Lack of Association Between Variations of PDE4D and Ischemic Stroke in the Japanese Population

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Background and Purpose—After the first genomewide association study of ischemic stroke identified PDE4D as a susceptible gene, many replication studies have been conducted. However, the validity of the association has remained controversial because of the heterogeneity of both genetic markers and phenotypes.

Methods—We investigated the association between variations of PDE4D and ischemic stroke by 3 methods: single-marker, haplotype, and tag-single nucleotide polymorphism (SNP) analyses. In the single-marker analysis, we evaluated the association using 2 large case–control samples (1112 cases and 1112 control subjects in a sample obtained from Kyushu, Japan, and 1711 cases and 1786 control subjects in BioBank Japan) and a prospective cohort with 14 years of follow-up. These samples were analyzed both separately and pooled. Haplotype and tag-SNP analyses were performed using the 2 case–control samples together.

Results—In single-marker association tests, we found no significant association in the same direction among the 6 SNP reported in the initial study and ischemic stroke subtypes. Haplotype analysis revealed no significant association between the region around the 5′-end of the gene and combined atherothrombotic and cardioembolic infarction. Rs7730070, a SNP located around the 3′-end of PDE4D, showed the lowest nominal probability value by tag-SNP analysis but was not significant after adjustment for multiple testing (adjusted probability value = 0.36).

Conclusions—These results suggest that variations in PDE4D are not associated with ischemic stroke risk in the Japanese population. (Stroke. 2009;40:1245-1251.)

Key Words: cerebral infarct ■ genetics ■ PDE4D

Stroke is one of the most common causes of death and long-term disability around the world. Ischemic stroke is the most common form of stroke and is further subdivided into lacunar, atherothrombotic (ATI), and cardioembolic infarction (CEI). As for genetic contributions to the pathogenesis of ischemic stroke, twin and family studies1,2 suggested that stroke risk was mediated by both environmental and genetic factors. The first genomewide association study of ischemic stroke reported the phosphodiesterase 4D gene (PDE4D) as a susceptible gene using 864 cases and 908 control subjects in an Icelandic population.3 This study showed that the microsatellite marker AC008818−1 and 6 single nucleotide polymorphisms (SNPs) located in the 5′-end of the gene (SNP41, SNP45, SNP56, SNP87, SNP89, and SNP83) were significantly associated with ATI or with the combined ATI and CEI phenotype. Haplotype blocks B and C, which covered 260 kb around the 5′-end of the gene, were also associated, and the combination of the G allele of SNP45, the 0 allele of AC008818−1, and a common haplotype in block C led to the classification of individuals into at-risk, wild-type, and protective groups. Although the authors of the study showed that the affected individuals with the G0 haplotype had lower expression levels of some PDE4D isoforms, they could not find causative SNPs or haplotypes. Moreover, the biological role of PDE4D in ischemic stroke or the underlying atherosclerosis remained uncertain.

To our knowledge, 15 replication studies have been published on the association between SNPs in PDE4D and ischemic stroke.4–18 However, the results are still controversial. Of the 4 studies that examined associations between the 2 markers (AC008818−1 and SNP45) and combined ATI and...
CEI, none replicated the original findings.4–7 Among the 14 studies that examined at least SNP45, 4–17 only one found a nominal association with combined ATI and CEI.6 Other groups reported significant associations between different phenotypes and different SNPs in the 1.5-Mb region of the gene.6–13,19 There are thought to be several reasons for these inconsistencies among the results. The sample sizes in most studies were too small and had insufficient power to detect associations.20 Sampling biases of cases and controls may have distorted true associations. Several positive findings in different SNPs might reflect associations among hidden causative variants linked to the SNPs or to the G0 haplotype. The association between variants in PDE4D and ischemic stroke risk might differ among ethnic groups.

According to the recent published criteria, replication studies should examine the same SNP or a SNP in perfect or very high linkage disequilibrium with the prior SNP on the same or a very similar phenotype. They also should show similar magnitude of effect and significance in the same direction.21 Therefore, we performed single-marker association tests between 6 SNPs and the same subtypes of ischemic stroke as in the initial study and used a sufficient sample size. We also performed haplotype analyses in blocks B and C using tag-SNPs selected from the same regions. To examine the possibility of hidden causative SNPs, we additionally genotyped 190 tag-SNPs selected from the same regions. To examine the possibility of hidden causative SNPs, we additionally genotyped 190 tag-SNPs selected from the same regions. To examine the possibility of hidden causative SNPs, we additionally genotyped 190 tag-SNPs selected from the same regions.

