MTHFR Gene Polymorphism as a Risk Factor for Silent Brain Infarcts and White Matter Lesions in the Japanese General Population

The NILS-LSA Study

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Background and Purpose—Silent brain infarcts (SBI) and white matter lesions are relatively common neuroimaging findings, especially in the elderly population. The genetic background for SBI and white matter lesions in a large Japanese general population was investigated.

Methods—Subjects were recruited from participants in the National Institute for Longevity Sciences, Longitudinal Study of Aging. Genotyping of methylenetetrahydrofolate reductase (MTHFR) C677T gene mutation and brain MRI examination were performed in 1721 subjects free of any history of stroke. SBI and white matter lesions were diagnosed from MRI findings.

Results—Of 1721 MRI examinations, SBI was observed in 178 (10.3%). The prevalence of SBI and white matter lesions increased with age. The prevalence of SBI was significantly higher in subjects with the MTHFR TT genotype compared with the TC/CC genotype (14.6% versus 9.5%; 42 of 288 versus 136 of 1433; $\chi^2=6.71; P=0.010$). The stage of white matter lesions was not significantly different. In subjects ≥60 years of age (n=849), the prevalence of SBI was significantly higher in TT than TC/CC (27.7% versus 16.6%; 36 of 130 versus 119 of 719; $\chi^2=9.16; P=0.002$). The prevalence of moderately advanced white matter lesions was also significantly higher in TT than TC/CC (60.7% versus 49.0%; 79 of 130 versus 352 of 719; $\chi^2=9.16; P=0.002$). After correction for other risk factors, the MTHFR TT genotype was independently associated with SBI (odds ratio [OR], 1.72; 95% CI, 1.10 to 2.68; $P=0.018$) and moderately advanced white matter lesions (OR, 1.58; 95% CI, 1.07 to 2.33; $P=0.02$).

Conclusions—These findings indicate that the MTHFR TT genotype is an independent risk factor for SBI and white matter lesions in the general Japanese population, especially in elderly subjects. (Stroke. 2003;34:1130-1135.)

Key Words: amine oxidoreductases ■ brain infarction ■ elderly ■ polymorphism ■ white matter

Silent brain infarcts (SBI) and white matter lesions, which are often incidentally identified during CT or MRI scanning in asymptomatic individuals, are relatively common neuroimaging findings, especially in the elderly population.1–3 However, the presence of SBI and white matter lesions has been identified as an independent risk factor for the development of future symptomatic stroke4,5 and dementia.6 Accordingly, the underlying mechanisms have been the focus of much research.1–3,7,8 Population-based studies have been performed to identify risk factors for SBI.2,3 It has been demonstrated that classic risk factors such as hypertension and smoking are involved in the development of SBI.1–3,7,8 The genetic predisposition to SBI has also been studied.9,10 However, a population-based study to identify a candidate gene for SBI has not been performed.

Methylenetetrahydrofolate reductase (MTHFR) catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which serves as a methyl donor in the reaction converting homocysteine (Hcy) to methionine.11,12 An increased plasma Hcy level has consistently been shown to be an independent risk factor for atherothrombotic disorders in several meta-analyses.11–13 On the other hand, a common mutation of MTHFR, which results in a mild increase in the plasma level of Hcy, has not been reported as a consistent risk factor for atherothrombotic disorders, including stroke.11,12,14 Although there could be several possible mechanisms underlying the discrepancy, the sampling of cases and controls might account for part of the discrepancy. Two studies have evaluated the association between MTHFR gene C677T mutations and SBI.9,10 Both studies

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failed to demonstrate significant associations. One evaluated subjects undergoing medical checkup. To evaluate the genetic predisposition to SBI, community-based sampling of subjects would be less biased in the selection of cases and controls and would eliminate regional differences. Another study evaluated community-dwelling elderly subjects; however, the number of subjects was too small to reach a conclusion. The National Institute for Longevity Sciences, Longitudinal Study of Aging (NILS-LSA) is a comprehensive study on aging in the Japanese general population. In this standardized cohort with >1700 subjects, we investigated the association between MTHFR C677T mutations and the prevalence of SBI. All participants underwent brain MRI examination and were genotyped for the MTHFR C677T mutation.

