Evaluation of Genetic Risk Factors for Silent Brain Infarction
Yoshitomo Notsu, MT; Toru Nabika, MD; Hyun-Young Park, MD; Junichi Masuda, MD; Shotai Kobayashi, MD

Background and Purpose—Silent brain infarction (SBI) is often found with white matter hyperintensity. A recent genetic study on elderly twins indicated that the susceptibility to white matter hyperintensity was largely determined by genetic factors, implying the existence of genetic susceptibility for SBI as well. We therefore studied 3 genetic polymorphisms in SBI, the deletion/insertion polymorphism of angiotensin-converting enzyme (ACE) gene, the apolipoprotein(a) [apo(a)] size polymorphism, and the T677C polymorphism of methylenetetrahydrofolate reductase (MTHFR) gene, by a case-control study.

Methods—By MRI, 147 subjects with SBI and 214 without cerebral infarctions (control group) were selected from participants of a health examination of the brain. Seventy-four patients with symptomatic subcortical infarction (SSI) from the same area were also included in the study. In addition to the control group, 2 more reference populations were recruited. Typing of the apo(a) size polymorphism was done by Western blotting with the use of an anti-apo(a) antibody. Genotypes of ACE and MTHFR were determined by polymerase chain reaction amplification of the genomic DNA and subsequent restriction enzyme digestion.

Results—The ACE polymorphism was not associated with either SBI or SSI. In contrast, the small apo(a) was associated with both SSI and SBI. The MTHFR polymorphism was associated only with SSI. The association of MTHFR and apo(a) was greater in the younger subjects.

Conclusions—Among the 3 genetic polymorphisms studied, only the apo(a) size polymorphism is a risk factor for SBI. (Stroke. 1999;30:1881-1886.)

Key Words: amine oxidoreductases n angiotensin converting enzymes n apolipoproteins n genetics n lacunar infarction

Silent brain infarction (SBI) is a unique entity of cerebrovascular disorders from both clinical and pathological points of view. MRI has shown that SBI is fairly common in the elderly and furthermore that subjects with SBI are more likely to develop symptomatic infarction. It is therefore important to identify risk factors for SBI as well as factors promoting the progression of SBI to other symptomatic forms of cerebrovascular disorders, although SBI itself does not cause any symptoms.

SBI consists mainly of small lacunar infarction. It is often found with other white matter lesions such as white matter hyperintensity (WMH) and periventricular hyperintensity, suggesting that these disorders share a common etiologic background. Since a recent study on elderly twins suggested that the genetic factor was a major determinant of the susceptibility to WMH, we hypothesized that genetic factors play significant roles in the pathogenesis of SBI as well. In the present study we selected 3 genes potentially important in cerebrovascular diseases and evaluated them in SBI to elucidate whether they have predisposing effects.

The genetic polymorphisms evaluated in this report were the apolipoprotein (a) [apo(a)] size polymorphism [so-called apo(a) phenotype], the T677C substitution in the methylenetetrahydrofolate reductase (MTHFR) gene, and the deletion/insertion (D/I) polymorphism in the angiotensin-converting enzyme (ACE) gene. We selected these polymorphisms because of their association with intermediate phenotypes that potentially contribute to the pathogenesis of cerebrovascular disorders; the ACE D/I polymorphism was shown to be associated with plasma ACE activity. In the same way, the T677C of MTHFR and the apo(a) size polymorphism were associated with the serum homocyst(e)in and lipoprotein (a) [Lp(a)] concentration, respectively.

