Elevated Plasma Homocysteine Was Associated With Hemorrhagic and Ischemic Stroke, but Methylenetetrahydrofolate Reductase Gene C677T Polymorphism Was a Risk Factor for Thrombotic Stroke: A Multicenter Case-Control Study in China

Zhaohui Li, MD; Li Sun, MD; Hongye Zhang, MD; Yuhua Liao, MD; Daowen Wang, MD; Bingrang Zhao, MD; Zhiming Zhu, MD; Jizong Zhao, MD; Aiqun Ma, MD; Yu Han, BS; Yibo Wang, BS; Yi Shi, BS; Jue Ye, BS; Rutai Hui, MD, PhD

Background and Purpose—It is still controversial whether elevated plasma homocysteine and the C677T polymorphism of methylenetetrahydrofolate reductase (MTHFR) gene are risk factors for stroke. The aim of the present study was to investigate the association between the 2 factors and stroke in Chinese in a large case-control study.

Methods—We recruited 1823 stroke patients (807 cerebral thrombosis, 513 lacunar infarction, 503 intracerebral hemorrhage) and 1832 controls. Total plasma homocysteine was determined by high-performance liquid chromatography. C677T polymorphism was genotyped by polymerase chain reaction and Hinfl digestion.

Results—Total plasma homocysteine levels were significantly higher in cases than controls (median, 14.7 versus 12.8 μmol/L; P<0.001) and associated with an increased risk of 1.87-fold (95% confidence interval [CI], 1.58 to 2.22) for overall stroke, 1.72-fold (95% CI, 1.39 to 2.12) for cerebral thrombosis, 1.89-fold (95% CI, 1.50 to 2.40) for lacunar infarction, and 1.94-fold (95% CI, 1.48 to 2.55) for intracerebral hemorrhage. The C677T mutation of the MTHFR gene was positively correlated with plasma homocysteine levels in both controls (β=0.250, P<0.001) and cases (β=0.272, P<0.001) and more frequently in cases than in controls (47.0% versus 44.2%, P=0.017). The TT genotype was associated with an increased risk for overall stroke (odds ratio, 1.27; 95% CI, 1.04 to 1.56) and thrombotic stroke (odds ratio, 1.37; 95% CI, 1.06 to 1.78).

Conclusions—The C677T polymorphism of the MTHFR gene was associated with increased risk of cerebral thrombotic stroke in Chinese. Total plasma homocysteine was correlated with both ischemic and hemorrhagic stroke, suggesting potential initiation of homocysteine-lowering therapy in this population. (Stroke. 2003;34:2085-2090.)

Key Words: amine oxidoreductases homocyst(e)ine polymorphism risk factors stroke
biochemical variables between groups. Plasma tHcy of 16 μmol/L was taken as the cutoff value, which represented approximately the top 75th percentile of the distribution of controls, and plasma tHcy was defined as a categorical variable in the χ² test and logistic regression model. Differences between groups were examined by the χ² test or unpaired Student’s t test when appropriate. Relative risk (estimated as odds ratio [OR]) analysis was carried out with 2×2 cross-tabulation (crude OR) and a binary logistic regression model (adjusted OR) for the adjustment of age, sex, blood pressure, body mass index, cigarette smoking, glucose, TG, and log-transformed TG. Glomerular filtration rate (calculated with plasma creatinine according to the Cockcroft Gault formula) was also included in the association analysis between tHcy and stroke. The relation between genotype and log-transformed tHcy (dependent variable) was determined by multiple linear regression analysis to exclude the influence of age, sex, smoking, alcohol intake, and glomerular filtration rate. All statistics were performed with the SPSS 10.0 package. A value of P<0.05 was taken as significant (2 tailed).

Results

The characteristics of cases and controls are shown in Table 1. The mean ± SD age was 60.3 ± 9.4 years in cases and 59.6 ± 8.8 years in controls. Men accounted for 63.5% of cases and 57.4% of controls. As expected, cases had a higher prevalence of conventional risk factors for vascular disease, including aging, cigarette smoking, alcohol intake, higher blood pressure, glucose, and TG, whereas TC was lower in cases, especially in those with hemorrhage.

Compared with controls, plasma tHcy was significantly higher in cases (median, 14.7 versus 12.8 μmol/L; P<0.001) and the 3 subtypes of stroke (Table 2). Elevated plasma tHcy was associated with a crude OR of 2.09 (95% confidence interval [CI], 1.59 to 2.40) for overall stroke. After adjustment, the positive association was only weakly attenuated (OR, 1.87; 95% CI, 1.58 to 2.22). The adjusted ORs were 1.72 (95% CI, 1.39 to 2.12) for thrombosis, 1.89 (95% CI, 1.50 to 2.40) for lacunar, and 1.94 (95% CI, 1.48 to 2.55) for hemorrhage.