**Methods**

**Subject Selection**
All participants were independent residents in Obu and Higashiura in the Aichi prefecture in central Japan. Residents 40 to 79 years of age were randomly selected from the resident register in cooperation with the local government. They were stratified by both age and sex. Randomly selected men and women were invited by mail to attend an explanatory meeting. At that meeting, the procedures for each examination and the follow-up schedule were fully explained. Written, informed consent to the entire procedure was obtained from each participant. Participants in the present study were recruited from subjects examined in 1997 to 1999. In total, 1758 subjects completed the entire procedure. Among them, 1721 subjects, 876 men and 845 women, free of any history of stroke, including transient ischemic attack, were evaluated in the present study. The ethics committee of the Chubu National Hospital approved all procedures of the NILS-LSA.

**Research Area**
The residential area of the present study is in the south of Nagoya. It is a commuter town and an industrial area for the Toyota group but still has many orchards and farms; thus, it has both urban and rural characteristics. This research area is in the center of Japan, and the climate is average for Japan. We examined a representative sample of the area’s population via a national postal questionnaire of the prefecture-stratified random samples of 3000 households from all prefectures in Japan and showed that the lifestyle of this area was the most typical of all areas in Japan.

**Risk Factor Evaluation**
Details of physical examinations were published elsewhere. In brief, lifestyle, medical history, and prescribed drugs were examined by questionnaires. Anthropometric and blood pressure measurements were performed by a physician. Venous blood was collected early in the morning after at least a 12-hour fast for measurement of serum lipids and plasma glucose. Blood pressure was measured twice at >5-minute intervals in subjects in the sitting position by doctors using a standard sphygmomanometer. The mean of 2 determinations was obtained for each participant. As risk factors for stroke, hypertension, glucose intolerance, hyperlipidemia, and smoking status were evaluated. Hypertension was defined as systolic blood pressure ≥140 mm Hg, diastolic blood pressure ≥90 mm Hg, and/or the use of an antihypertensive drug. Glucose intolerance was defined as fasting plasma glucose ≥110 mg/dL, HbA1c ≥5.8%, and/or the use of medication for lowering blood glucose. Hyperlipidemia was defined as serum total cholesterol ≥230 mg/dL, serum triglycerides ≥170 mg/dL, and/or the use of a lipid-lowering drug.

**Brain MRI Gene and Silent Brain Infarcts**
Brain MRI imaging was performed in all participants in the present study with a 1.5-T scanner (Toshiba Visart) at the NILS. The head position was oriented in the scanner and stabilized during the scanning procedure by use of a head support. To establish slice orientation, the first scanning sequence consisted of a T1-weighted sagittal series (repetition time [TR], 500 ms; echo time [TE], 15 ms; matrix, 256×256) centered in the midline to define the orbitomeatal line. The second series of T1-weighted axial images (TR, 500 ms; TE, 15 ms; thickness, 8 mm; gap, 1.5 mm; matrix, 256×256) and T2-weighted axial images (TR, 4000 ms; TE, 120 ms; thickness, 8 mm; gap, 1.5 mm; matrix, 320×320) were oriented parallel to the orbitomeatal line. Fourteen slices were taken at each examination.

An infarct was defined as a lesion ≥0.3 cm in diameter shown as a low-signal-intensity area on T1-weighted images that was also visible as a hyperintense lesion on T2-weighted images.16,17 Small lesions (<1.5 cm) were diagnosed as lacunae. White matter lesions, depicted on T2-weighted images, were classified into 5 grades: grade 0, no abnormality; grade 1, minimal periventricular signal hyperintensities in the form of caps confined exclusively to the anterior horns; grade 2, hyperintensities in both the anterior and posterior horns of the lateral ventricles, periventricular unifocal patches, or rims lining the ventricles; grade 3, multiple periventricular hyperintense punctuate lesions reaching early confluence in the periventricular region; and grade 4, diffuse lesions. A neurologist (F.M.) blinded to the clinical status of the subjects interpreted all MRI series.

