Methylenetetrahydrofolate Reductase Gene Polymorphism and Ischemic Stroke in Japanese


Abstract—Hyperhomocyst(e)inemia has been identified as an independent risk factor for atherosclerotic and thromboembolic diseases such as coronary artery disease, cerebral artery disease, and venous thrombosis. Recently, the alanine/valine (A/V) gene polymorphism of 5,10-methylenetetrahydrofolate reductase (MTHFR), one of the key enzymes that catalyzes the remethylation of homocysteine, was reported. The VV genotype is correlated with increased plasma homocyst(e)ine levels as a result of the reduced activity and increased thermolability of this enzyme. In this study, we examined the association between the V allele of the MTHFR gene and ischemic stroke in an elderly Japanese population. The diagnosis of cerebral infarction of all study patients was confirmed by CT of the brain. The MTHFR genotype was analyzed by polymerase chain reaction followed by HindI digestion. In 256 stroke patients and 325 control subjects, the frequencies of the V allele were 0.45 and 0.32, respectively. The odds ratios for ischemic stroke were, respectively, 1.51 (1.02 to 2.23) for the AV genotype and 3.35 (1.94 to 5.77) for the VV genotype compared with the AA genotype. Both of these effects were statistically significant (P=0.041 and P<0.001, respectively). In patients with multiple infarcts in particular, the allele frequency of the V mutation was 0.56, and the association between the V allele and stroke was highly significant. Plasma homocyst(e)ine levels were significantly higher in patients with the VV genotype than in patients with the AA or AV genotype, especially those with low plasma folate levels. The V allele of the MTHFR gene was significantly associated with cerebral infarction in an elderly Japanese population in a codominant manner. The VV genotype may contribute to risk for ischemic stroke through a predisposition to increased plasma homocyst(e)ine levels, and dietary folate supplementation may be of benefit, particularly to patients with this genotype. (Arterioscler Thromb Vasc Biol. 1998;18:1465-1469.)

Key Words: genetics ■ homocysteine ■ methylenetetrahydrofolate reductase ■ stroke ■ risk factors

Stroke is a frequent cause of death in the elderly throughout the world. It is the sixth leading cause of death in the United States, and the leading cause of death in the elderly. It is the third most common cause of death in Japan, and the second most common cause of death in the elderly. In Japan, the incidence of stroke is higher than that of coronary artery disease in Western countries.1-3 Only a few determinants of stroke risk have been identified. Age, smoking, hypertension, and glucose intolerance have been established as independent risk factors for stroke.4-6 In Japan, ischemic stroke is much more prevalent than hemorrhagic stroke.1-3 In particular, hypertensive vascular lesions are thought to be responsible for the occurrence of lacunar infarction, which is the most prevalent type of ischemic stroke in the Japanese.3

Previous studies have shown that the plasma homocyst(e)ine level is associated with the risk for atherosclerosis and thrombotic diseases.7-14 Homocystinuria, a rare autosomal recessive disease due to cystathionine β-synthase deficiency, is characterized by markedly elevated plasma homocyst(e)ine concentrations. Typical clinical manifestations of homocystinuria include premature atherosclerosis and thromboembolism, as well as ocular, skeletal, and neurogenic complications.15 Milder homocyst(e)inemia has also been shown to increase the risk of cerebrovascular disease.16-18 Recently, a common mutation of 5,10-methylenetetrahydrofolate reductase (MTHFR), 1 of the key enzymes that catalyzes the remethylation of homocysteine, was reported to be associated with decreased enzyme activity and increased plasma homocyst(e)ine levels.19 Subsequent studies, including ours, suggest that this polymorphism is a potential coronary risk factor, although its status as such is still controversial.20-25 It is also controversial whether the MTHFR A/V polymorphism is associated with ischemic stroke.26,27 In this study, we examined whether this polymorphism is a genetic risk factor for ischemic stroke in an elderly Japanese population.