As seen in the Figure, the TT and CT genotypes were associated with higher plasma tHcy levels, and TT homozygote corresponded to the highest level in both controls and cases. Similar results were found in all subtypes of stroke (data not shown). After adjustment for age, sex, smoking status, alcohol intake, and glomerular filtration rate, the association remained. The standardized coefficient, β, was 0.250 (P<0.001) for controls and 0.272 (P<0.001) for overall stroke.

The frequency of MTHFR genotypes in cases and controls is shown in Table 3. Among 3655 individuals, 46.2% were

Biochemical Variables and tHcy Assay

Blood samples were collected after at least a 12-hour fast. The biochemical variables were measured with an automatic analyzer (Hitachi 7060, Hitachi). If hemolysis was identified, plasma was recollected. tHcy was analyzed with high-performance liquid chromatography (HP1100, Hewlett Packard) coupled to a fluorescence detector (HP1045, Hewlett Packard) according to the method of Durand et al.23 In cases of acute stroke or any other emergencies, blood samples were collected 6 weeks later.

Genotyping of MTHFR C677T Polymorphism

Genomic DNA was isolated from frozen packed white blood cells as described previously.24 A 198-bp fragment encompassing the region around nucleotide 677 was amplified by polymerase chain reaction (PCR), with the following primers: 5'–TGAAAGGAGAAGGGTCTGCGGGA–3' and 5'–AGGACGGTGCCGTGAAGAGTT–3'. The C677T mutation creates a HinfI restriction site, so digestion of the PCR product of the mutant allele by this enzyme generates 2 fragments (175 and 23 bp) that could be fractionated on 3% agarose gel electrophoresis. Genotyping results were confirmed by direct sequencing of the PCR products with a DNA sequencer (ABI prism 377, Perkin Elmer).

Statistical Analysis

Because the levels of plasma tHcy and TG were highly skewed, the Mann-Whitney U test was used to examine the differences in these variables between groups. Plasma tHcy of 16 μmol/L was taken as the cutoff value, which represented approximately the top 75th percentile of the distribution of controls, and plasma tHcy was defined as a categorical variable in the χ² test and logistic regression model. Differences between groups were examined by the χ² test or unpaired Student’s t test when appropriate. Relative risk (estimated as odds ratio [OR]) analysis was carried out with 2×2 cross-tabulation (crude OR) and a binary logistic regression model (adjusted OR) for the adjustment of age, sex, blood pressure, body mass index, cigarette smoking, glucose, TC, and log-transformed TG. Glomerular filtration rate (calculated with plasma creatinine according to the Cockcroft Gault formula23) was also included in the association analysis between tHcy and stroke. The relation between genotype and log-transformed tHcy (dependent variable) was determined by multiple linear regression analysis to exclude the influence of age, sex, smoking, alcohol intake, and glomerular filtration rate. All statistics were performed with the SPSS 10.0 package. A value of P<0.05 was taken as significant (2 tailed).

Subjects

This is a multicenter study for assessment of risk factors of stroke sponsored by the Ministry of Science and Technology of China (973 project). The study protocol was reviewed and approved by the review board of Ministry of Public Health, Ministry of Science and Technology of China and was approved by the ethics committees at all participating hospitals. Informed consent was obtained from all individuals.

Cases and controls were recruited simultaneously from the same geographic area through November 2000 to November 2001 from 7 clinical centers located in Yanzhou, Xi'an, Chongqing, Wuhan, Beijing, and Tianjin. We recruited only patients with 1 of 3 subtypes of stroke: cerebral thrombosis (thrombosis), lacunar infarction (lacunar), and intracerebral hemorrhage (hemorrhage). Other types of stroke (transient ischemic attack, subarachnoid hemorrhage, embolic brain infarction, brain tumors, and cerebrovascular malformation) and severe systemic diseases (collagenosis, endocrine, and metabolic disease [except for diabetes mellitus, DM]; inflammation, liver, neoplastic, or renal diseases) were in the range of exclusion. Diagnosis of stroke was based on the results of strict neurological examination—CT (89.8%), MRI (5%), or both (5.2%)

Diagnosis of stroke was based on the results of strict neurological examination—CT (89.8%), MRI (5%), or both (5.2%)—according to the International Classification of Diseases, ninth revision. Controls were selected from inpatients (21.5%) with minor illnesses from the departments of ophthalmology, gastroenterology, otorhinolaryngology, and orthopedics and from community-based inhabitants (78.5%) free of neurological diseases following the same exclusion criteria as cases. At each local community, both men and women 35 to 74 years of age in the range of selection were grouped by age (5-year range each group), and when 1 case was enrolled, 1 control was randomly selected from the corresponding group.