In vitro and in vivo studies have suggested that these phenotypic changes play pathophysiological roles in cerebrovascular diseases; a histochemical study indicated that the cerebral vasculature is rich in ACE. Since ACE is thought to play an important role in the vascular remodeling that is observed both in the arteries of the stroke-prone spontaneously hypertensive rat and in white matter lesions of...
humans, increased ACE activity may play a key role in the pathophysiological process in lacunar infarction. Homocysteinemia has been identified as a strong risk factor for atherothrombotic disorders through studies on a mendelian form of the cystathionine  β-synthase deficiency. A high concentration of serum Lp(a) caused atherosclerosis in a transgenic mouse, indicating that high Lp(a) was a risk factor for atherosclerosis. The apo(a) molecule, however, was recently shown to interact directly with vascular components, suggesting that apo(a) plays a role in the pathological processes disturbing vascular wall integrity through mechanisms independent of lipid accumulation. These physiological observations prompted us to evaluate these genetic polymorphisms in SBI.

Considering the major role of small lacunar infarction in SBI, we additionally compared symptomatic subcortical infarctions (SSI), which are radiologically defined lacunar infarctions with symptoms, with SBI. The size and number of infarctions in addition to their location are the most important factors determining whether a lesion manifests symptoms. We therefore expected SSI to include more severe forms of lacunar infarctions. In addition, a recent large-scale population-based study suggested some difference of risk factors between symptomatic and silent lacunar infarctions. In SBI, the size and number of lacunes (SSI), which are radiologically defined lacunar infarctions in addition to their location are the most important factors determining whether a lesion manifests symptoms. We therefore expected SSI to include more severe forms of lacunar infarctions. In addition, a recent large-scale population-based study suggested some difference of risk factors between symptomatic and silent lacunar infarctions. We report here that the apo(a) size polymorphism was a common genetic risk factor for SSI and SBI, whereas the T677C in MTHFR was a risk only for SSI.

**Subjects and Methods**

**Subjects**

Among people who visited the Shimane Institute of Health Science for a health screening examination between January 1995 and December 1997, 147 consecutive subjects diagnosed with SBI (SBI group) were recruited in the study. The diagnosis of SBI was made by MRI examination (0.2 T, Siemens). The criteria for SBI were as follows: (1) spotty areas ≥3 mm in diameter in the areas supplied by deep perforating arteries, showing high intensity in the T2-weighted images coinciding with low intensity in the T1-weighted images of MRI; (2) absence of neurological signs and symptoms corresponding to the lesions; and (3) no past history of cerebral stroke, including transient ischemic attack. Most of our SBI patients had lacunes of ≤1 cm. Two hundred fourteen subjects without evidence of SBI on MRI were selected from the same population (control group). For the control group, we picked 4 separate 3-month periods in the entire course of our study and continuously chose participants during these periods. Genotype frequencies of the 3 genetic polymorphisms in the control group were not different from those in the reference groups (REF1 and REF2; see below) or from those in previous reports in Japan. We thus concluded that the control group represented the general population well (see Table 2). Seventy-four SBI patients treated consecutively were selected from outpatients at Shimane Medical University in the same period (SSI group). The criteria for SSI were as follows: (1) lacunar infarction of <1.5 cm in the subcortical area supplied by perforating arteries; (2) no apparent atherosclerotic lesions in either bilateral carotid arteries or major cerebral arteries; and (3) neurological signs or symptoms corresponding to the lesions. Histories of hypertension and diabetes mellitus were obtained through an interview. A smoker was defined as a subject with a smoking index (number of cigarettes per day × years) ≥200. Total cholesterol, HDL cholesterol, triglycerides, and Lp(a) were measured in all subjects of the SSI, SBI, and control groups.

In addition to the control group, 2 reference populations were used in the analysis. For the apo(a) phenotype analysis, among the employees of Shimane Medical University who attended a health examination, sera of the first 100 participants were used (REF1 group). DNA from 176 students of Shimane Medical University who voluntarily donated their blood was used for genotype analysis of ACE and MTHFR (REF2 group). All participants gave informed consent.