**MTHFR Genotype Analysis**
Genomic DNA was extracted from peripheral blood lymphocytes by the standard procedure. MTHFR C677T mutation was determined by allele-specific primer–polymerase chain reaction (ASP-PCR) method. The single nucleotide polymorphism region of the gene was amplified by PCR with 2 ASPs (C-specific primer, 5′-GAAGGTGCTCGGGAXCC-3′; T-specific primer, 5′-GAAGGTGCTCGGGAXCT-3′) and a biotin-labeled common antisense primer (5′-biotin-GAATGTGTCAGCCTAAAG-AAA-3′). Amplified allele-specific DNA products were used for colorimetric genotyping. For MTHFR genotyping, 2 types of wells conjugated with the allele-specific C-type probe (5′-TCTGGCGGGAAXCCATTCTCAT-3′) or T-type probe (5′-TCTGGCGGGAAXCGATTTCAT-3′) or T-type probe (5′-TCTGGCGGGAAXCTGATTTCAT-3′) were prepared. The amplified DNA product was denatured with NaOH and added to each well. Then, it was hybridized at 37°C for 30 minutes with hybridization buffer containing formamide. After the wells were washed thoroughly, alkaline phosphatase–conjugated streptavidin was added to each well, and the plate was incubated at 37°C for 15 minutes. After the wells were washed, 0.8 mmol/L WST-1 [2-(4-iiodophenyl)-3-(4-nitrophenyl)-5-(2,4-dih- sulfophenyl)-2H-tetrazolium, monosodium salt] and 0.4 mmol/L BCIP (5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt), a substrate for alkaline phosphatase, was added, and colorimetry was performed. The genotypes were identified by the absorbance signal ratio between C type–specific and T type–specific wells. The validity of the ASP-PCR method was confirmed with genotyped DNA samples obtained by the standard method reported by Frosst et al. KOD polymerase derived from Thermococcus kodakaraensis KOD1 was used. The fidelity of our method is 3.4 times higher than that with Taq DNA polymerase. The mutation rate with this method is 0.35%.

**Statistical Analysis**
All values are expressed as mean±SD if not specified. The association between SBI and white matter lesions and MTHFR genotype were analyzed by χ² test. Logistic regression analysis was used to explore the independence of the effect of the MTHFR TT genotype on the prevalence of SBI and the presence of white matter lesions. All statistical analyses were performed with SAS software (SAS Institute Inc). A value of P<0.05 was considered statistically significant.
TABLE 1. Background Characteristics of Subjects With Silent Brain Infarcts and Moderate White Matter Lesions

<table>
<thead>
<tr>
<th></th>
<th>Silent Brain Infarcts</th>
<th>White Matter Lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Grades 0–1</td>
<td>Grades 2–4</td>
</tr>
<tr>
<td>n</td>
<td>1543</td>
<td>178</td>
</tr>
<tr>
<td>Male, %</td>
<td>50</td>
<td>64</td>
</tr>
<tr>
<td>Age, y</td>
<td>58±11</td>
<td>69±8‡</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>22.9±3.0</td>
<td>23.2±3.3</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>123±19</td>
<td>134±19‡</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>75±11</td>
<td>80±11‡</td>
</tr>
<tr>
<td>T-Chol, mg/dL</td>
<td>220±35</td>
<td>220±34</td>
</tr>
<tr>
<td>HDL-Chol, mg/dL</td>
<td>62±15</td>
<td>59±17*</td>
</tr>
<tr>
<td>Triglyceride, mg/dL</td>
<td>122±67</td>
<td>126±60</td>
</tr>
<tr>
<td>Blood glucose, mg/dL</td>
<td>103±21</td>
<td>108±23*</td>
</tr>
<tr>
<td>Hyper tension, %</td>
<td>31</td>
<td>66‡</td>
</tr>
<tr>
<td>Hyperlipidemia, %</td>
<td>48</td>
<td>52</td>
</tr>
<tr>
<td>Glucose intolerance, %</td>
<td>21</td>
<td>35‡</td>
</tr>
<tr>
<td>Current smoker, %</td>
<td>23</td>
<td>24</td>
</tr>
</tbody>
</table>

