Tagging SNPs in the MTHFR Gene and Risk of Ischemic Stroke in a Chinese Population

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Received: 10 March 2014; in revised form: 7 May 2014 / Accepted: 8 May 2014 / Published: 20 May 2014

Abstract: Stroke is currently the leading cause of functional impairments worldwide. Folate supplementation is inversely associated with risk of ischemic stroke. Methylenetetrahydrofolate reductase (MTHFR) is an important enzyme involved in folate metabolism. The aim of this study is to examine whether genetic variants in MTHFR gene are associated with the risk of ischemic stroke and fasting total serum homocysteine (tHcy) level. We genotyped nine tag SNPs in the MTHFR gene in a case-control study, including 543 ischemic stroke cases and 655 healthy controls in China. We found that subjects with the rs1801133 TT genotype and rs1801131 CC genotype had significant increased risks of ischemic stroke (adjusted odds ratio (OR) = 1.82, 95% confidence interval (CI): 1.27–2.61, \( p = 0.004 \); adjusted OR = 1.99, 95% CI: 1.12–3.56, \( p = 0.01 \)) compared with subjects with the major alleles. Haplotype analysis also found that carriers of the MTHFR CTTTCGA haplotype (rs12121543-rs13306553-rs9651118-rs1801133-rs2274976-rs1801131) had a significant reduced risk of ischemic stroke (adjusted OR = 0.53, 95% CI: 0.35–0.82) compared with those with the CTTTTGA haplotype. Besides, the MTHFR rs1801133 and rs9651118 were significantly associated with serum levels of tHcy in healthy controls (\( p < 0.0001 \) and \( p = 0.02 \)). These findings suggest that variants in the MTHFR gene may influence the risk of ischemic stroke and serum tHcy.
Keywords: ischemic stroke; MTHFR; polymorphism; homocysteine

1. Introduction

Stroke is a devastating neurological disease that mainly caused by abnormal perfusion of brain tissue, which ranked the second leading cause of death in people older than 60 years worldwide [1]. Over the last 4 decades (1970–2008), stroke incidence declined 42% in the developed countries, while the incidence rates in developing countries have increased by >100% and exceed that of the developed countries by 20% [2]. According to recent statistics, the annual stroke mortality rate in China is approximately 157 per 100,000, which has become the leading cause of death and adult disability [3]. Ischemic stroke is the most common type of stroke in China. Data from 1996 to 2000 showed that ischemic stroke accounts for about two thirds of strokes in China [4].

The major risk factors for stroke in China include hypertension, dyslipidemia, obesity, diabetes and smoking [5]. Data have also shown that certain nutrients and vascular endothelial regulation factors, such as folic acid and Kruppel-like factor 2, are protective factor for ischemic stroke [6]. Folic acid intake appears to reduce the risk of stroke through the regulation of plasma homocysteine concentration, which was a strong and independent risk factor for stroke [7]. It was reported that a 25% lower usual homocysteine level was associated with a 19% lower risk of stroke [8]. Several polymorphisms in homocysteine regulatory genes, such as MTRR, SHMT1 and TCN2, have also been found to have impacts on plasma homocysteine level and ischemic stroke risk [9]. MTHFR (methyleneetrahydrofolate reductase), a folate-dependent enzyme, plays an important role in the conversion of the amino acid homocysteine to another amino acid, methionine, by converting 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. Studies have found that specific genetic mutations in the MTHFR gene could lead to change of MTHFR enzyme activity [10,11]. Previous epidemiologic studies have shown that genetic mutations in MTHFR gene may be related to cancers, type 2 diabetes, cardiovascular diseases, hypertension, etc. [12–14]. However, data on MTHFR genotypes and susceptibility to ischemic stroke in Chinese population are relatively rare, lack consistency and mainly focused on the most studied SNPs (C677T and A1298C) [15–19].

To help clarify whether the MTHFR variants are associated with susceptibility of ischemic stroke and total serum homocysteine (tHcy) level, we examined nine tag SNPs (single nucleotide polymorphism) in the MTHFR gene (rs12121543, rs13306561, rs13306553, rs9651118, rs1801133, rs2274976, rs4846048, rs1801131, rs17037396) in a case-control study in the Chinese population.

