Association of Phosphodiesterase 4D Polymorphisms With Ischemic Stroke in a US Population Stratified by Hypertension Status

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Background and Purpose—Phosphodiesterase 4D (PDE4D) underlies the STRK1 linkage peak for stroke on chromosome 5q12 identified in Iceland. We tested association of 13 single-nucleotide polymorphisms (SNPs) and 1 microsatellite in a nested case-control sample of elderly white women (>65 years of age) from the Study of Osteoporotic Fractures (SOF) in the United States.

Methods—The genotypes of 248 women who experienced an incident ischemic stroke during an average of 5.4 years of follow-up were compared with 560 controls.

Results—Marginal associations with stroke ($P$<0.10) were found for 3 polymorphisms. Stratification of the population by hypertension markedly strengthened the association. SNPs 9 (hazard ratio [HR], 0.48; 95% CI, 0.26 to 0.91), 42 (HR, 1.73; 95% CI, 1.10 to 2.70), 219 (HR, 1.73; 95% CI, 1.13 to 2.64), and 220 (HR, 1.56; 95% CI, 1.05 to 2.32) showed significant association with stroke ($P$<0.05) under a dominant model in subjects without hypertension at baseline, and SNP 175 was significantly associated with stroke under an additive model (HR, 0.76; 95% CI, 0.59 to 0.98) in subjects with hypertension. Furthermore, the microsatellite AC008818-1 showed association with stroke only in the nonhypertensive subjects. Based on results in Iceland, specific haplotypes were tested in SOF, and stratification by hypertension also affected these association results.

Conclusion—These data are consistent with an association of the PDE4D gene with stroke in a non-Icelandic sample and suggest an effect of hypertension status. (Stroke. 2006;37:1385-1390.)

Key Words: genetics ■ hypertension ■ polymorphism ■ stroke

Recently, the phosphodiesterase 4D (PDE4D) gene has been identified as a risk factor for ischemic stroke.1 Gretarsdottir et al conducted a whole genome scan and found a linkage peak (log of odds = 4.4) on chromosome 5q12.1 Fine-mapping and association studies identified PDE4D as the gene linked to ischemic stroke in an Icelandic population.2 Furthermore, mRNA levels of several PDE4D isoforms in transformed B lymphocytes varied significantly between stroke cases and controls.3

PDE4D is a large gene that spans 1.5 Mb and has ≥22 exons and 8 splice variants.3–4 PDE4D hydrolyzes cAMP and is expressed in multiple tissues, including brain, lung, kidney, macrophages, monocytes, B and T lymphocytes, and vascular smooth muscle cells,4,5,9 with varying expression patterns of the splice variants.3 Several studies have reported PDE4D involvement in inflammation, cell proliferation, and migration, processes implicated in stroke occurrence.6–10

The association of specific PDE4D single-nucleotide polymorphisms (SNPs) and haplotypes with stroke was initially identified in an Icelandic population.2 We looked for associations in a more diverse population, the Study of Osteoporotic Fractures (SOF),11 a prospective study begun in 1986. All of the subjects are women of European ancestry living in 1 of 4 US cities who were ≥65 years of age at time of recruitment. We used a nested case-control design comparing individuals with incident ischemic stroke with approximately twice as many controls and genotyped 13 SNPs and 1 microsatellite (Figure 1). These polymorphisms map to 2 regions of the gene and were chosen based on the strength of association in the Icelandic population. The A region, at the 5’ end of the gene, includes the putative promoter and first intron. The D region includes introns near the middle of the gene and may overlap regulatory elements for the shorter splice variants.

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Materials and Methods

Subjects

SOF recruited ambulatory women between 1986 and 1988 from 4 clinical centers (Portland, Ore; Minneapolis, Minn; Baltimore, Md; and the Monongahela Valley, Pa). The study group consists of 9704 white women without bilateral hip replacement or earlier hip fracture at time of recruitment. Cases experienced incident adjudicated ischemic strokes (n = 248) before the censor date in November 1998. Controls, 2:1 to cases, did not have a prevalent stroke or during a mean follow-up of 5.4 years and were randomly chosen, including as many as possible with biomarker information (n = 560). Hypertension was defined at baseline as supine systolic blood pressure (BP) >160 mm Hg or diastolic BP >90 mm Hg or the use of thiazide diuretics. Although current guidelines recommend 140/90 as the hypertension definition, we used the original SOF definition to increase stringency of the definition. Individuals who died during follow-up were included. The study was approved by the institutional review boards, and all women provided written informed consent.

The Icelandic population has been described previously. For comparison with SOF stroke, some unpublished Icelandic data are summarized in supplemental Table I, available online at http://stroke.ahajournals.org (see Table 4).

Genotyping

Genotypes for microsatellite AC008818-1 were generated using Applied Biosystems (AB) Genescan and Genotyper software after allele determination by fragment sizing capillary electrophoresis on an AB 3100 Genetic Analyzer (Applied Biosystems). Eight SNPs were genotyped using allele-specific real-time polymerase chain reaction (PCR) and detection by SYBR Green (Molecular Probes) on an AB 5700 using a modification of Germer et al. The samples underwent extensive quality control.