MTHFR indicates methylenetetrahydrofolate reductase; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; T-Chol, total cholesterol; HDL-Chol, high density lipoprotein cholesterol.

Values are mean±SD. *P<0.05, †P<0.001, ‡P<0.0001 versus corresponding controls [silent brain infarcts (–) and white matter lesions 0–1].

Results

Background Characteristics of Participants and Prevalence of SBI

Table 1 summarizes the backgrounds of all participants subdivided by the presence of SBI and moderately advanced white matter lesions. The presence of SBI and white matter lesions was associated with advanced age, hypertension, and glucose intolerance. The prevalence of SBI and white matter lesions significantly increased with age (the Figure). In subjects ≥60 years of age, SBI was observed in 18.3% (155 of 849).

MTHFR Gene Mutation and SBI and White Matter Lesions

The breakdown of the total 1721 subjects by MTHFR C677T gene mutation was as follows: CC genotype, 623; CT genotype, 810; and TT genotype, 288. The distribution of MTHFR genotypes was consistent with published reports on Japanese subjects18–20 and was in agreement with the Hardy-Weinberg proportion (P=0.80).

The prevalence of SBI and the grade of white matter lesions are shown in Table 2. The prevalence of SBI was significantly higher in subjects with the MTHFR TT genotype compared with C carriers (CT+CC). However, there was no significant association between MTHFR genotype and grade of white matter lesions. Because SBI and white matter lesions were prevalent only after the age of 60 years, the associations between MTHFR genotype and prevalence of SBI and white matter lesions were evaluated in subjects ≥60 years of age. In this population, the MTHFR genotype was significantly associated with the presence of SBI and white matter lesions (Table 3).

To further investigate whether the MTHFR genotype is associated with SBI independently of other known risk factors, logistic regression analysis for SBI was performed with 3 models including the following risk factors: hypertension, hyperlipidemia, glucose intolerance, smoking, and...
marginal increase in Hcy was associated with a modest increase in plasma Hcy (2.6 µg/mL) by MTHFR TT mutation is 1.10 to 1.15, it is expected that a larger population would be necessary to prove a significant association with the MTHFR TT genotype.11 These findings indicate that the genetic association between MTHFR TT genotype and atherosclerotic disorders is not conclusive.

The association between the MTHFR gene mutation and SBI has been studied in the Japanese population.9,10 Those studies reported a lack of association between MTHFR and SBI. In a study by Notsu et al.9 SBI patients were recruited from consecutive patients who underwent brain MRI examination for a health screening examination. Accordingly, these subjects were neither randomly selected nor community-based samples. One of the concerns in the recruitment of participants in medical checkups is that the possibility that some could have subtle symptoms prompting them to have a medical checkup, including brain MRI, cannot be excluded. To evaluate the genetic predisposition to SBI, community-based sampling of subjects would be less biased in the selection of cases and controls and would eliminate regional differences. Another study reported by Matsui et al10 was a community-based study. Although they did not find a positive association, the number (38 cases) was too small to reach a conclusion. To respond to these concerns, large, population-based, random sampling is advisable. NILS-LSA is a community-based random sample study in central Japan. Our preliminary study indicated that the area is representative of the total Japanese population. Region-dependent differences in folic acid21 could also be eliminated. All participants underwent brain MRI, and genotyping was performed. The advantage of a community-dwelling study is minimal bias in the selection of cases and controls. On the other hand, a disadvantage is that the number of cases of concern is small,