2. Results

Characteristics of the study subjects are shown in Table 1. Cases and controls were evenly matched by age and gender. Subjects were more likely to smoke cigarettes (41.3% vs. 32.8%), have diabetes (25.2% vs. 12.4%) and hypertension (72.4% vs. 33.9%). Besides, the subjects have significantly lower levels of serum HDL-C and heart rate, and higher levels of serum LDL-C than that of the controls.
Table 1. Selected characteristics of cases and controls.

| Characteristics          | Cases (n = 543) | Controls (n = 655) | p value |
|--------------------------|-----------------|-------------------|---------|
| Age (year)               | 66 (61–70)      | 66 (60–70)        | 0.76    |
| Sex (Male/Female)        | 346/197         | 402/253           | 0.42    |
| Smoking (Yes/No)         | 224/319         | 215/440           | 0.003   |
| Diabetes (Yes/No)        | 137/406         | 81/574            | <0.001  |
| Hypertension (Yes/No)    | 293/250         | 222/433           | <0.001  |
| BMI (kg/m²)              | 24.3 (22.9–25.8)| 24.3 (23.0–25.7)  | 0.55    |
| Total cholesterol (mmol/L)| 4.70 (4.09–5.48)| 4.14 (3.60–5.04)  | 0.82    |
| HDL-C (mmol/L)           | 1.20 (1.09–1.51)| 1.24 (1.08–1.56)  | 0.008   |
| LDL-C (mmol/L)           | 2.82 (2.45–3.29)| 2.48 (2.16–3.03)  | <0.001  |
| Heart rate (bmp)         | 72 (67–76)      | 74 (68–78)        | <0.001  |

The associations of MTHFR variants and risk of ischemic stroke are presented in Table 2. The genotype distributions of these nine polymorphisms showed no deviation from the expected Hardy–Weinberg equilibrium among controls (p > 0.05). Of these SNPs, TT genotype of rs1801133 and CC genotype of rs1801131 conferred significant higher risk of ischemic stroke (OR = 1.64, 95% CI: 1.16–2.31, p = 0.004; OR = 1.97, 95% CI: 1.13–3.45, p = 0.01) compared with CC and AA genotypes. These associations were still significant after adjustment for other risk factors (age, smoking, hypertension and diabetes). None of the other SNPs examined was associated with the risk of ischemic stroke.

Table 2. Odds ratios (ORs) and 95% confidence intervals (CIs) for ischemic stroke in relation to polymorphisms of the methylenetetrahydrofolate reductase (MTHFR) gene.