**Determination of the Apo(a) Size Polymorphism**

The apo(a) size polymorphism was determined by Western blot analysis of fractionated serum protein with the use of a commercial kit (Lp(a) phenotype analysis kit, Sanwa Chemical Co). According to the size standards included in the kit, the apo(a) molecule was divided into 5 alleles as described previously. Alleles I to V corresponded to apo(a) proteins whose kringle-IV repeats are <16, 16 to 18, 19 to 21, 22 to 28, and >29, respectively. The detection limit of Lp(a) by this assay system was 4 mg/dL, and thus Lp(a) concentrations below this level were categorized as null (N) alleles. Since alleles I to III were rare (<1%), alleles I to IV were combined and analyzed as 1 allele (designated as v). Accordingly, 3 alleles, N, V, and v, were used in the subsequent analysis. The apo(a) allele frequencies were calculated from the genotype frequencies as described.

**Genotype Determination of MTHFR and ACE**

Genotypes of MTHFR and ACE were determined as described previously. For MTHFR, polymerase chain reaction (PCR) was performed with primers used in a previous report. PCR products were then digested with HinfI (New England Biolabs) and analyzed on 3% NuSieve 3:1 gels (FMC Bioproducts). For ACE, 2 separate PCR reactions, one for both D and I alleles and the other for the I allele, were performed to avoid misreading between DI heterozygotes and DD homozygotes.

**Assay Procedures**

Serum Lp(a) levels were measured by the latex immunooagglutination method (Daichi Chemical Co) with a mouse anti-human apo(a) monoclonal antibody. Serum total cholesterol and triglyceride levels were determined with a commercial enzyme assay kit (Kyouwa Medex Co). Serum HDL cholesterol was determined directly with an enzyme assay kit (Kyouwa Medex Co). LDL cholesterol was calculated according to the Friedewald equation, as described previously.

**Statistical Analyses**

Since the distribution of Lp(a) concentrations was highly skewed, the log of the Lp(a) concentration was used to calculate the geometric means and 95% CIs of the Lp(a) levels, as was done in a previous report. The calculated means and CIs were represented as real values transformed back from log [Lp(a)]. The χ² and Student’s t tests were used to compare the clinical parameters among the studied populations. The differences in allele frequencies among the populations were tested by the χ² test. Multiple logistic analysis of risk factors was performed with the SPSS package. The difference was considered statistically significant at P<0.05.

**Results**

The clinical profiles of the 3 populations tested are summarized in Table 1. The SSI and SBI groups showed a greater mean age and a higher frequency of hypertension than did the control group, as reported previously. When compared with the control group, the frequency of diabetes mellitus was significantly higher only in the SSI group. The total cholesterol and LDL cholesterol concentrations were significantly lower in the SSI group than in the control group, probably because of therapeutic manipulation. The Lp(a) concentration and other lipid data were not significantly different among the
3 groups. No significant difference was observed in the frequency of smokers between the groups.

Table 2 summarizes genotype and allele frequencies of the 3 genes. Each population was in Hardy-Weinberg equilibrium, indicating that no apparent bias was observed in these populations. The ACE allele frequencies were not different among SSI, SBI, control, and REF1, whereas the incidence of the minor allele of MTHFR in SSI was significantly higher than in SBI and control groups. The allele frequency of apo(a) in SSI was also significantly different from that in the control group. Since an association of allele \(v\) with higher Lp(a) concentration was evident (20.4 ± 2.9 mg/dL [with \(v\)] versus 5.4 ± 3.0 mg/dL [without \(v\)]; \(P<0.001\)), we categorized the apo(a) size polymorphism into 2 entities, with and without allele \(v\). SSI and SBI groups had more \(v\) alleles than did REF1 (Table 2).