Initially, 2000 cases and 2000 controls were recruited. Before data assessment, 345 subjects (177 cases, 168 controls) were excluded at different experimental stages because of lack of a definite diagnosis (24 cases), absence of plasma (76 cases, 93 controls), and failure of genotyping (77 for cases, 75 controls). Among the 1823 cases, 807 were diagnosed as thrombosis; 513 as lacunar; and 503 as hemorrhage. In addition to neurological history and family history of hypertension and DM, the following vascular risk factors for each individual were also recorded: cigarette smoking, body mass index, systolic blood pressure, diastolic blood pressure, blood glucose, total plasma cholesterol (TC), and triglycerides (TG). Hypertension was defined as a mean of 3 independent measures of blood pressure ≥140/90 mm Hg or the use of antihypertensive drugs. DM was diagnosed when the subject had a fasting glucose ≥7.8 mmol/L, ≥11.1 mmol/L at 2 hours after oral glucose challenge, or both.

Results

The characteristics of cases and controls are shown in Table 1. The mean ± SD age was 60.3 ± 9.4 years in cases and 59.6 ± 8.8 years in controls. Men accounted for 63.5% of cases and 57.4% of controls. As expected, cases had a higher prevalence of conventional risk factors for vascular disease, including aging, cigarette smoking, alcohol intake, higher blood pressure, glucose, and TG, whereas TC was lower in cases, especially in those with hemorrhage.

Compared with controls, plasma tHcy was significantly higher in cases (median, 14.7 versus 12.8 μmol/L; P<0.001) and the 3 subtypes of stroke (Table 2). Elevated plasma tHcy was associated with a crude OR of 2.09 (95% confidence interval [CI], 1.82 to 2.40) for overall stroke. After adjustment, the positive association was only weakly attenuated (OR, 1.87; 95% CI, 1.58 to 2.22). The adjusted ORs were 1.72 (95% CI, 1.39 to 2.12) for thrombosis, 1.89 (95% CI, 1.50 to 2.40) for lacunar, and 1.94 (95% CI, 1.48 to 2.55) for hemorrhage.

As seen in the Figure, the TT and CT genotypes were associated with higher plasma tHcy levels, and TT homozygote corresponded to the highest level in both controls and cases. Similar results were found in all subtypes of stroke (data not shown). After adjustment for age, sex, smoking status, alcohol intake, and glomerular filtration rate, the association remained. The standardized coefficient, β, was 0.250 (P<0.001) for controls and 0.272 (P<0.001) for overall stroke.

The frequency of MTHFR genotypes in cases and controls is shown in Table 3. Among 3655 individuals, 46.2% were
CT heterozygotes, and 22.5% were TT homozygotes. Controls and cases demonstrated T-allele frequencies of 44.2% and 47% (P < 0.017), respectively. Compared with the wild type of CC, the TT genotype was associated with a 1.27-fold increased risk (95% CI, 1.04 to 1.56) for overall stroke. The positive association was only found in thrombosis (OR, 1.37; 95% CI, 1.06 to 1.78). When further stratified by sex, the association was identified only in men (OR, 1.45; 95% CI, 1.04 to 2.02) (Table 4).

Discussion

The principal findings of this study were that (1) elevated plasma tHcy was associated with not only ischemic but also hemorrhagic stroke, (2) the MTHFR C677T polymorphism contributed to higher plasma tHcy in both cases and controls, and (3) the TT genotype was associated with thrombotic stroke in Chinese.

Results from epidemiologic studies on the relation between homocysteine and stroke conflict greatly, which might be a result of different study designs or ethnic groups. Two recent meta-analyses, which support our findings, concluded that homocysteine was associated with stroke.10,11 One study indicated that the association was causal.11 To the best of our knowledge, the present study included almost the largest number of cases and demonstrated a positive association between elevated plasma tHcy and hemorrhagic stroke. It is