| SNP       | Genotypes | Cases, n (%) | Controls, n (%) | OR (95% CI) | OR (95% CI) | P trend |
|-----------|------------|--------------|-----------------|-------------|-------------|---------|
| rs12121543| CC         | 362 (66.8)   | 449 (68.7)      | 1.00        | 1.00        |         |
|           | CA         | 159 (29.3)   | 185 (28.3)      | 1.04 (0.80–1.36) | 0.99 (0.75–1.30) |         |
|           | AA         | 21 (3.9)     | 20 (3.1)        | 1.42 (0.75–2.71) | 1.45 (0.74–2.84) | 0.61    |
|           | CA + AA    | 180 (33.2)   | 205 (31.4)      | 1.08 (0.84–1.39) | 1.03 (0.79–1.34) |         |
| rs13306561| TT         | 432 (79.7)   | 529 (80.9)      | 1.00        | 1.00        |         |
|           | TC         | 106 (19.6)   | 121 (18.5)      | 1.03 (0.76–1.39) | 0.95 (0.69–1.29) |         |
|           | CC         | 4 (0.7)      | 4 (0.6)         | 1.27 (0.30–5.37) | 1.36 (0.30-6.18) | 0.67    |
|           | TC + CC    | 110 (20.3)   | 125 (19.1)      | 1.03 (0.77–1.39) | 0.96 (0.70–1.30) |         |
| rs13306553| TT         | 435 (80.3)   | 534 (81.7)      | 1.00        | 1.00        |         |
|           | TC         | 105 (19.3)   | 116 (17.6)      | 1.06 (0.78–1.44) | 0.97 (0.70–1.33) |         |
|           | CC         | 2 (0.4)      | 4 (0.6)         | 0.62 (0.11–3.60) | 0.71 (0.11–4.55) | 0.54    |
|           | TC + CC    | 107 (19.7)   | 120 (18.3)      | 1.05 (0.78–1.41) | 0.96 (0.70–1.31) |         |
| rs9651118 | TT         | 230 (42.4)   | 273 (41.7)      | 1.00        | 1.00        |         |
|           | TC         | 233 (43.0)   | 291 (44.5)      | 0.93 (0.72–1.20) | 0.93 (0.71–1.21) |         |
|           | CC         | 79 (14.6)    | 90 (13.8)       | 1.05 (0.73–1.51) | 1.00 (0.69–1.46) | 0.66    |
|           | TC + CC    | 312 (57.6)   | 381 (58.3)      | 0.96 (0.75–1.21) | 0.95 (0.74–1.21) |         |
| rs1801133 | CC         | 160 (29.5)   | 242 (37.0)      | 1.00        | 1.00        |         |
|           | CT         | 270 (49.8)   | 308 (47.1)      | 1.34 (1.03–1.76) | 1.31 (0.99–1.73) |         |
|           | TT         | 112 (20.7)   | 104 (15.9)      | 1.64 (1.16–2.31) | 1.82 (1.27–2.61) | 0.04    |
|           | CT + TT    | 382 (70.5)   | 412 (63.0)      | 1.42 (1.10–1.83) | 1.44 (1.10–1.87) |         |
| rs2274976 | GG         | 433 (79.9)   | 542 (82.9)      | 1.00        | 1.00        |         |
|           | GA         | 104 (19.2)   | 107 (16.4)      | 1.17 (0.86–1.60) | 1.10 (0.80–1.52) |         |
|           | AA         | 5 (0.9)      | 5 (0.8)         | 1.34 (0.37–4.82) | 1.50 (0.39–5.69) | 0.21    |
|           | GA + AA    | 109 (20.1)   | 112 (17.1)      | 1.18 (0.87–1.60) | 1.12 (0.81–1.53) |         |
Table 2. Cont.

| SNP      | Genotypes | Cases, n (%) | Controls, n (%) | OR (95% CI) † | OR (95% CI) ‡ | P trend |
|----------|-----------|--------------|-----------------|---------------|---------------|---------|
| rs4846048 | AA        | 424 (78.5)   | 538 (82.3)      | 1.00          | 1.00          |         |
|          | AG        | 108 (20.0)   | 111 (17.0)      | 1.23 (0.90–1.66) | 1.19 (0.87–1.64) |         |
|          | GG        | 8 (1.5)      | 5 (0.8)         | 2.60 (0.81–8.38) | 2.82 (0.83–9.54) | 0.12    |
|          | AG + GG   | 116 (21.5)   | 116 (17.7)      | 1.28 (0.95–1.72) | 1.25 (0.92–1.70) |         |
| rs1801131 | AA        | 333 (61.4)   | 448 (68.5)      | 1.00          | 1.00          |         |
|          | AC        | 174 (32.1)   | 182 (27.8)      | 1.27 (0.97–1.64) | 1.19 (0.91–1.57) |         |
|          | CC        | 35 (6.5)     | 24 (3.7)        | 1.97 (1.13–3.45) | 1.99 (1.12–3.56) | 0.03    |
|          | AC + CC   | 209 (38.6)   | 206 (31.5)      | 1.35 (1.05–1.73) | 1.29 (0.99–1.66) |         |
| rs17037396| CC        | 418 (77.1)   | 500 (76.3)      | 1.00          | 1.00          |         |
|          | CT        | 112 (20.7)   | 142 (21.7)      | 0.95 (0.71–1.27) | 0.96 (0.71–1.30) |         |
|          | TT        | 12 (2.2)     | 13 (2.0)        | 1.22 (0.54–2.79) | 1.13 (0.49–2.62) | 0.71    |
|          | CT + TT   | 124 (22.9)   | 155 (23.7)      | 0.97 (0.73–1.29) | 0.97 (0.73–1.31) |         |

† Adjusted for age; ‡ Adjusted for age, cigarette smoking, diabetes and hypertension.