### TABLE 3. Prevalence of Silent Brain Infarcts and White Matter Lesions in 3 MTHFR Genotypes in Subjects Aged 60 or Over

<table>
<thead>
<tr>
<th>MTHFR (n)</th>
<th>CC (304)</th>
<th>CT (415)</th>
<th>TT (130)</th>
<th>CC+CT (719)</th>
<th>Total (849)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No SBI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>257</td>
<td>343</td>
<td>94</td>
<td>600</td>
<td>694</td>
</tr>
<tr>
<td>SBI</td>
<td>47</td>
<td>72</td>
<td>36</td>
<td>119</td>
<td>155</td>
</tr>
</tbody>
</table>

\[ \chi^2 = 9.58 \quad \chi^2 = 9.16^* \]

\[ P = 0.008 \quad P = 0.002 \]

<table>
<thead>
<tr>
<th>Lacunae</th>
<th>44</th>
<th>63</th>
<th>32</th>
<th>107</th>
<th>139</th>
</tr>
</thead>
</table>

\[ \chi^2 = 8.08 \quad \chi^2 = 8.10^* \]

\[ P = 0.018 \quad P = 0.004 \]

**White matter lesions**

| Grades 0–1 | 155      | 212      | 51       | 367         | 418         |
| Grade 2    | 78       | 114      | 42       | 192         | 234         |
| Grades 3–4 | 71       | 89       | 37       | 160         | 197         |

\[ \chi^2 = 6.69 \quad \chi^2 = 6.20^* \]

\[ P = 0.015 \quad P = 0.045 \]

<table>
<thead>
<tr>
<th>Grades 2–4</th>
<th>149</th>
<th>203</th>
<th>79</th>
<th>352</th>
<th>431</th>
</tr>
</thead>
</table>

\[ \chi^2 = 6.15 \quad \chi^2 = 6.15^* \]

\[ P = 0.046 \quad P = 0.013 \]

**SBI indicates silent brain infarct.**

*Comparison with MTHFR TT genotype.

MTHFR genotype in subjects ≥60 years of age (Table 4). It revealed that the MTHFR TT genotype was independently associated with SBI, asymptomatic lacunar infarctions, and white matter lesions of grade 2 or higher.

### Discussion

Meta-analyses have revealed a consistent association between the plasma level of Hcy and atherosclerotic disorders.11,13 Boushey et al13 reported that the odds ratio (OR) for coronary arterial disease with a 5-µmol/L Hcy increment was 1.6 (95% CI, 1.4 to 1.7) for men and 1.8 (95% CI, 1.3 to 1.9) for women in their meta-analysis. Recently, it has also been reported that a high Hcy level was significantly associated with SBI and white matter lesions.16–20 Although MTHFR C677T mutation is a major cause of mild hyperhomocysteinemia, Brattstrom et al14 showed in their meta-analysis that the mutation did not increase cardiovascular risk in their 5869 controls and 6644 cases. They suggested that the modest increase in plasma concentration of Hcy found in patients with cardiovascular disease is an epiphenomenon, a consequence of the well-established standard risk factors for vascular disease and renal function, and that it is not directly causal. However, Ueland et al11 further extended the interpretation of the findings of meta-analysis. Because the estimated relative risk for atherosclerotic disorders associated with a modest increase in plasma Hcy (2.6 µg/mL) by MTHFR TT mutation is 1.10 to 1.15, it is expected that a larger population would be necessary to prove a significant association with the MTHFR TT genotype.11 These findings indicate that the genetic association between MTHFR TT genotype and atherosclerotic disorders is not conclusive.

