Homozygous C677T mutation in the MTHFR gene as an independent risk factor for multiple small-artery occlusions


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Abstract

Introduction: Hyperhomocysteinemia is an independent risk factor for cerebrovascular disease and the homozygous C677T mutation in the methylenetetrahydrofolate reductase (MTHFR) gene can induce hyperhomocysteinemia. However, the association between this 677TT genotype and ischemic stroke still remains controversial. Therefore, we carried out this study to determine whether the MTHFR TT genotype is associated with certain subtypes of ischemic stroke.

Materials and methods: We enrolled 195 ischemic stroke patients and 198 healthy individuals and checked their fasting plasma homocysteine levels and analyzed the C677T polymorphism in the MTHFR gene.

Results: Our findings concur with previous reports that stroke occurrence is associated with hyperhomocysteinemia, but not with the 677TT genotype. However, when we re-analyzed the data based on a subtype classification, the adjusted odds ratio (AOR) and 95% confidence intervals (CI) of the 677TT genotype were found to be significantly higher in patients with small-artery occlusion than that in controls (AOR, 2.92; 95% CI, 1.01–8.48). Moreover, the AOR of the 677TT genotype was found to be much bigger in patients with multiple small-artery occlusions (AOR, 6.90; 95% CI, 1.70–27.99), but not in those with single small-artery occlusion (AOR, 1.19; 95% CI, 0.27–5.35).

Conclusions: The homozygous C677T mutation in the MTHFR gene is associated with multiple small-artery occlusions, but not with single small-artery occlusion. Our findings suggest a genetic basis for certain subtypes of ischemic stroke.

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Keywords: MTHFR; Hyperhomocysteinemia; Small-artery occlusion; Multiple small-artery occlusions

1. Introduction

Epidemiological evidence indicates that moderately elevated plasma homocysteine levels constitute an important risk factor in ischemic stroke [1–4]. The results of prior studies show a strong association between the homozygous C677T mutation in the methylenetetrahydrofolate reductase (MTHFR) gene and hyperhomocysteinemia [5,6]. However, most of these studies [7,8] and meta-analyses [9,10] on the MTHFR gene have failed to confirm an association between the MTHFR 677TT genotype and ischemic stroke. A possible explanation of this may be because the 677TT genotype is only associated with certain etiological subtypes of ischemic stroke. Regarding subtypes, the association between elevated homocysteine levels and the subtypes of ischemic stroke remains controversial [11–15]. Faßbender et al. [11] found that hyperhomocysteinemia was associated with cerebral microangiopathy, but not with the macroangiopathic mechanism, however, Eikelboom et al. [15] reported that hyperhomocysteinemia was associated with large-artery atherosclerosis. In addition, no association was found between the 677TT genotype and the stroke subtypes [16,17]. However, up until now, no study has classified patients with small-artery occlusion into those with single small-artery occlusion and those with multiple small-artery occlusions, the latter of which may be related to different types of vascular dementia [18–21]. Moreover, if the pathogenesis of plasma homocysteine is associated with cerebral vessel diameter, the 677TT genotype should be
associated as well. Therefore, under the presumption that the MTHFR TT genotype and hyperhomocysteinemia are closely related to vessel diameter, our study was aimed to determine whether the 677TT genotype is associated with a subtype of ischemic stroke.

2. Materials and methods

2.1. Study population

The study population was enrolled from November 2000 to May 2002 in our department at Pundang CHA General Hospital, Pochon CHA University, by consecutive referral. Ischemic stroke was defined as a stroke—a clinical syndrome characterized by rapidly developing clinical symptoms and signs of focal and at times global loss of brain function—with evidence of a cerebral infarction in the clinically relevant area of the brain by brain imaging study.

The diagnosis of ischemic stroke was made when neurological deficits were accompanied by corresponding abnormal magnetic resonance imaging (MRI) findings of the brain, which had been interpreted by two independent experienced neurologists. Ischemic stroke was excluded when researchers did not agree with each other, and also patients with cerebral hemorrhage were excluded in advance. Examinations were performed with a 1.5-T superconducting magnet system (Siemens Magnetom Symphony), and the whole brain was scanned with a slice thickness of 7 mm and a 2-mm interslice gap, producing 16 axial images. The imaging protocol consisted of T2-weighted spin echo (TR/TE = 3700/90 ms), T1-weighted spin echo (TR/TE = 560/14 ms), Proton (TR/TE = 3700/22 ms), and FLAIR (TR/TE = 9000/105 ms, inversion time 2500 ms) images. All study subjects underwent brain MRI and electrocardiography, and MR angiography was performed about 72%. Transcranial Doppler and four-vessel conventional cerebral angiography were performed in approximately 88%. Echocardiography was performed about 72%. Transcranial Doppler and four-vessel conventional cerebral angiography were performed at the discretion of the responsible clinician.