Six SNPs in the MTHFR gene (rs12121543, rs13306553, rs9651118, rs1801133, rs2274976, rs1801131) were in linkage disequilibrium with D' ranging from 0.61 to 0.99 and \( r^2 \) ranging from 0.03 to 0.84. Subjects carrying the MTHFR CTTCGA haplotype had a significant reduced risk of ischemic stroke (OR = 0.55, 95% CI: 0.36–0.84) compared with those carrying the CTTTGA haplotype. This association was still significant after adjustment for other risk factors (Table 3).

Table 3. Association of haplotypes in the MTHFR gene with risk of ischemic stroke.

| Haplotype * | Cases, % | Controls, % | OR (95% CI) † | OR (95% CI) ‡ |
|-------------|----------|-------------|---------------|---------------|
| CTTTGA      | 38.7     | 38.7        | 1.00          | 1.00          |
| CTCCGA      | 33.6     | 35.7        | 0.96 (0.79–1.17) | 0.92 (0.76–1.12) |
| ATTCGC      | 6.8      | 8.7         | 0.79 (0.56–1.12) | 0.76 (0.54–1.08) |
| ACTCAC      | 7.5      | 7.7         | 0.94 (0.67–1.33) | 0.88 (0.62–1.24) |
| CTTCGA      | 3.2      | 6.6         | 0.55 (0.36–0.84) | 0.53 (0.35–0.82) |

* In the order rs12121543, rs13306553, rs9651118, rs1801133, rs2274976, rs1801131; † Adjusted for age; ‡ Adjusted for age, cigarette smoking, diabetes and hypertension.

Table 4. Association between MTHFR polymorphisms and serum tHcy levels in healthy control.

| SNP         | M/m   | tHcy (mmol/L) | p   |
|-------------|-------|---------------|-----|
|             | MM    | Mm            | mm  |     |
| rs12121543  | C/A   | 12.4 (10.1–14.6) | 12.1 (9.7–13.7) | 10.8 (8.5–13.4) | 0.26 |
| rs13306561  | T/C   | 12.1 (10.0–14.4) | 12.3 (10.2–14.1) | 9.1 (8.1–11.8) | 0.45 |
| rs13306553  | T/C   | 12.1 (10.0–14.4) | 12.4 (10.1–14.2) | 9.1 (8.0–11.8) | 0.45 |
| rs9651118   | T/C   | 12.5 (9.8–14.9) | 12.0 (10.0–13.8) | 11.5 (9.5–14.1) | 0.02 |
| rs1801133   | C/T   | 10.9 (9.2–12.8) | 12.8 (10.9–14.6) | 13.1 (10.0–16.8) | <0.0001 |
| rs2274976   | G/A   | 12.1 (10.0–14.4) | 12.3 (10.0–13.9) | 9.8 (8.4–13.7) | 0.57 |
| rs4846048   | A/G   | 12.3 (10.1–14.4) | 11.6 (9.4–13.6) | 11.1 (10.3–11.6) | 0.36 |
| rs1801131   | A/C   | 12.4 (10.0–14.6) | 12.2 (10.0–13.8) | 10.4 (8.6–13.4) | 0.36 |
| rs17037396  | C/T   | 12.2 (10.0–14.4) | 11.9 (9.9–14.4) | 11.7 (11.4–12.9) | 0.94 |

M indicates major alleles; m indicates minor alleles.
Finally, we investigated the associations between the \textit{MTHFR} SNPs and serum tHcy levels in the control population. Carriers of the mutant alleles of rs1801133 were significantly associated with increased serum level of tHcy (C/T: 12.8 (10.9–14.6) mmol/L; T/T: 13.1 (10.0–16.8) mmol/L) compared with carriers of the CC genotype (12.5 (9.8–14.9) mmol/L). Rs9651118 T/C and C/C genotypes (12.0 (10.0–13.8) mmol/L; 11.5 (9.5–14.1) mmol/L) conferred significant decreased serum level of tHcy in controls compared with TT genotype (10.9 (9.2–12.8) mmol/L). These associations were also significant in multivariate ANCOVA analyses. None of the other studied SNPs were associated with serum tHcy level (Table 4).