This observation suggested modest effects of apo(a) and MTHFR on the pathogenesis of lacunar infarction. Since subjects with genetic predisposition were expected to have earlier onset of diseases, we then stratified the populations by the age of onset. When the populations were stratified into 3 subpopulations according to their ages, the younger subjects

<table>
<thead>
<tr>
<th>TABLE 1. Demographic Data of the Populations Studied</th>
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<tbody>
<tr>
<td>Age, y</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>66* (64, 68)</td>
</tr>
<tr>
<td>Sex, M/F</td>
</tr>
<tr>
<td>Hypertension, %</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
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<tr>
<td>Smoker, %</td>
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<tr>
<td>Total cholesterol, mmol/L</td>
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<td>HDL cholesterol, mmol/L</td>
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<td>LDL cholesterol, mmol/L</td>
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<tr>
<td>Triglycerides, mmol/L</td>
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<tr>
<td>Lp(a), mg/dL</td>
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</tbody>
</table>

Values in parentheses are 95% CIs of the mean.
*\(P<0.05\) vs control by \(t\) test or \(\chi^2\) test.

<table>
<thead>
<tr>
<th>TABLE 2. Genotype and Allele Frequencies of ACE, MTHFR, and Apo(a)</th>
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<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>ACE</td>
</tr>
<tr>
<td>II</td>
</tr>
<tr>
<td>DI</td>
</tr>
<tr>
<td>DD</td>
</tr>
<tr>
<td>Allele D</td>
</tr>
<tr>
<td>MTHFR</td>
</tr>
<tr>
<td>CC</td>
</tr>
<tr>
<td>CT</td>
</tr>
<tr>
<td>TT</td>
</tr>
<tr>
<td>Allele T</td>
</tr>
</tbody>
</table>

Apo(a) size polymorphism

<table>
<thead>
<tr>
<th>Genotype frequency</th>
<th>SSI (n=74)</th>
<th>SBI (n=147)</th>
<th>Control (n=214)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NN</td>
<td>0 (0)</td>
<td>3 (0.02)</td>
<td>8 (0.04)</td>
</tr>
<tr>
<td>VW/VN</td>
<td>42 (0.60)</td>
<td>85 (0.59)</td>
<td>129 (0.67)</td>
</tr>
<tr>
<td>Vv</td>
<td>21 (0.30)</td>
<td>37 (0.26)</td>
<td>43 (0.22)</td>
</tr>
<tr>
<td>vV/vV</td>
<td>7 (0.10)</td>
<td>19 (0.13)</td>
<td>14 (0.07)</td>
</tr>
<tr>
<td>Without v†</td>
<td>42 (0.60)</td>
<td>88 (0.61)</td>
<td>137 (0.71)</td>
</tr>
<tr>
<td>With v</td>
<td>28 (0.40)</td>
<td>56 (0.39)</td>
<td>57 (0.29)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Allele frequency‡</th>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>0.09</td>
<td>0.17</td>
<td>0.18</td>
</tr>
<tr>
<td>V</td>
<td>0.68</td>
<td>0.61</td>
<td>0.66</td>
</tr>
<tr>
<td>v</td>
<td>0.23</td>
<td>0.22</td>
<td>0.16</td>
</tr>
</tbody>
</table>

*SSI vs control, \(P=0.03\) by \(\chi^2\) test.
†SSI vs REF1, \(P=0.01\); SBI vs REF1, \(P=0.005\) by \(\chi^2\) test.
‡SSI vs control, \(P=0.02\) by \(\chi^2\) test.
TABLE 3. Age-Dependent Change of the Apo(a) Allele ν Frequency

<table>
<thead>
<tr>
<th>Age, y</th>
<th>≤60</th>
<th>61–70</th>
<th>≥71</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30 (34/113)</td>
<td>33 (17/51)</td>
<td>20 (6/30)</td>
</tr>
<tr>
<td>SBI*</td>
<td>53 (17/32)</td>
<td>41 (22/54)</td>
<td>29 (17/58)</td>
</tr>
<tr>
<td>SSI*</td>
<td>63 (12/19)</td>
<td>35 (9/26)</td>
<td>28 (7/25)</td>
</tr>
</tbody>
</table>

Percentages of the subjects with allele ν are shown, with absolute values in parentheses (the number of the subjects with allele ν/the number of the total subjects) for 3 age groups.