On the basis of clinical manifestations and neuroimaging data, we classified all ischemic strokes into five etiologic subtypes according to the criteria used in the Trial of Org 10172 in Acute Stroke Treatment (TOAST) [22] as follows: (1) Large-artery atherosclerosis: an infarction lesion greater than 15 mm in diameter on MRI, or significant (>50%) stenosis of a major brain artery or a branch cortical artery on four-vessel conventional cerebral angiography with symptoms associated with that arterial territory. (2) Small-artery occlusion: an infarction lesion less than 15 mm and more than 5 mm in diameter on MRI, and traditional clinical lacunar syndrome without evidence of cerebral cortical dysfunction, and potential cardiac sources for embolism should be absent. (3) Cardioembolism: arterial occlusions presumably due to an embolus arising in the heart as detected by echocardiography. (4) Other determined etiology: rare causes of stroke, regardless of size or location. (5) Undetermined etiology: the cause of stroke could not be determined with any degree of confidence.

The association between the MTHFR C677T mutation and small-artery occlusion was further studied in terms of the radiological findings of the lesions. For patients with small-artery occlusion, single and multiple (two or more lesions) small-artery occlusions were distinguished by brain MRI scan. The size and site of cerebral infarction were documented only by MRI.

As control subjects, we selected healthy individuals matched for sex and age from those that presented at Pundang CHA hospital for the health examination, during the same period, who were free from a history of cerebrovascular disease or myocardial infarction. Baseline demographic data and a history of conventional vascular risk factors were obtained for each control subject. The other exclusion criteria were the same as those applied in ischemic stroke patients, as mentioned above.

Some members of both ischemic stroke patients and controls were diagnosed as having hypertension or diabetes mellitus when the diagnostic criteria were fulfilled at the time of enrollment. Relevant information on past medical history and smoking habits were obtained from all subjects. Informed consent was obtained from all study participants after receiving a full explanation of the study. The study populations were in Hardy–Weinberg equilibrium, and the institutional review board of Pundang CHA hospital approved this study in June 2000.

2.2. Laboratory analysis

All samples were collected and processed according to a standardized protocol. Fasting homocysteine levels were

### Table 1
Baseline characteristics and conventional risk factors in ischemic stroke patients and control subjects

<table>
<thead>
<tr>
<th></th>
<th>Ischemic patients (n = 195)</th>
<th>Control subjects (n = 198)</th>
<th>OR</th>
<th>95% CI</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean ± S.D.)</td>
<td>61.4 ± 10.9</td>
<td>60.8 ± 11.2</td>
<td>...</td>
<td>...</td>
<td>0.570</td>
</tr>
<tr>
<td>No. of male (ratio)</td>
<td>106 (54.4%)</td>
<td>111 (56.1%)</td>
<td>...</td>
<td>...</td>
<td>0.735</td>
</tr>
<tr>
<td>Hypertension</td>
<td>127 (65.1%)</td>
<td>78 (39.4%)</td>
<td>2.87</td>
<td>1.91–4.33</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>62 (31.8%)</td>
<td>44 (22.2%)</td>
<td>1.63</td>
<td>1.04–2.56</td>
<td>0.033</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>45 (23.1%)</td>
<td>39 (19.7%)</td>
<td>1.22</td>
<td>0.75–1.98</td>
<td>0.414</td>
</tr>
<tr>
<td>Smoking</td>
<td>57 (29.2%)</td>
<td>36 (18.2%)</td>
<td>1.72</td>
<td>1.07–2.78</td>
<td>0.025</td>
</tr>
</tbody>
</table>

* Chi-square test for the categorical data, and two-sample t test for the continuous data.
measured in all subjects (IMx, Abbott Laboratories). Fasting venous blood samples were collected in tubes containing trisodium EDTA. Samples were promptly centrifuged after collection and stored at \(-20^\circ \text{C}\). Plasma homocysteine levels were determined as total homocysteine by fluorescence polarization immunoassay (FPIA).