<table>
<thead>
<tr>
<th>Groups</th>
<th>Subjects (n)</th>
<th>Median (Range)</th>
<th>Elevated (≥16), %</th>
<th>Crude OR (95% CI)</th>
<th>Adjusted OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1832</td>
<td>12.8 (123.2)</td>
<td>25.7</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Case</td>
<td>1823</td>
<td>14.7 (207.8)§</td>
<td>41.9</td>
<td>2.09 (1.82–2.40)§</td>
<td>1.87 (1.58–2.22)§</td>
</tr>
<tr>
<td>Thrombosis</td>
<td>807</td>
<td>14.7 (207.8)§</td>
<td>42.3</td>
<td>2.12 (1.78–2.53)§</td>
<td>1.72 (1.39–2.12)§</td>
</tr>
<tr>
<td>Lacunar</td>
<td>513</td>
<td>14.8 (115.4)§</td>
<td>41.7</td>
<td>2.07 (1.69–2.55)§</td>
<td>1.89 (1.50–2.40)§</td>
</tr>
<tr>
<td>Hemorrhage</td>
<td>503</td>
<td>14.6 (124.6)§</td>
<td>41.6</td>
<td>2.06 (1.68–2.53)§</td>
<td>1.94 (1.48–2.55)§</td>
</tr>
</tbody>
</table>

*Mann-Whitney U test.
‡2×2 cross-tabulation.
§Multivariate logistic regression analysis adjusted for age, sex, blood pressure, body mass index, cigarette smoking, glucose, TC, TG, and glomerular filtration rate.
\§P<0.001 vs control.
generally accepted that elevated tHcy has procoagulative effects and induces endothelial damage, which may lead to thrombotic vascular disease. The mechanism of high tHcy underlying hemorrhagic stroke has not yet been fully elucidated. Recently, Hofmann et al.26 showed that induction of high homocysteine in apolipoprotein E–null mice enhanced the expression and activity of key participants in vascular inflammation, atherogenesis, hypercoagulation status, and vulnerability of established atherosclerotic plaque. The level and activity of tissue destructive enzymes such as MMP-9 have been shown to be increased in mice with hyperhomocysteinemia. The enzymes, present in atherosclerotic plaque, might promote lesion instability and rupture.27,28 Thus, higher homocysteine could cause either ischemic stroke through its hypercoagulative effect or hemorrhagic stroke by promoting plaque rupture. These findings, together with the present observations, raise the possibility that suppression of high plasma tHcy level in vivo could stabilize atherosclerotic plaque and prevent it from rupture.

We confirmed previous findings that the MTHFR C677T polymorphism was associated with plasma tHcy. It has been indicated that the influence of this polymorphism on plasma homocysteine is more apparent in subjects with lower folate.18,29,30 Unfortunately, plasma vitamin B_{12} and folate levels were not determined, which might be potential limitations of this study.

The MTHFR C677T polymorphism was associated with overall stroke independently of the well-documented vascular risk factors in Chinese. This is consistent with studies conducted in Japanese15 and Italians.16 Frosst et al.31 first suggested that the C677T polymorphism in the MTHFR gene was a candidate risk factor for vascular disease. However, subsequent results are controversial. Most studies, including a meta-analysis, failed to confirm this association.18–22 Several reasons might account for the conflicts among these studies. First, the frequencies of genotypes and alleles may differ among ethnic groups. In this study, the TT homozygote was much more frequent than reported in European and North American whites (3.9% to 17%, and 30% in only 1 group) in a meta-analysis on coronary heart disease.32 Second, the sample size in most previous studies is small, and it is hard to escape selection bias. As suggested by Cardon and Bell,33 to generate robust data in genetic association studies, especially for further analysis in individual subgroups, a much larger sample involving >1000 individuals for each group might be required. Thus, the number of cases in our study was large enough to permit detailed cause-specific analysis, which might strengthen the results. Third, the environmental factors that contribute to stroke are difficult to control. Particularly, the effects of unrecognized risk factors are easily ignored. Last is the methodologic limitation. In case-control studies, including the present one, cases are limited to survivors of the targeted disease, which might confound the real role of candidate modifiers.

After stratification, the association between the C677T polymorphism and stroke was different among stroke subtypes and sexes. Such a discrepancy might be due to the complexity of the disease and modified tHcy metabolism by folate and other factors suggested by Klerk et al.32 Additionally, effects of the statistical process could not be ignored. Valid stratification can diminish the effects of confounding factors. However, at the same time, sample size is reduced, which makes the boundary effect more difficult to be detected. In this case, the results should be interpreted cautiously. It is not surprising that the TT genotype was only