3. Discussion

In this molecular epidemiologic study, polymorphisms in the \textit{MTHFR} gene were fully studied for their association with susceptibility to ischemic stroke and serum tHcy levels. We demonstrated that two genetic mutations (rs1801133 and rs1801131) in \textit{MTHFR} gene were significantly associated with risk of ischemic stroke, and rs1801133 and rs9651118 were significantly associated with serum tHcy levels in our study population.

The \textit{MTHFR} gene is located on chromosome 1 location p36.3 in humans. To date, over 40 point mutations in \textit{MTHFR} gene have been identified, of which C677T (rs1801133) and A1298C (rs1801131) seem to have the most clinical significance. The \textit{MTHFR} rs1801133 polymorphism involving C to T substitution at position 677 (C677T) that results in the conversion of alanine to valine. This missense mutation results in approximately 70% and 35% reduction of normal MTHFR enzyme activity in TT and CT genotype carriers, respectively [10]. MTHFR activity has an adverse effect on tHcy level. It was also reported that 677TT allele was associated with elevated tHcy levels, predominantly in individuals who have a low plasma folate level [20]. Higher homocysteine concentration has a negative effect on stroke [8]. Thus, our findings are biologically possible.

Although many studies focused on the association of \textit{MTHFR} C677T mutation and risk of stroke, the results varied, which may partially due to different population groups and sample size. A meta-analysis showed that TT genotype of C677T had a 1.84-fold significantly increased risk of hemorrhagic stroke compared to CC genotype, and subgroup analyses by ethnicity further proved that this association existed both in Asian and Caucasian populations [21]. However, another meta-analysis including 152,797 individuals failed to found any relation between C677T and risk of ischemic stroke (OR = 1.23 95% CI: 0.61–1.47) [22]. Besides, data from a meta-analysis did not show any association between the \textit{MTHFR} C677T molecular variant and risk of carotid dissection, which is recognized as a cause of stroke [23]. In our study, we found a positive correlation between C677T mutation and ischemic stroke risk, and the risk alleles were associated with significant higher level of tHcy in healthy controls.

A1298C (rs1801131) is a common mutation in the \textit{MTHFR} gene that results in the conversion of adenine to cytosine, and this mutation also leads to a reduction in MTHFR enzyme activity. Several studies have been performed to evaluate the effects of \textit{MTHFR} A1298C mutation on the risk of ischemic stroke, but obtained conflicting results [24–27]. The association between A1298C and serum homocysteine level was also controversial. Kumar \textit{et al.} found that CC genotype conferred significant increased level of homocysteine (16.3 μmol/L) compared with AA genotype (14.4 μmol/L) in an Indian population, while this association was not found in a Scottish population [28,29]. Although we found
significant association between A1298C and risk of ischemic stroke, we failed to find its relation with homocysteine level. The reason for these results still needs to be clarified.

Rs9651118 is located in the intron region of MTHFR gene, and the genetic function of this polymorphism is still unclear. It was reported that mutant allele of rs9651118 was associated with reduced lung cancer risk in never smokers, and a nominally significant association with schizophrenia in the form of haplotypes (rs1801133, rs17421511, rs17037396, and rs9651118) in the MTHFR gene in a Japanese population [30,31]. Moreover, the rs9651118 TT genotype conferred a significantly elevated level of tHcy compared with CC genotype in a population-based CoLaus study [32]. In our research, rs9651118 was not associated with risk of ischemic stroke, but we found carriers of TT genotype had significant higher level of tHcy relative to CC genotype carriers, which was in accordance with the previous study.