*The linear association of allele ν frequency with the age group was significant by the Mantel-Haenszel test (SBI, P<0.03; SSI, P=0.02).

of both SBI and SSI had more allele ν of apo(a). In contrast, no such tendency was observed in the control group (Table 3). In support of this result, the relative risks were higher for both SBI and SSI in the younger subpopulations (Table 4). Although a similar tendency was seen for MTHFR in SSI (Table 4), no such age-dependent alterations were observed for the ACE polymorphism; the D allele frequency was 0.42, 0.37, and 0.38 for age ≤60, age 61 to 70, and age ≥71 years, respectively (P=0.66 by χ² test).

Multivariate estimation of risk factors by logistic regression analysis showed that age and hypertension were both potent risks for SBI and SSI. Allele ν of apo(a) had modest but still significant effects on both. MTHFR and diabetes mellitus were risks only for SSI (Table 5).

Discussion

Of the 3 genetic polymorphisms studied in this report, we found that the apo(a) size polymorphism was an independent risk factor for both SBI and SSI. In contrast, there was no evidence of association of the ACE D/I polymorphism with either SSI or SBI. The MTHFR polymorphism was associated only with SSI.

The D/I polymorphism in the ACE gene was repeatedly shown to be associated with plasma ACE activity, although the mechanisms involved are still unknown. A similar result was seen for MTHFR in SSI (Table 4), no such age-dependent alterations were observed for the ACE polymorphism; the D allele frequency was 0.42, 0.37, and 0.38 for age ≤60, age 61 to 70, and age ≥71 years, respectively (P=0.66 by χ² test).

Multivariate estimation of risk factors by logistic regression analysis showed that age and hypertension were both potent risks for SBI and SSI. Allele ν of apo(a) had modest but still significant effects on both. MTHFR and diabetes mellitus were risks only for SSI (Table 5).

Infarction. Markus et al28 studied this polymorphism in a small series of lacunar infarction cases (n=18) in which there was a positive association. However, this result was not replicated in another study using a larger population of lacunar infarction patients (n=130).29 In regard to SBI, 2 studies in Japan gave conflicting results. Watanabe et al21 showed no association of the ACE polymorphism with SBI in a smaller population (n=36), which is consistent with the present result. The other study on a Japanese population found that, in hypertensive patients only, the ACE D allele was associated with SBI.31 We could not replicate this finding; the D allele frequencies for SBI and SSI were 0.40 and 0.34, respectively, in our population (Table 2). The frequencies were not significantly changed after the stratification by the history of hypertension (0.41 and 0.30, respectively), and furthermore, they did not differ significantly from that for control (P=0.91 and P=0.08, respectively). Many factors, such as geographic location of the populations studied, differences in the categorization of hypertensives, and technical issues in genotyping, potentially account for the inconsistency. A well-controlled study on larger populations from different areas of Japan is necessary to obtain conclusive results.

Because of the age-dependent nature of the penetrance in cerebrovascular diseases, subjects with positive genetic risks might be mixed in control since it had a younger mean age.
Consequently, the effect of the ACE polymorphism on the infarctions might be masked. However, this was not likely in the present study because the allele frequency of the ACE D/I polymorphism in control did not change significantly according to age (see Results).

The present study showed that, in contrast to ACE, the apo(a) size polymorphism was a common risk factor for both SBI and SSI. High Lp(a) has been established as a risk factor for atherothrombosis, and therefore dyslipidemic function of Lp(a) might contribute to the pathogenesis of lacunar infarction as well. However, recent in vitro studies noted that apo(a) itself could interact with vascular endothelial cells,\textsuperscript{3,19–32} monocytes,\textsuperscript{33} and vascular smooth muscle cells,\textsuperscript{16} evoking various pathophysiological reactions. Although these functions of apo(a) were related to atherogenic events, they may disturb the integrity of the small artery wall, inducing lipohyalinotic or fibrinoid necrotic changes as well.