For the prospective cohort study, we used a cohort population of 1112 cases of ischemic stroke and 1112 age- and sex-matched control subjects. Details on this population were described previously.22 Briefly, patients with ischemic stroke were recruited from 7 medical centers in and around Fukuoka City, Japan, in 2004. These included 491 cases of lacunar infarction, 369 of ATI, 136 of CEI, and 116 of undetermined subtype. Age (within 5 years) and sex-matched control subjects were selected from the 3328 participants of the Hisayama screening survey between 2002 and 2003. All case subjects were diagnosed by stroke neurologists on the basis of detailed clinical features and ancillary laboratory examinations such as brain imaging. The subtypes of ischemic stroke were determined on the basis of the Classification of Cerebrovascular Disease III proposed by the National Institute of Neurological Disorders and Stroke (NINDS-III).23

Another case–control sample was selected from the BioBank Japan project.24 This project was started in 2003 to collect a total of 300 000 cases who have at least one of 47 diseases by a collaborative network of 66 hospitals located throughout Japan. The registration of cases was based on diagnoses made by physicians at the affiliated hospitals. From June 2003 to March 2006, 7974 cases with ischemic stroke were registered. We selected 1711 cases diagnosed with ischemic stroke subtypes by brain imaging, the same as with the Kyushu sample. The subtypes included 1143 with lacunar infarction, 355 with ATI, and 213 with CEI. Control subjects were randomly selected from the subjects who were registered with BioBank Japan for other diseases.

For the prospective cohort study, we used a cohort population of the Hisayama study established in 1988.25 In this cohort, 2634 Hisayama residents aged ≥40 years and who had no history of stroke or coronary heart disease were enrolled in 1988 and continuously followed up for 14 years until the occurrence of cardiovascular disease or death. Among them, 1656 subjects participated in the examination between 2002 and 2003 and were used in the present study. During the 14-year follow-up, 67 events of first-ever ischemic stroke were observed.

Written informed consent was obtained from all study subjects. The study was approved by the ethics committees of the Graduate School of Medical Sciences at Kyushu University and the Institute of Physical and Chemical Research.

Clinical characteristics of 2 case–control samples are shown in Supplemental Table I, available online at http://stroke.ahajournals.org. In both samples, hypertension was defined as systolic blood pressure of ≥140 mm Hg or diastolic blood pressure of ≥90 mm Hg or current treatment with hypertensive medication.

SNP Selection and Genotyping

For the association study, we selected 6 SNPs that were significantly associated with ischemic stroke in the initial study: SNP41 (rs12153798), SNP45 (rs12188950), SNP56 (rs702553), SNP83 (rs966221), SNP87 (rs2910829), and SNP89 (rs1396476). In the haplotype analysis, we selected 16 additional tag-SNPs from the regions of blocks B and C defined in the initial study. For tag-SNP analysis, we selected 190 tag-SNPs from the 2.2-Mb region, including PDE4D. Tag-SNPs were selected from the Hapmap JPT data by the pairwise tagging method with the following criteria: r² > 0.8, minor allele frequency > 5%, and call rate > 75%.

Genomic DNA was extracted from peripheral blood leukocytes by a standard method. We genotyped SNPs using the multiplex polymerase chain reaction–based Invader assay26 (Third Wave Technologies) or TaqMan assays (Applied Biosystems) in a blind fashion to the clinical information of sample studies. All genotypes were called by visual inspection, and we determined genotype success as < 10 undetermined samples in a 384-well plate. When we failed to genotype more than one 384-well plate in a total of 16 plates, we excluded the SNP from further analyses. To validate the genotyping data, we genotyped 10 SNPs in 48 subjects using direct sequencing, and the concordance was 99.6%.

Statistical Analysis

We examined the association both by each population and by meta-analysis. We assessed case–control association analysis and Hardy-Weinberg equilibrium by χ² test or Fisher exact test, as appropriate. In the association analysis, we mainly used an additive model and also referenced dominant and recessive models. For an easy understanding of the risk direction, we calculated the OR and 95% CI of each SNP according to the risk allele in the initial study. In a meta-analysis of the single-marker association test, pooled estimates of the ORs for 2 case–control studies and one prospective study were obtained using a fixed-effect model. Heterogeneities across the population were estimated formally using Cochran’s Q test and the I² statistic. Haplotype analysis was performed using Haploview version 4.0 (Broad Institute). For the adjustment for multiple testing, we performed a random permutation test with 10 000 replications, linkage disequilibrium was calculated as D', and haplotype blocks were defined by Gabriel’s criteria.27