Methods

Study Population
The study population comprised 256 patients (123 males and 133 females) with clinically overt stroke and 325 control subjects (174...
TABLE 1. Baseline Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Patients (n=256)</th>
<th>Control Subjects (n=325)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of men, %</td>
<td>48</td>
<td>54</td>
</tr>
<tr>
<td>Age, y</td>
<td>70.3±8.6</td>
<td>67.7±7.5</td>
</tr>
<tr>
<td>Smokers, %</td>
<td>38*</td>
<td>24</td>
</tr>
<tr>
<td>TC, mg/dL</td>
<td>213.7±35.4</td>
<td>213.5±38.6</td>
</tr>
<tr>
<td>HDL-C, mg/dL</td>
<td>56.9±16.5</td>
<td>61.5±17.3</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>60*</td>
<td>31</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>21</td>
<td>18</td>
</tr>
<tr>
<td>Daily alcohol consumption, %</td>
<td>26</td>
<td>29</td>
</tr>
</tbody>
</table>

TC indicates total cholesterol; HDL-C, HDL cholesterol.

*Significantly different from control.

males and 151 females). All of the subjects were of Japanese ancestry, and none were first- or second-degree relatives. Two hundred ninety-seven patients with ischemic stroke were enrolled at the Kitamura Neurosurgery Clinic from September 1996 to May 1997. All patients were enrolled >2 months after the onset of stroke. The diagnosis of ischemic stroke was made when neurological deficits were accompanied by corresponding abnormal CT findings of the brain. Neurological and CT findings were interpreted by 2 or more independent experienced neurologists. Patients with cerebral hemorrhage were excluded in advance. The classification of stroke was based on the criteria proposed by the National Institute of Neurological Disorders and Stroke Ad Hoc Committee.24 Forty-one patients were excluded from this study because of renal dysfunction (6 cases), valvular heart disease (8), recent myocardial infarction (4), atrial fibrillation (24; 6 of 24 also had valvular heart disease; 4 of 24 also had a history of major cardiac surgery), complete atrioventricular block (5), or a history of major cardiac surgery (6). After exclusion of these cases, we enrolled 256 patients (age of onset, 46 to 91 years; mean±SD, 70.3±8.6 years) in this study. Volunteers without a clinical history of cerebrovascular disease or present neurological abnormalities (range, 46 to 89 years old; mean±SD, 67.7±7.5 years) were recruited as control subjects at the time of their annual health examination at the Institute for Adult Diseases Asahi Life Foundation and the Nikon Clinic, which are in the same area of the megalopolis as the Kitamura Neurosurgery Clinic. The exclusion criteria were the same as those in the patient group mentioned above. Volunteers were diagnosed as having hypertension or diabetes mellitus at enrollment when the World Health Organization diagnostic criteria for each disease were fulfilled. Relevant information on past medical history, current smoking habits, and alcohol consumption was obtained from all of the study subjects. Fasting venous blood samples were drawn for estimation of biochemical measurements at the time of enrollment. All of the female patients and control subjects were postmenopausal. Informed consent was obtained from every subject after a full explanation of the study, which was approved by the Ethics Committee of the University of Tokyo.

Genetic Analysis

Venous blood samples were collected in tubes containing disodium EDTA and applied to genomic DNA extracting columns (QIAamp blood kit, Qiagen) according to the manufacturer’s protocol. Polymerase chain reaction (PCR) was performed on the genomic DNA samples with a GeneAmp PCR kit (Perkin-Elmer Cetus) and primers as previously reported.25 The amplified fragments were cut with Hinfl, which can recognize the C→T substitution in the fragments. Because this 1 nucleotide substitution corresponds to the conversion of the Ala to Val residue in the MTHFR encoding region, the 2 different alleles were designated A (Ala) and V (Val). The 198-bp fragment derived from the A allele is not digested by Hindl, whereas the fragment of the same length from the V allele is digested by Hindl into 175- and 23-bp fragments. The Hindl-treated PCR fragments were electrophoresed in 9.6% polyacrylamide gels and stained with ethidium bromide.

Measurement of Plasma Levels of Homocyst(e)ine and Folate

In the randomly selected subgroup of 141 patients with cerebral infarction, we measured the plasma levels of homocyst(e)ine and folate. Fasting venous blood samples were collected in tubes containing disodium EDTA. Samples were promptly centrifuged after collection and stored at –20°C. Plasma homocyst(e)ine levels were determined as total homocysteine by high-performance liquid chromatography with fluorescence detection as previously described.26 Plasma folate levels were measured by use of commercially available radioimmunoassay kits.