Venous blood samples were collected in tubes containing trisodium EDTA, and genomic DNA was obtained by column extraction (QIAamp blood kit, Qiagen), according to the manufacturer’s protocol. Polymerase chain reaction (PCR) was performed on the genomic DNA samples using a GeneAmp PCR kit (Perkin-Elmer Cetus) and the previously reported primers [23]. The amplified fragments were cleaved with \(Hin\text{II}\), which recognizes C–T substitution. This one nucleotide substitution corresponds to the conversion of the Ala residue to Val in the MTHFR encoding region. The 198-bp fragment containing the Ala allele is not digested by \(Hin\text{II}\), whereas the fragment containing the Val allele is digested by \(Hin\text{II}\) into 175- and 23-bp fragments. The \(Hin\text{II}\)-treated PCR fragments were electrophoresed in 3.0% agarose gels and stained with ethidium bromide.

### 2.3. Statistical analysis

Odds ratio (OR) and 95% confidence intervals (CI) were used to estimate the relative risk associated with subtypes of ischemic stroke and a particular MTHFR genotype. To analyze baseline characteristics, we used the chi-square test for the categorical data (sex, hypertension, diabetes mellitus, hypercholesterolemia, and smoking) and the two-sample \(t\) test for the continuous data (age and fasting plasma homocysteine concentrations) when comparing patient and control baseline data. For multivariate analysis, logistic regression was used to adjust the effects for possible confounders—i.e., age, sex, hypertension, diabetes mellitus, and smoking. SPSS for Windows, version 11.0 (SPSS, Chicago, IL, USA), was used to calculate the adjusted odds ratio (AOR) and 95% CI.

### 3. Results

The baseline characteristics of ischemic stroke patients (\(n = 195\)) and of controls (\(n = 198\)) are shown in Table 1. Stroke patients had a significantly higher prevalence of all conventional vascular risk factors with the exception of hypercholesterolemia than controls. Fasting homocysteine levels in plasma were significantly higher in ischemic stroke patients than that in controls. Moreover, plasma homocysteine levels were much more significant in patients with small-artery occlusion than that in controls. However, these were not significant for patients with single small-artery occlusion, but for those with multiple small-artery occlusions (Table 2).

To evaluate the pure effects of the MTHFR genotypes on ischemic stroke, we adjusted the odds ratio for age, sex, hypertension, diabetes mellitus, and smoking. The AOR of the 677TT genotype was compared to both normal (the 677CC genotype) and non-homozygous mutations including the 677CC and the 677CT (heterozygous) genotypes in both patients and controls. Identical results were obtained on comparing both normal and non-homozygous mutations with the 677TT genotype.

We found that the AOR of the 677TT genotype was not significantly higher in ischemic stroke patients than that in controls. However, the AOR of the 677TT genotype was significantly higher in patients with small-artery occlusion.

### Table 2

| Fasting plasma homocysteine concentrations in the subtypes of ischemic stroke |
|---------------------------------|-----------------|-----------------|
| Homocysteine (\(\mu\text{mol/l}\)) | \(p^*\)          |
| Control subjects (\(n = 198\))   | 8.99 ± 2.57     | ...             |
| Ischemic stroke patients (\(n = 195\))   | 10.38 ± 6.18   | 0.004           |
| Large-artery atherosclerosis (\(n = 55\)) | 10.19 ± 7.18   | 0.052           |
| Small-artery occlusion (\(n = 72\))   | 11.27 ± 6.00   | 0.003           |
| Cardioembolism (\(n = 41\))   | 9.88 ± 6.29    | 0.887           |
| Other determined etiology (\(n = 6\))   | 10.15 ± 3.39   | 0.444           |
| Undetermined etiology (\(n = 21\))   | 8.86 ± 4.06    | 0.882           |
| Single small-artery occlusion (\(n = 32\))   | 9.59 ± 4.87    | 0.504           |
| Multiple small-artery occlusions (\(n = 40\)) | 12.61 ± 6.52   | 0.001           |

Based on brain MRI findings, small-artery occlusion was divided into two groups: single small-artery occlusion and multiple small-artery occlusions.

* Two-sample \(t\) test was used for continuous data.