Although the Lp(a) concentration is determined largely by genetic factors, it is also known that several environmental factors, such as sex hormones\textsuperscript{34,35} and acute inflammations,\textsuperscript{34,36} influence its concentration. In addition, long-term storage of serum resulted in degradation of Lp(a), which caused problems in this kind of study.\textsuperscript{37} These unexpected noises might account for the failure to obtain a significant difference of Lp(a) among SSI, SBI, and control groups in the present study despite different allele \( \nu \) frequencies among the groups (Table 2). Actually, previous studies showed that mean Lp(a) concentrations were always slightly higher in lacunar infarctions than in controls, although the difference did not reach a significant level.\textsuperscript{26,27,38–40} Environmental noise in the Lp(a) concentration may explain, at least in part, such observations. In contrast, the apo(a) size polymorphism is fully determined by a genetic factor and is not affected by environmental factors at all. Since the apo(a) size polymorphism is a major determinant of Lp(a) concentration, it is probably a good marker for the long-term average Lp(a) concentration. In addition, Kang et al\textsuperscript{41} reported recently that apo(a) of different sizes showed different abilities to bind with mononuclear cells in vitro, implying that the apo(a) size itself may also be responsible for different pathophysiological outcomes caused by Lp(a).

The MTHFR protein with C677 allele was found to be labile to heat, causing mild homocyst(e)inemia.\textsuperscript{6} When it is inferred from the observation that severe homocyst(e)inemia is a strong risk factor for thrombotic disorders, this polymorphism has been assumed to be a risk factor for myocardial infarction and cerebral stroke.\textsuperscript{6–8} Accordingly, we selected the MTHFR polymorphism as a negative control in this study because it had been related to atherosclerotic infarction rather than lacunar infarction.\textsuperscript{42} However, we obtained an unexpected positive association of the MTHFR polymorphism with SSI. Potential heterogeneity of SSI might account for this association; as larger infarctions tend to present symptoms,\textsuperscript{43} they are more likely to be categorized in SSI. Consequently, some infarctions caused by the microatheroma-induced arterial occlusion at the proximal origin of perforating arteries were mixed with lipohyalinotic lesions in SSI, as suggested previously.\textsuperscript{43,44} SBI may be a useful entity in this respect because it is expected to consist of smaller lesions and thus to be more homogeneous in lacunar infarction.

A positive association of diabetes mellitus with lacunar infarction was observed.\textsuperscript{45,46} In addition, diabetes mellitus was also shown to be associated with SBI in a previous study.\textsuperscript{1} The inconsistency between the present and previous results can probably be explained by the different mean age of the subjects in the studies. When we took subpopulations of younger cases, the relative risk of those with diabetes mellitus for SBI increased from 1.1 (for the whole population) to 1.8 (for those aged \( \leq 65 \) years) and 2.8 (for those aged \( \geq 60 \) years). This implies that diabetes mellitus is a risk factor for SBI, especially in a younger population, as are the other genetic factors.

Case-control studies are known to be sensitive to sampling biases.\textsuperscript{47} To avoid stratification, we recruited reference populations in addition to a control. Genotype frequencies of the 3 polymorphisms were similar between control and REF; and REF\textsubscript{2} implying that these populations represent the general population well. In addition, the fact that there were greater effects of MTHFR and apo(a) in the younger populations supports the hypothesis that these genes have predisposing effects (Table 4). However, the effects were quite modest, and therefore we need to replicate the results in larger populations and/or under different study designs that are less vulnerable to sampling biases.\textsuperscript{48}

Despite these limitations, this study is unique in that it focused on the genetic risks for SBI, a clinically interesting entity in relation to vascular dementia. We proposed that the apo(a) size polymorphism was a risk factor for SBI, especially in younger patients. Pathophysiological backgrounds for these risk factors should be clarified in future studies.

Acknowledgments

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1886 Stroke September 1999


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