Results

Single-Marker Association Test

We initially performed single-marker association tests between the 6 SNPs reported in the initial study and the same ischemic stroke subtypes (Table 1). SNP45 and SNP41, which showed the most significant association in
the initial study, were monomorphic, and all individuals were homozygotes of the risk alleles in our population. In all samples, SNP83 showed no significant association with ATI. For the combined ATI and CEI subtypes, we found SNP56 to be significantly associated in the prospective cohort ($P = 0.02$; OR, 2.17; 95% CI, 1.14 to 4.11), but it was not associated in the 2 case–control samples. In the meta-analysis, we could not find a significant association between SNP56 and the combined ATI and CEI phenotypes. SNP89 showed a significant association in the Kyushu sample, but its risk was in the opposite direction of the effect ($P = 0.03$; OR, 0.62; 95% CI, 0.40 to 0.97). SNP89 was not significantly associated in the BioBank Japan sample and the prospective cohort, and we found no

<table>
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<tr>
<th>Table 2. Association Between SNPs Reported in the Initial Study and the Subtypes of Ischemic Stroke Among Japanese</th>
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Allele 1 indicates the risk allele in the initial study; AF, allele frequency of allele 1; Meta-analysis was performed using a fixed-effect model.

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Allele 1 indicates the risk allele in the initial study; Frequency, risk allele frequency; Due to the lack of hypertension status data, 22 ATI cases, 10 CEI cases, and 371 control subjects were excluded in the stratified analysis.
significant association with SNP89 in the meta-analysis. SNP87 was not associated with the combined ATI and CEI phenotypes in any of the samples. We also examined the associations of these SNPs with ischemic stroke or other subtypes in the 2 case–control samples (Supplemental Table II, available online at http://stroke.ahajournals.org). SNP56 showed nominal association with ATI in the Kyushu sample (P=0.02; OR, 1.27; 95% CI, 1.03 to 1.57) but was not associated in the BioBank Japan sample. The meta-analysis showed no significant association between ATI and SNP56. No other SNPs showed a significant association with any phenotype in the same direction as the initial study.

Stratified Analysis by Hypertension Status

Some replication studies showed significant associations between the SNPs in \textit{PDE4D} and ischemic stroke in subjects without hypertension.\textsuperscript{11,17} Thus, we evaluated the association between the 6 SNPs and the subtypes of ischemic stroke among the combined samples stratified by hypertension status (Table 2). However, none of the SNPs were associated with ATI or the combined ATI and CEI phenotypes even in the subjects without hypertension.

Haplotype Analysis

Because SNP45 and SNP41, which are key SNPs for haplotype construction in block B, were monomorphic in our population, we constructed haplotypes using SNP56 and 16 additional tag SNPs selected from the regions of blocks B and C (Table 3). In block B, none of the haplotypes were significantly associated with the combined ATI and CEI phenotypes. In block C, the most common haplotype, G-C-C-A-G, showed the lowest probability value, but the association was not significant after adjustment for multiple testing (adjusted \(P=0.33\)). There was no significant haplotype in the combined region of blocks B and C (data not shown).

Tag-SNP Analysis

To determine the possibility of a hidden causative SNP, we attempted to examine the associations between tag-SNPs in \textit{PDE4D} and ischemic stroke. We selected 190 additional tag-SNPs from the 2.2-Mb region that included \textit{PDE4D} and genotyped in combined samples of 2823 cases and 2898 control subjects. Because 14 SNPs did not pass our criteria, we finally analyzed 198 SNPs (the 6 reported in the initial study and 192 tag-SNPs). The genomic structure, case–control results, and linkage disequilibrium map of the 2.2-Mb region are shown in the Figure. Although the initial study showed a strong association around the region of blocks A to C, none of the SNPs in this region showed any association. The rs7730070 SNP, located around the 3’-end of \textit{PDE4D}, showed the lowest probability value (OR, 1.21; 95% CI, 1.06 to 1.37; \(P=0.0037\)). However, this SNP was not linked to the 5’-end of the gene that was the causative region in the initial study (Figure, C). Moreover, this association was not significant after adjustment for multiple testing (adjusted \(P=0.36\)).