<table>
<thead>
<tr>
<th>Groups</th>
<th>Subjects (n)</th>
<th>T, %</th>
<th>P*</th>
<th>CC, %</th>
<th>CT, %</th>
<th>TT, %</th>
<th>P*</th>
<th>OR (95% CI)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1832</td>
<td></td>
<td></td>
<td>33.3</td>
<td>45.0</td>
<td>21.7</td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>Case</td>
<td>1823</td>
<td></td>
<td></td>
<td>29.3</td>
<td>47.5</td>
<td>23.2</td>
<td>0.033</td>
<td>1.27 (1.04–1.56)</td>
</tr>
<tr>
<td>Thrombosis</td>
<td>807</td>
<td>47.8</td>
<td>0.014</td>
<td>28.9</td>
<td>46.6</td>
<td>24.5</td>
<td>0.056</td>
<td>1.37 (1.06–1.78)</td>
</tr>
<tr>
<td>Lacunar</td>
<td>513</td>
<td>45.7</td>
<td>0.385</td>
<td>30.4</td>
<td>47.8</td>
<td>21.8</td>
<td>0.432</td>
<td>1.17 (0.87–1.57)</td>
</tr>
<tr>
<td>Hemorrhage</td>
<td>503</td>
<td>45.0</td>
<td>0.137</td>
<td>28.8</td>
<td>48.7</td>
<td>22.5</td>
<td>0.156</td>
<td>1.27 (0.91–1.79)</td>
</tr>
</tbody>
</table>

*χ² test versus control.
†Multivariate logistic regression controlling for age, sex, blood pressure, body mass index, cigarette smoking, glucose, TC, and TG.
weakly associated with stroke, considering the multifactorial backgrounds of the pathogenesis of the disease. Because stroke is quite a common disorder in China, the population-attributable risk may be high even for genetic variants conferring a modestly increased relative risk. Although the relative risk was small, its influence can still be considerably important. On the other hand, the penetrance of a gene may be dependent on certain permissive backgrounds on an individual level. The development of a complex disease may need the epistatic interactions of >1 predisposing gene. Examination of other permissive genetic modifiers might be useful in identifying the subpopulation susceptible to stroke.

In conclusion, the present study has shown that elevated plasma tHcy was associated with both ischemic and hemorrhagic stroke and that the MTHFR C677T polymorphism might be a genetic modifier for stroke in Chinese. The TT genotype was associated with an increased risk for thrombotic stroke. The present results might serve as an additional incentive for the initiation of an intervention trial with homocysteine-lowering therapy.

Acknowledgments

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References


### TABLE 4. Frequency of MTHFR C677T Genotype and T Allele and Relative Risk of Stroke for MTHFR 677 TT versus CC Genotype in Men (Women)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Subjects, n</th>
<th>T, %</th>
<th>P</th>
<th>CC, %</th>
<th>CT, %</th>
<th>TT, %</th>
<th>P</th>
<th>OR†</th>
<th>95% CI†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1052 (780)</td>
<td>43.9 (44.6)</td>
<td>33.6 (32.6)</td>
<td>44.5 (45.8)</td>
<td>21.7 (21.7)</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case</td>
<td>1157 (666)</td>
<td>47.3 (46.4)</td>
<td>0.025 (0.321)</td>
<td>28.5 (30.6)</td>
<td>48.4 (45.9)</td>
<td>23.1 (23.4)</td>
<td>0.026 (0.631)</td>
<td>1.30 (1.24)</td>
<td>1.00–1.69 (0.88–1.74)</td>
</tr>
<tr>
<td>Thrombosis</td>
<td>513 (294)</td>
<td>48.7 (46.4)</td>
<td>0.013 (0.436)</td>
<td>27.1 (32.0)</td>
<td>48.5 (43.2)</td>
<td>24.4 (24.8)</td>
<td>0.026 (0.526)</td>
<td>1.45 (1.25)</td>
<td>1.04–2.02 (0.81–1.93)</td>
</tr>
<tr>
<td>Lacunar</td>
<td>326 (187)</td>
<td>46.0 (44.9)</td>
<td>0.343 (0.948)</td>
<td>29.7 (31.6)</td>
<td>48.2 (47.1)</td>
<td>22.1 (21.4)</td>
<td>0.364 (0.948)</td>
<td>1.16 (1.27)</td>
<td>0.80–1.70 (1.77–2.08)</td>
</tr>
<tr>
<td>Hemorrhage</td>
<td>318 (185)</td>
<td>46.2 (47.8)</td>
<td>0.304 (0.253)</td>
<td>29.6 (27.6)</td>
<td>48.4 (49.2)</td>
<td>22.0 (23.2)</td>
<td>0.331 (0.422)</td>
<td>1.29 (1.27)</td>
<td>0.84–1.98 (0.72–2.25)</td>
</tr>
</tbody>
</table>

* x² test versus control.
† Multivariate logistic regression controlling for age, blood pressure, body mass index, cigarette smoking, glucose, TC, and TG.


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