Statistical Analysis

Means (±SDs) and proportions for baseline risk factors were computed for patients and control subjects. Allele and genotype frequencies among the patients and control subjects were compared by the χ² test with Hardy-Weinberg predictions. Statistical analysis was carried out without adjustment and after adjustment for parameters that may contribute to the risk for ischemic stroke—age, sex, hypertension, diabetes, and smoking—by a multiple logistic regression model. Relative risks of stroke (estimated as the odds ratios [ORs]) for the AV and VV genotypes compared with the AA genotype were calculated and are presented with their corresponding 95% confidence intervals (CIs). Plasma homocyst(e)ine and folate levels were analyzed by univariate analysis with the Mann-Whitney rank-sum test, and multiple linear regression analysis was used to examine the determinants of plasma homocyst(e)ine levels. A 2-tailed value of P<0.05 was considered significant. Statistical analysis was done with SAS software (Statistical Analysis System).27

Results

Distribution of the MTHFR Genotype in Control Subjects and Stroke Patients

The baseline characteristics of the patients (n=256) and control subjects (n=325) are shown in Table 1. The patients had a significantly higher prevalence of hypertension and smoking, which are well known as major risk factors for stroke, than did the control subjects.

In these subjects, the 3 MTHFR genotypes for the Ala→Val mutation (ie, AA, AV, and VV) were diagnosed by digestion of the 198-bp PCR products by Hindl, as described

TABLE 2. Distribution of MTHFR Genotypes in Control Subjects and Stroke Patients and the Relative Risk of Stroke Associated With MTHFR Genotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Patients (n=256)</th>
<th>Control Subjects (n=325)</th>
<th>Adjusted OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>153 (47)</td>
<td>80 (31)</td>
<td>1</td>
</tr>
<tr>
<td>AV</td>
<td>139 (43)</td>
<td>121 (47)</td>
<td>1.67 (1.16–2.40)</td>
</tr>
<tr>
<td>VV</td>
<td>33 (10)</td>
<td>55 (21)</td>
<td>3.19 (1.92–5.30)</td>
</tr>
<tr>
<td>A/V Frequencies</td>
<td>0.68/0.32</td>
<td>0.55/0.45</td>
<td></td>
</tr>
</tbody>
</table>

*Adjusted for age, sex, hypertension, diabetes, and smoking.
TABLE 3. Association Between MTHFR Genotypes and Number of Stroke Lesions on CT Findings

<table>
<thead>
<tr>
<th></th>
<th>Control Subjects (n=325)</th>
<th>All Patients (n=256)</th>
<th>With a Single Lesion on CT Findings (n=182)</th>
<th>With 2 or More Lesions on CT Findings (n=74)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA, %</td>
<td>47</td>
<td>31</td>
<td>35</td>
<td>22</td>
</tr>
<tr>
<td>AV, %</td>
<td>43</td>
<td>47</td>
<td>48</td>
<td>45</td>
</tr>
<tr>
<td>VV, %</td>
<td>10</td>
<td>21</td>
<td>16</td>
<td>34</td>
</tr>
<tr>
<td>OR (95% CI), VV vs AA</td>
<td>1.0</td>
<td>3.19 (1.92–5.30)</td>
<td>2.17 (1.22–3.86)</td>
<td>7.24 (3.49–15.1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.35 (1.94–5.77)*</td>
<td>2.10 (1.14–3.87)*</td>
<td>8.88 (3.97–19.9)*</td>
</tr>
<tr>
<td>OR (95% CI), VV vs AA</td>
<td>1.0</td>
<td>3.33 (1.56–7.15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.87 (1.74–8.60)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AV/V frequencies</td>
<td>0.68/0.32</td>
<td>0.55/0.45</td>
<td>0.59/0.41</td>
<td>0.44/0.56</td>
</tr>
</tbody>
</table>

*Adjusted for age, sex, hypertension, diabetes, and smoking.

in a previous report by Frosst et al. The distribution of the MTHFR genotypes in both the control and patient groups is shown in Table 2. The allele frequency of the V mutation was significantly higher in the stroke group than in the control group (0.45 versus 0.32, P<0.001). Genotype frequencies were 47.1% for AA, 42.8% for AV, and 10.2% for VV in the control subjects and 31.3% for AA, 47.3% for AV, and 21.5% for VV in the patients. The genotype distributions of both groups were compatible with Hardy-Weinberg equilibrium. The crude ORs and 95% CIs were 1.51 (1.02 to 2.23) for AA, 4.47 (1.92 to 5.30) for AV, and 2.10 (1.14 to 3.87) and 8.88 (3.97 to 19.9), respectively. In this analysis, we also found that age, hypertension, and smoking were other determinants of the risk for stroke.