Discussion

We examined the association between variations of \textit{PDE4D} and ischemic stroke using 2 independent large case–control samples and a population-based cohort. Using these samples, we tried to replicate the previous reports in 3 ways: a single-marker association test, haplotype analysis in blocks B and C, and tag-SNP analysis, which covered the entire \textit{PDE4D} gene region. Using 2 case–control samples consisting of 2823 cases and 2898 control subjects and a prospective cohort consisting of 1656 subjects, we found no significant association between the same SNPs and the same ischemic stroke subtypes in the single-marker tests. Similarly, no haplotypes in blocks B and C were found to be associated with the combined ATI and CEI phenotypes. Tag-SNP analysis could not find the hidden causative SNP in \textit{PDE4D}. From these results, we suggest that the common variants of \textit{PDE4D} did not confer risk for ischemic stroke, at least in the Japanese population.

Among the replication studies that examined variations of \textit{PDE4D} and ischemic stroke, the most probable reason for the inconsistent findings is that the small sample sizes
missed true associations of modest effect. Assuming our sample size, the allele frequencies of the SNPs in our control subjects, and the relative risks of the SNPs in the initial study, the power to detect associations at a significance level of 0.05 would be greater than 99% for SNP83 and SNP56, 98.3% for SNP87, and 69.7% for SNP89 in the case–control samples. In contrast, the statistical power of the prospective cohort was <30% for the 6 SNPs. However, a meta-analysis of these samples should increase the statistical power to detect the association. Therefore, if a true association exists, our study could detect the association between SNPs or haplotypes in PDE4D and ischemic stroke with high probability. A recent meta-analysis of 5216 cases and 6615 control subjects also showed that allele 0 of AC008818 and haplotype G0 carriers were associated with increased risk of ischemic stroke, but these associations become nonsignificant after exclusion of the initial study.28 These results indicate that the effect size of PDE4D variants on ischemic stroke, if it exists, may be small.

Because the initial study could not determine a causative SNP or haplotype in PDE4D, many replication studies have reported positive associations between different SNPs in PDE4D and various ischemic stroke subtypes.19 This indicates the possibility that hidden causative SNPs for ischemic stroke might exist in PDE4D. We analyzed a total of 198 tag-SNPs that covered the 2.2-Mb region, including PDE4D, but none of the SNPs were significant after adjustment for multiple testing. Because we selected tag-SNPs according to strict criteria, this analysis was able to capture the most common SNPs in PDE4D. Therefore, the previous positive findings of different SNPs may be attributable to chance.

One possible reason for the lack of association between PDE4D and ischemic stroke in our study was the difference in the ethnic background. Indeed, SNP45 and SNP41, which showed the most significant association with the combined ATI and CEI phenotypes in an Icelandic population, were monomorphic and all of the Japanese populations studied were homozygotes of the risk alleles in both SNPs. If SNP45 or SNP41 or absolutely linked variations are causative, we cannot estimate the effects of these variations on ischemic stroke, because all causative variations are homozygotes of risk alleles in both cases and control subjects.

Several limitations of this study should be discussed. First, we did not genotype the microsatellite marker, AC008818-1, in this study. However, we genotyped 16 tag-SNPs selected

Table 3. Continued

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Figure. Genomic structure, case–control results, and linkage disequilibrium map of the 2.2-Mb region, including PDE4D. A, Genomic structure around PDE4D. The white arrow indicates PDE4D reported by the initial study. B, Case–control association results for ischemic stroke among Japanese. The log 10-transformed probability values calculated by the Cochran-Armitage trend test are plotted on the y axis. “A” indicates block A; “B,” block B; “C,” block C in the initial study. C, Pairwise linkage disequilibrium map between SNPs. The strength of the linkage disequilibrium increases from white to black. A black inverse triangle indicates the location of rs7730070 in the map.
from the regions of blocks B and C according to strict criteria. Therefore, we believe that the effect of AC008818-1 could be sufficiently covered by haplotype analysis using tag-SNPs. Second, we could use only 1656 of 2634 subjects in the prospective cohort. Subjects who developed ischemic stroke would have a higher mortality rate than subjects who did not, and this may have resulted in the lower participation rate in this study. There is a possibility that the results of the prospective cohort might have been distorted by a survivorship bias. Third, the criteria used for classifying ischemic stroke were different between the initial study and ours. For classification of ischemic stroke, the initial study used the Trial of Org 10172 in Acute Stroke Treatment research criteria29 and we used NINDS-III. 23 However, these 2 classifications are similar to each other, and we diagnosed the subtypes of ischemic stroke by adequate laboratory examinations. We believe that there is no large difference in the phenotype definition.

In conclusion, although we performed a replication study between the variations of PDE4D and ischemic stroke risk using 2 independent large case–control samples and a population-based prospective cohort, we failed to replicate the associations. We suggest that variations of PDE4D do not confer risk for ischemic stroke in the Japanese population.

Acknowledgments

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Disclosures

None.

References

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