### Table 3

<table>
<thead>
<tr>
<th>MTHFR</th>
<th>Controls ((n = 198))</th>
<th>Total patients ((n = 195))</th>
<th>Large-artery* ((n = 55))</th>
<th>Small-artery** ((n = 72))</th>
<th>Cardioembolism ((n = 41))</th>
<th>Other etiology ((n = 6))</th>
<th>Unknown ((n = 21))</th>
</tr>
</thead>
<tbody>
<tr>
<td>677 CC</td>
<td>73 (36.9)</td>
<td>62 (31.8)</td>
<td>20 (36.4)</td>
<td>20 (27.8)</td>
<td>12 (29.3)</td>
<td>2 (33.3)</td>
<td>8 (38.1)</td>
</tr>
<tr>
<td>677 CT</td>
<td>100 (50.5)</td>
<td>97 (49.7)</td>
<td>30 (54.5)</td>
<td>32 (44.4)</td>
<td>22 (53.6)</td>
<td>3 (50.0)</td>
<td>10 (47.6)</td>
</tr>
<tr>
<td>677 TT</td>
<td>25 (12.6)</td>
<td>36 (18.5)</td>
<td>5 (9.1)</td>
<td>20 (27.8)</td>
<td>7 (17.1)</td>
<td>1 (16.7)</td>
<td>3 (14.3)</td>
</tr>
<tr>
<td>AOR (CI)**</td>
<td>1.00</td>
<td>1.06 (0.49–2.29)</td>
<td>1.29 (0.31–5.34)</td>
<td>2.92 (1.01–8.48)</td>
<td>3.60 (0.80–16.26)</td>
<td>0.48 (0.02–11.35)</td>
<td>4.41 (0.63–31.04)</td>
</tr>
</tbody>
</table>

* Large-artery atherosclerosis.

** Small-artery occlusion.

*** Adjusted odds ratio and 95% confidence intervals, adjusted for age, sex, hypertension, diabetes mellitus, and smoking.
than that in controls. The AOR was not significant in large-artery atherosclerosis, cardioembolism, other determined etiology, or undetermined etiology (Table 3).

For the further study of small-artery occlusion, we distinguished single small-artery occlusion from multiple small-artery occlusions by brain MRI. In the case of multiple small-artery occlusions, the AOR of the 677TT genotype was found to be significantly higher than that in controls. However, in single small-artery occlusion, the AOR was not significantly higher. Moreover, the AOR of multiple small-artery occlusions was much greater value than that of small-artery occlusion (Table 4).

4. Discussion

Hyperhomocysteinemia is a risk factor for ischemic stroke, but the MTHFR 677TT genotype has not been proven to be associated with ischemic stroke [1,9]. The possible explanation of this contradiction may be a consequence of the fact that previous studies have overlooked the heterogeneity of ischemic stroke. Due to different etiologies, risk factors, and outcomes, ischemic stroke is perhaps one of the most heterogeneous clinical syndromes of all neurological diseases [24]. Some have considered its etiological heterogeneity and focused on the subtypes of stroke [15–17]. However, none has classified patients with small-artery occlusion into those with single small-artery occlusion and those with multiple small-artery occlusions according to brain MRI scan results. There were some studies that different pathophysiological mechanisms may exist for single small-artery occlusion and multiple small-artery occlusions [18–21].

Without making the above subtype classification, our results correspond to previous reports that ischemic stroke patients have higher homocysteine levels, but no greater prevalence of the TT genotype than controls [9,10]. However, when we re-analyzed the data by subtype, we discovered that both the AOR of the TT genotype and plasma homocysteine levels were significantly higher in patients with small-artery occlusion than those in controls. Also, these were much more significant in patients with multiple small-artery occlusions. So, our findings reveal that multiple small-artery occlusions are closely correlated with the presence of the homozygous C677T mutation in the MTHFR gene and suggest that this 677TT genotype increases the risk of cerebral microangiopathy.

Our results are supported by previous reports, which showed that small-artery occlusion was associated with hyperhomocysteinemia [11–13]. Though some reports have shown an association between hyperhomocysteinemia and large-artery atherosclerosis, we did not find significantly high plasma homocysteine levels in patients with large-artery atherosclerosis, but rather only observed an increasing tendency [14,15]. Shimizu et al. [25] found that plasma homocysteine levels were significantly higher in lacunar infarctions, but that homocysteine levels were only marginally different in atherothrombotic infarctions. In addition, it has been reported that there was no significant difference in plasma homocysteine levels and in the prevalence of the TT genotype in large-artery atherosclerosis [26]. These are consistent with our results. We also found that small-artery occlusion was significantly associated with both the TT genotype and elevated homocysteine levels. Considering the discordance among reports on this topic, it is possible that homocysteine may play a differential role in the pathogenesis of vascular disorders, and that its effect on the vascular system may be varied. However, our results are believed to be more reasonable considering the fact that, if increased plasma homocysteine levels are associated with certain subtypes of ischemic stroke, the 677TT genotype must be associated with these subtypes, because the TT genotype is accepted to be a strong hereditary factor for hyperhomocysteinemia.