To evaluate the effects of confounding factors, we calculated the OR according to the MTHFR genotype adjusted for age, sex, hypertension, diabetes, and smoking on the basis of the clinical characteristics of the 3 genotype subgroups of the 256 patients and 325 control subjects. The adjusted ORs and 95% CIs were 1.51 (1.02 to 2.23) for AV and 3.35 (1.94 to 5.77) for VV (Table 2). Both of these effects were statistically significant (P=0.006 and P<0.001, respectively), suggesting a codominant effect of the V allele on the risk for stroke.

MTHFR Genotype and Multiple Infarcts

The association of this mutation with ischemic stroke was further studied in terms of the radiological findings of the lesions. In stroke patients with CT-proven multiple (2 or more) infarcts, the V allele frequency was 0.56 (Table 3). The adjusted ORs (adjusted for risk factors of age, sex, hypertension, diabetes, and smoking) and 95% CIs for single- and multiple-infarct groups with respect to the control group for comparison of the VV genotype with the AA genotype were 2.10 (1.14 to 3.87) and 8.88 (3.97 to 19.9), respectively. The association between the VV genotype and ischemic stroke was significantly higher in the patients with single lesions as well as those with multiple lesions on CT findings than in control subjects (P=0.018 and P<0.001, respectively). Moreover, the adjusted OR and 95% CI for comparing the VV with the AA genotype for the multiple-infarct group with respect to the single-infarct group was 3.87 (1.74 to 8.60). The association between the VV genotype and ischemic stroke was significantly higher in the multiple-infarct group than in the single-infarct group (P<0.001).

MTHFR Genotype and Plasma Homocyst(e)ine Levels

The plasma levels of homocyst(e)ine and folate were determined in 141 of the 256 patients with cerebral infarction (Table 4). The plasma homocyst(e)ine levels were 10.3±3.7 for AA, 11.5±3.7 for AV, and 14.1±5.3 μmol/L for VV. The homocyst(e)ine levels were significantly higher in patients with the VV genotype than in those with the AA or AV genotype. (P=0.004 and P=0.044, respectively) Folate levels in patients with the VV genotype tended to be lower than in those with the AA or AV genotype (4.3±2.1 for AA, 3.8±1.2 for AV, and 3.5±0.9 ng/mL for VV), but these differences were not statistically significant.

We further examined the effects of folate on the relation between genotypes and homocyst(e)ine levels. Plasma homocyst(e)ine levels were significantly higher in patients with the VV genotype than in those with the AA or AV genotype when patients with folate levels <4.0 were selected; in contrast, in those with folate levels ≥4.0, homocyst(e)ine levels were similar regardless of MTHFR genotype (Table 4).

A multiple regression model was used to estimate the independent effects of this gene polymorphism and several

TABLE 4. Comparison of Plasma Homocyst(e)ine and Folate Levels Among MTHFR Genotypes

<table>
<thead>
<tr>
<th>MTHFR Genotype</th>
<th>Plasma Homocyst(e)ine, μmol/L</th>
<th>Folate &lt;4</th>
<th>Folate ≥4</th>
<th>Folate, ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA (n=41)</td>
<td>10.3±3.7</td>
<td>11.3±4.0</td>
<td>8.9±2.7</td>
<td>4.3±2.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n=24)</td>
<td>(n=17)</td>
<td></td>
</tr>
<tr>
<td>AV (n=75)</td>
<td>11.5±3.7</td>
<td>12.2±3.8</td>
<td>10.4±3.4</td>
<td>3.8±1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n=48)</td>
<td>(n=27)</td>
<td></td>
</tr>
<tr>
<td>VV (n=25)</td>
<td>14.1±5.3†</td>
<td>15.1±5.3†</td>
<td>9.9±2.6</td>
<td>3.5±0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n=20)</td>
<td>(n=5)</td>
<td></td>
</tr>
</tbody>
</table>

*P<0.01, AA vs VV.
†P<0.05, AV vs VV.
‡P<0.03, AA vs VV.
other factors on plasma homocyst(e)ine levels. As shown in Table 5, age, sex, plasma folate levels, and the MTHFR VV genotype were independent factors significantly associated with plasma homocyst(e)ine levels. In contrast, smoking and alcohol consumption were not significantly associated with plasma homocyst(e)ine levels in the current study.