### TABLE 4. Odds Ratio for Presence of Silent Brain Infarcts, Lacunar Lesions, and White Matter Lesions in Subjects Aged 60 or Over

<table>
<thead>
<tr>
<th></th>
<th>Model I</th>
<th>Model II</th>
<th>Model III</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBI</td>
<td>1.93</td>
<td>1.82</td>
<td>1.72</td>
</tr>
<tr>
<td></td>
<td>1.25–2.97</td>
<td>1.18–2.82</td>
<td>1.10–2.68</td>
</tr>
<tr>
<td></td>
<td>0.0028</td>
<td>0.0073</td>
<td>0.018</td>
</tr>
<tr>
<td>Lacunae</td>
<td>1.91</td>
<td>1.81</td>
<td>1.71</td>
</tr>
<tr>
<td></td>
<td>1.22–3.00</td>
<td>1.15–2.86</td>
<td>1.08–2.73</td>
</tr>
<tr>
<td></td>
<td>0.0049</td>
<td>0.011</td>
<td>0.024</td>
</tr>
<tr>
<td>White matter lesions</td>
<td>1.62</td>
<td>1.63</td>
<td>1.58</td>
</tr>
<tr>
<td>Grades 2–4</td>
<td>1.10–2.37</td>
<td>1.11–2.39</td>
<td>1.07–2.33</td>
</tr>
<tr>
<td></td>
<td>0.013</td>
<td>0.013</td>
<td>0.02</td>
</tr>
</tbody>
</table>

OR indicates odds ratio; CI, 95% confidence interval; SBI, silent brain infarct. Model I, no correction for other risk factors; model II, correction for sex, smoking, hyperlipidemia, and glucose intolerance; model III, correction for sex, smoking, hyperlipidemia, glucose intolerance, and hypertension.
and a large population is necessary to have enough cases for analysis. One way to overcome this disadvantage is to use a surrogate condition such as SBI for the cases.

Asymptomatic brain infarction is reported to be common in the elderly population. Although neurologically asymptomatic, MRI-proven abnormal findings have been shown to be associated with several disorders.16,17,22–25 Accordingly, it is conceivable that the subjects with SBI might not have been completely free of symptoms in the present study. In our previous studies, SBI was shown to be associated with hypertensive end-organ damage24 and abnormal diurnal changes in blood pressure.17,25 Although we did not analyze cognitive function in the present study, an association between impaired cognitive function and SBI and white matter lesions has also been reported.14,22 In several studies, the MTHFR TT genotype has also been demonstrated to be a risk factor for dementia,26,27 although conflicting results have also been reported.28,29 These findings suggest a possible association between the MTHFR TT genotype and cognitive impairment in the general population. However, this issue needs to be confirmed in a large community population.

Although the origin of the TT variant is not known, the high prevalence of this polymorphism in most populations could indicate that the TT variant might represent an ancestral genetic adaptation to living.12 Enhanced homocysteine, cell proliferation, and tissue repair have been postulated as underlying mechanisms.12 The MTHFR TT variant shows reduced enzymatic activity, resulting in an increase in Hcy concentration. It has been reported that hyperhomocysteinemia is a possible causal factor in free radical generation during the acute phase of thrombotic cerebrovascular stroke.30 Recently, it has also been reported that the MTHFR TT genotype was associated with reduced superoxide dismutase activity.31 Because DNA can be damaged in oxidative stress such as ischemia-reperfusion injury in the brain, DNA repair after oxidative stress could also be altered by the TT mutation. Furthermore, it has been reported that errors in DNA repair caused by oxidative stress occur preferentially at GC sequences in the brain,32,33 suggesting that the TT mutation could be a hallmark of oxidative stress in the brain. These findings suggest that damage caused by oxidative stress such as reperfusion could be enhanced in subjects with the MTHFR TT mutation, which may account for their higher prevalence of SBI. However, this hypothesis requires further study.

In summary, in a large, randomly selected, community-based population, the MTHFR TT genotype was an independent risk factor for both SBI and white matter lesions. Because the prevalence of SBI is relatively high, an intervention approach could be useful in reducing the future risk of developing symptomatic stroke and cognitive decline.

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