4. Material and Methods

4.1. Study Participants

In this study, we consecutively enrolled 543 ischemic stroke patients in Tianjin first center hospital, aged from 45 to 89 years, between June 2011 and June 2013. Clinical diagnoses of ischemic stroke were made by CT or MRI scans of the brain. Six hundred and fifty five healthy control subjects were selected during the same period and from the same hospital, and were frequency matched to the cases by age (5-year age groups) and gender. All controls were individuals free of ischemic stroke that determined by medical history, clinical examinations, or ultrasound screening. At enrollment, demographic characteristics, anthropometric measures, medical histories were collected from each subject by a trained interviewer using a structured questionnaire. Blood samples were collected after a 12-h overnight fast and then separated into serum, red blood cells, and buffy coat. Written informed consent was obtained from all enrolled participants and this study was approved by the Ethics Committee of Tianjin first center hospital.

4.2. SNP Selection

Tag SNPs were selected by searching Han Chinese data from the HapMap project using the Tagger program. The following criteria were used to identify tag SNPs: (a) SNPs located in the gene or within the 2-kb region flanking the gene; (b) a minor allele frequency \(\geq 0.1\); (c) other unselected SNPs could be captured by one of the tagging SNPs with a linkage disequilibrium of \(r^2 \geq 0.90\); and (d) SNPs significantly associated with stroke in previous studies were preferred. As a result, a total of nine tag SNPs were identified.

4.3. Laboratory Tests

Serum total cholesterol (TC), low density lipoprotein-cholesterol (LDL-C), high density lipoprotein-cholesterol (HDL-C) and fasting total homocysteine (tHcy) levels were determined by commercial kits from the Nanjing Jiancheng Bio-company (Nanjing, China). Genomic DNA was extracted from buffy coat using DNA Extraction Kit (Qiagen, Hilden, Germany). Genotyping was performed using the TaqMan assay on the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA), with dual fluorescent reporter probes VIC and
FAM. The genotyping call rate was >95%, and the completion rate was >99%. The quality and potential misclassification of the genotyping were assessed by re-genotyping 5% of duplicate DNA samples that were randomly selected from the whole population and placed within the same reaction plates used for the study subjects. The concordance rate for the quality control samples was 100%.

4.4. Statistical Analysis

We used SAS software (version 9.3; SAS Institute, Inc., Cary, NC, USA) for the statistical analyses. \( \chi^2 \) statistics and the \( t \) test were used to evaluate case-control differences in the distribution of risk factors. Variables were tested for normality with Shapiro–Wilk statistics. Skewed data, including age, BMI, TC, LDL-C, HDL-C, tHcy and heart rate were log transformed and expressed as medians and interquartile ranges. The odds ratios (ORs) and 95% confidence intervals (CIs) for the associations between the SNPs and disease risk were estimated by unconditional logistic regression. Hardy–Weinberg equilibrium for genotypic distribution and linkage disequilibrium between loci were assessed by HaploView version 4.0 (Daly Lab at the Broad Institute, Cambridge, MA, USA) [33]. Associations between haplotypes (>1% frequency) and the risk of ischemic stroke were evaluated by computing OR and 95% CI using HAPSTAT, assuming an additive model, using the most common haplotype as the referent category [34]. Both univariate ANOVA and multivariate ANCOVA analyses adjusting for age, smoking, diabetes and hypertension were performed to determine the effects of the MTHFR polymorphisms on serum tHcy levels in healthy controls. A two tailed \( p \)-value of 0.05 was considered statistically significant.

5. Conclusions

In conclusion, the present study suggests \textit{MTHFR} rs1801133 and rs1801131 were associated with ischemic stroke risk and \textit{MTHFR} rs1801133 and rs9651118 may affect serum tHcy levels.

Author Contributions

Yi-Pin Zhou contributes to study conception and design; Bao-Sheng Zhou and Guo-Yun Bu contribute to data collection, biological experiments and drafting the article; Mu Li and Bin-Ge Chang contribute to biological experiments and data analysis.

Conflicts of Interest

The authors declare no conflict of interest.

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