               | 150 (5.85)        | 1.267 (0.992–1.618)               | .057       | 1.132 (1.001–1.281)               | .049       |
| Dominant model   | 931 (47.33)              | 1138 (44.37)      | 1.127 (1.002–1.268)               | .047       | 1.116 (0.991–1.257)               | .071       |
| recessive model  | 1829 (92.98)             | 2415 (94.15)      | 1.215 (0.956–1.543)               | .110       | 1.237 (0.971–1.575)               | .085       |
| Allele           |                          |                   |                                   |             |                                    |             |
| C                | 2865 (72.83)             | 3842 (74.89)      | 1.000 (reference)                 |            |                                    |            |
| T                | 1059 (27.17)             | 1268 (25.11)      | 1.113 (1.013–1.223)               | .026       |                                    |            |
| HWE              |                          |                   | \(\chi^2 = 0.681; P = .409\)     |             | \(\chi^2 = 1.507; P = .220\)     |             |

\(^\dagger\) Calculated for carriers of the polymorphism (heterozygous and homozygous) versus wild type.

The dominant model \((TT + CT \text{ vs } CC)\) and the recessive model \((TT \text{ vs } CT + CC)\) were also analyzed.

The \(P\) values calculated for the analysis were calculated.

1. Adjusted for age and sex in a logistic regression model.

4. Discussion

The mortality and morbidity of ischemic stroke remain very high in different districts. Ischemic stroke is a complex multifactorial disorder, resulting from the interaction between genetic and environmental factors.\(^4,8\) Previous study has shown that the MTHFR C677T polymorphisms were associated with ischemic stroke. It has also been proposed that known genetic abnormality associated with ischemic stroke may not be the same in individuals with different genetic backgrounds.\(^20,22\) Till now, little is known about the association of MTHFR C677T polymorphisms and the risk of ischemic stroke in the Chinese Hakka population. Therefore, in the present study, we have detected such association in a southern Chinese Hakka population. In our study population, the homozygous TT
genotype significantly increases the risk of ischemic stroke after adjustment for age and sex ($P = 0.049$, OR $1.132$, 95% CI: $1.001–1.281$). The positive association was found in dominant model without adjustment for age and sex ($P = 0.047$, OR $1.127$, 95% CI $1.002–1.268$). We also showed a significantly higher frequency of T allele in ischemic stroke patients ($P = 0.026$, OR $1.113$, 95% CI $1.013–1.223$). In addition, other factors, including the presence of age, hypertension, diabetes mellitus, dyslipidemia, and hyperhomocysteinemia, were associated with the risk of ischemic stroke ($P < .01$). Meanwhile, the carrier status of the $MTHFR$ T allele was identified as an independent risk factor for ischemic stroke even after the adjustment for conventional risk factors ($P = 0.047$, OR $1.109$, 95% CI $0.964–1.225$).

The prevalence of the mutations of $MTHFR$ C677T varies substantially different in distinct geographical region, races, and the ethnicity populations. For example, the frequency of T allele was reported to be high in European, North Americans, and East Asians, but low in African populations. Interestingly, a north-to-south cline of increase in allele frequency has also been observed in Europe, but a reverse trend geographical gradients purely originated from Central Plain—a vast region containing the current Shansi and Henan Provinces. The $MTHFR$ population is characteristic of their unique culture including some features in dialects, life styles, customs, and habits. For instance, the famous architectural type of Hakka—Round-Dragon House—is suggested to be derived from northern Han courtyard house. However, the Hakka population is fond of foods high in saturated fat and sodium such as preserved meat. These diet habits could also contribute to the development of a series of functional disorder such as hypertension, dyslipidemia, and hyperhomocysteinemia in the Hakka population, and eventually lead to ischemic stroke.

In this study, by multivariate logistic regressions analysis, we found that age, hypertension, diabetes mellitus, dyslipidemia, and hyperhomocysteinemia were statistically significant risk factors for ischemic stroke, whereas sex was served as a protective factor. Hakka is an intriguing Han population that mainly inhabits in southern China, but with northern Han cultural traditions and linguistic influences. It has been proposed that Hakka population living in Meizhou area purely originated from Central Plain—a vast region containing the current Shansi and Henan Provinces. The $MTHFR$ Hakka population is characteristic of their unique culture including some features in dialects, life styles, customs, and habits. For instance, the famous architectural type of Hakka—Round-Dragon House—is suggested to be derived from northern Han courtyard house. However, the Hakka population is fond of foods high in saturated fat and sodium such as preserved meat. These diet habits could also contribute to the development of a series of functional disorder such as hypertension, dyslipidemia, and hyperhomocysteinemia in the Hakka population, and eventually lead to ischemic stroke.

In this study, our data also confirm that homozygous T carriers possessed higher homocysteine plasma levels. Moreover, the carrier status of the $MTHFR$ T allele was identified as a moderate risk factor for ischemic stroke, even after the adjustment for traditional risk factors. These findings suggest that the possible effects of $MTHFR$ C677T mutation on ischemic stroke may mediate through elevated homocysteine plasma levels in the study population. Our study has several advantages. Our population was enrolled from Meizhou, which is a small town located in the northeast of Guangdong Province. The region has a high geographic stability, which could significantly reduce the potential confounding effects of the heterogeneous participants in the study. However, some limitations of this study should be pointed out. First, there may be differences in the results of patients from different regions and races. Therefore, these results need to be verified in other populations. Second, the present study
is limited by the fact that the lack of supplement information on dietary folate intake and plasma folate concentrations was not evaluated, and that the effects of MTHFR genotype may be modulated by folate status. Third, we chose to study only the polymorphisms of MTHFR C677T genes. Because stroke is a complex disease, there is a lack of conviction to study only 1 gene in individuals. A cumulative effect of multiple genotypes and an interaction between specific genetic and environmental may contribute to the emergence of an ischemic stroke event.

5. Conclusions
In summary, we provide evidence that homozygous TT genotype carriers of MTHFR had significantly higher plasma homocysteine levels and increased risk factor for ischemic stroke in the southern Chinese Hakka population. Given the hypothesis that MTHFR C677T mutation is a risk factor for ischemic stroke, identification of high-risk populations with genetic predisposition to ischemic stroke will promote the development of strategies for prevention and treatment of ischemic stroke in our study population.

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Author contributions
Contributions: Pingsen Zhao conceived and designed the experiments; Jingyuna Hou recruited subjects and collected clinical data; and conducted the laboratory testing. Xing Zeng, Yunquan Xie and Heming Wu helped to analyze the data. Pingsen Zhao and Jingyuna Hou prepare the manuscript. Pingsen Zhao reviewed the manuscript.

Conceptualization: Pingsen Zhao.

Data curation: Jingyuna Hou, Yunquan Xie, Hesen Wu, Pingsen Zhao.

Formal analysis: Pingsen Zhao.

Funding acquisition: Jingyuna Hou, Pingsen Zhao.

Investigation: Jingyuna Hou, Pingsen Zhao.

Methodology: Jingyuna Hou, Xing Zeng, Yunquan Xie, Hesen Wu, Pingsen Zhao.

Project administration: Pingsen Zhao.

Resources: Jingyuna Hou, Xing Zeng, Yunquan Xie, Hesen Wu, Pingsen Zhao.

Software: Jingyuna Hou, Xing Zeng, Yunquan Xie, Hesen Wu, Pingsen Zhao.

Supervision: Pingsen Zhao.

Validation: Pingsen Zhao.

Visualization: Pingsen Zhao.

Writing – original draft: Jingyuna Hou, Pingsen Zhao.

Writing – review & editing: Pingsen Zhao.

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