For more advanced analysis, the subjects with small-artery occlusion were sub-classified according to their having single or multiple lesions by brain MRI. Evers et al. [13] found that hyperhomocysteinemia is associated with multiple cerebral infarctions, but not with a single infarction. In addition, several papers have been found that the 677TT genotype is associated with multiple infarctions [27,28]. We found that there was a strong association between hyperhomocysteinemia and the C677T genotype in multiple small-artery occlusions, but not in single small-artery occlusion. These findings are of interest because the pathology of these types of stroke

<table>
<thead>
<tr>
<th>MTHFR</th>
<th>Controls (n = 198)</th>
<th>Single small-artery* (n = 32)</th>
<th>Multiple small-artery** (n = 40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>677 CC</td>
<td>73 (36.9)</td>
<td>11 (34.4)</td>
<td>9 (22.5)</td>
</tr>
<tr>
<td>677 CT</td>
<td>100 (50.5)</td>
<td>17 (53.1)</td>
<td>15 (37.5)</td>
</tr>
<tr>
<td>677 TT</td>
<td>25 (12.6)</td>
<td>4 (12.5)</td>
<td>16 (40.0)</td>
</tr>
<tr>
<td>AOR (CI),***TT vs. CC</td>
<td>1.00</td>
<td>1.19 (0.27–5.35)</td>
<td>6.90 (1.70–27.99)</td>
</tr>
<tr>
<td>AOR (CI),***TT vs. CC/CT</td>
<td>1.00</td>
<td>1.14 (0.31–4.17)</td>
<td>5.57 (1.97–15.72)</td>
</tr>
</tbody>
</table>

* Single small-artery occlusion.
** Multiple small-artery occlusions.
*** Adjusted odds ratio and 95% confidence intervals, adjusted for age, sex, hypertension, diabetes mellitus, and smoking.
has been suggested to be different in microatheroma, which produces stenosis or occlusion of arterioles in hypertensive individuals according for single small-artery occlusion, and a more diffuse arteriopathy according for multiple small-artery occlusions [18–21]. In most cases, a single lacunar infarction was caused only slight residual disability, but an increasing number of recurrent infarctions might represent an increasingly significant clinical deficit, including subcortical ischemic vascular dementia [20,29]. Recently, the consensus is growing that small-artery occlusion have a more important role in the field [18,19]. The frequency and the severity of cerebral infarction may be associated with the 677TT genotype and with elevated homocysteine levels. So, the TT genotype may be a predictor of the risk for multiple small-artery occlusions. Accordingly, it seems reasonable that the MTHFR genotype and plasma homocysteine levels be determined when assessing the probability of multiple small-artery occlusions.

This study has some limitations. It is a hospital-based case control study, and has a relatively small sample size. However, our data seem to be reliable because our study population was diagnosed by brain MRI, which is both more sensitive and more specific than brain CT in terms of differentiating the sub-groups of small-artery occlusion [30]. The racial distribution in our study also differed from previous studies, because all subjects were Korean, which could be viewed as a form of selection bias. Moreover, the frequency of the homozygous C677T mutation in the MTHFR gene is known to be ethnic group dependent [31]. For example, the prevalence of the C677T mutation is lower in Africa than it is in Asia [32]. In addition, Kim et al. [33] reported that both MTHFR 677 T allele ratio and 1298 C allele ratio in Koreans are similar to that found in other Asian populations, but that T allele ratio is higher and the C allele ratio lower than found in the European populations. It is indeed surprising to find higher C677T and lower A1298C frequencies in the Asian population, which as a distinctively lower prevalence of ischemic vascular disease than the European population [31]. Other possible limitations would include different environmental influences, such as daily folate intake, which might have influenced plasma homocysteine concentration and stroke occurrence.

It has been reported that patients with the 677TT genotype have higher folate requirements than those with the CC or the CT genotypes, and daily folate intake can reduce plasma homocysteine concentration [34,35]. Our cross-sectional study has insufficient persuasive power to allow firm conclusions to be drawn. However, our results suggest that it may be possible to reduce the frequency of certain subtypes of ischemic stroke, such as small-artery occlusion, and especially multiple small-artery occlusions, by reducing their homocysteine levels in patients with the MTHFR 677TT genotype.

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