### Discussion

The current study showed the association between the A/V polymorphism of the MTHFR gene and cerebral infarction in a codominant manner. A multivariate analysis demonstrated that this association was independent of other factors possibly related to stroke risk, such as age, sex, hypertension, diabetes, and smoking. In addition, this genetic effect on stroke was more predominant among patients with CT-proven multiple infarcts than in those with a CT-proven single infarct.

Many previous reports have shown that hyperhomocyst(e)inemia is closely associated with the occurrence of stroke. For example, Perry et al. measured serum total homocyst(e)ine levels in middle-aged British men and identified hyperhomocyst(e)inemia as a strong and independent risk factor for stroke with a graded response. Brattstrom et al. and Coull et al. reported that moderate hyperhomocyst(e)inemia was an independent risk factor for stroke and was independent of the type of stroke. However, it has been uncertain whether common ischemic stroke is affected by a genetic mutation(s) causing a mild elevation of plasma homocyst(e)ine levels, although hereditary homocystinuria due to cystathionine β-synthase deficiency has been known to cause early onset of stroke. Here we clearly demonstrated that the V allele of the MTHFR gene is associated with a high risk for common ischemic stroke. Recently, we reported that the V allele of the MTHFR gene is significantly associated with coronary heart disease. Taken together, these findings indicate that this mutation can be regarded as a genetic risk factor for systemic atherosclerosis and thrombosis.

In this study, we observed higher plasma homocyst(e)ine levels in patients with the VV genotype than in those with the AA or AV genotype, mainly among subjects with low folate levels, which is consistent with findings in some other studies. This result suggests that patients with the VV genotype may be more susceptible to the hyperhomocysteinemic effect of poor folate levels. After adjustment was made for the other factors, we found that patients with the VV genotype had homocyst(e)ine levels that were 2.7 µmol/L higher than patients with the AA genotype. Plasma folate levels, age, and sex also remained key determinants in the current model. The independent effect of plasma folate levels on plasma homocyst(e)ine levels supports the evidence that the effect of the VV genotype might be compensated for by increased folate intake. Concordantly, a structural homology between MTHFR and dihydrofolate reductase in a putative folate-binding domain indicates that folate may stabilize MTHFR to increase its enzymatic activity. Although the effect of the MTHFR A/V polymorphism on plasma homocyst(e)ine levels was recessive, its effect on risk for stroke was codominant in the current study. This discrepancy cannot be clearly explained. Further studies are needed to determine whether heterozygosity for the V allele could affect homocysteine metabolism.

Smoking has been regarded as a possible determinant of plasma homocyst(e)ine levels. However, the influence of smoking on plasma homocyst(e)ine levels was not significant in our current study. Although the cause of this inconsistency remains unknown, differences in the characteristics of the study population, such as ethnicity, may account for it.

While we were preparing this manuscript, Markus et al. demonstrated no association between this polymorphism and cerebrovascular disease in a population of the United Kingdom. The discrepancy between their result and ours may be due to ethnic or environmental differences. The other possible explanation is the methodological limitation of the current study. First, this study was limited to survivors of ischemic stroke. Second, the diagnosis of ischemic stroke was made on the basis of abnormal findings on the brain CT, which is a more prevalent and convenient but less sensitive method than is magnetic resonance imaging for diagnosis of stroke. A bias due to these limitations may influence the result of such a genetic association study in stroke.

Recently, vitamin supplements have received attention as a therapeutic strategy for vascular diseases and, indeed, the homocysteine-lowering effects of folate and vitamins B₆ and B₁₂ have been extensively studied. At present, the beneficial effects of lowering plasma homocyst(e)ine by vitamins on the risk of vascular diseases have not yet been established. Identification of the V allele of the MTHFR gene may give insight into its mechanism and provide a genetic marker to permit early therapeutic intervention in subjects at high risk for ischemic stroke.

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References
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