Five SNPs were genotyped by an immobilized probe-based assay. We developed multitarget genotyping assays for candidate markers from various pathways implicated in atherosclerotic and thrombotic disease, including SNPs in the PDE4D gene. Briefly, 15 ng of genomic DNA were amplified in a multiplex PCR with 5'-biotinylated primers. The PCR products were hybridized to a linear array of immobilized oligonucleotide sequence-specific probes and detected by horseradish peroxidase–mediated colorimetry. SNP nomenclature is as described in supplemental Table II in Gretarsdottir et al.

Statistical Analyses

Genotype and allele frequencies were determined by counting, and a χ² or exact test was used to assess departure from Hardy–Weinberg equilibrium (HWE). SNPs were tested for association with incident stroke using the Cox proportional hazard model. Adjusted analyses included age, weight, diabetes, and smoking as covariates. Hypertension is a major risk factor for stroke and may obscure lesser genetic effects. In consideration of that potential effect, hypertension stratification was decided on a priori, and statistical interaction testing was conducted for completeness. Pairwise linkage disequilibrium (LD) was calculated by the expectation-maximization algorithm (EM). Analyses were performed using SAS statistical software (version 8.2; SAS Institute Inc).

The association of multiple linked SNPs (within-gene haplotypes) with case/control status in the SOF sample was tested with the likelihood ratio test after frequencies were estimated by EM. SNP haplotype associations in Iceland were computed by EM as imple-
Stratification by Hypertension

After stratification by hypertension, 4 SNPs showed significant association (P<0.05) with stroke in women without hypertension (SNPs 9, 42, 219, and 220 under additive or dominant models; SNP 42 under a recessive model). SNP 175 was significantly associated with stroke in hypertensive subjects (Table 2). The data presented were adjusted for age, diabetes, smoking, and weight. Unadjusted results were very similar (data not shown). Supplemental Table II shows allele frequencies after stratification by hypertension status.

None of the microsatellite AC008818-1 alleles were significantly associated with stroke in the unstratified SOF population, but 2 alleles were significant after stratification: alleles 0 (10-repeats; relative risk [RR], 0.62; P=0.038) and −4 (9 repeats; RR, 1.35; P=0.031; Table 3).

Haplotype Analyses

For comparison with the Icelandic2 results, we examined the association between the SNP 45/microsatellite haplotype and stroke. In contrast to the Icelandic results (RR, 2.07; P=7.2×10^{-4} for G/0 relative to A/X; X=not 0 allele), the haplotype was significantly associated with stroke only in SOF subjects without hypertension (G/0 relative to A/X; RR, 0.46; P=0.003), and the association was in the opposite direction. However, the haplotype provided little change in

| TABLE 2. Associations Between PDE4D SNPs and Stroke in SOF After Stratification by Hypertension and Adjustment for Age, Diabetes, Smoking, and Weight |
|-----------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| SNP              | Additive HR  | P Value | HR  | P Value | HR  | P Value | HR  | P Value | HR  | P Value | HR  |
|                  | Dominant     | Without Hypertension |  | With Hypertension |  |  |  |  |  |  |
|                  |  |  |  |  |  |  |  |  |  |  |  |  |
| Case Control     |  |  |  |  |  |  |  |  |  |  |  |  |
| Baseline age (years) | 73.9±5.9 | 248 | 70.3±4.5 | 560 | 1.59* | <0.0001 |  |
| Weight (kg) | 67.6±12.2 | 248 | 68.1±12.9 | 560 | 0.998* | 0.969 |  |
| Diabetes | 19.2% | 245 | 4.3% | 558 | 2.70 | <0.0001 |  |
| Hypertensive | 56.5% | 248 | 32.7% | 560 | 1.71 | <0.0001 |  |
| Current smokers | 8.5% | 246 | 7.9% | 559 | 1.27 | 0.296 |  |

*Per 5 years or per 10 kg.
the estimated odds ratio (OR) or P value more than the microsatellite alone in the SOF sample.

Estimated haplotypes for 6 SNPs in the D region and 5 SNPs in the A region were examined for association with stroke in the SOF sample, based on results in Iceland. The Icelandic haplotypes were not significantly associated in the SOF sample; the converse was also true. In the A region, 1 haplotype, GATAA, which is the same as the stroke-associated Icelandic haplotype except for SNP 9 (A allele in Iceland; G allele in SOF), achieved a P value ≤0.05 (Table 4). The direction of association was opposite in the 2 populations. After stratification by hypertension, the GATAA haplotype remained the sole significant one (P=0.015; OR, 0.43) in nonhypertensives, and no haplotypes were significantly associated with stroke in hypertensives.

For region D, several haplotypes in SOF stroke showed association with a nominal P value ≤0.05 but only 1 with frequency >1% in both cases and controls (Table 4). This haplotype differed from the Icelandic haplotype at 3 positions, but both conferred increased risk. In nonhypertensive subjects, 3 haplotypes had frequencies >1% and P<0.05. In hypertensive subjects, 1 haplotype had a frequency >1% and P value <0.05.

We investigated LD patterns in the SOF stroke population compared with the Icelandic stroke population. Figure 2 shows LD observed in controls of each population. Some SNP pairs exhibited LD differences between populations as measured by r². For example, the r² between SNP 42 and 45 was 0.49 in Iceland and 0.32 in SOF. Interestingly, both SNP 42 and 45 were significantly associated with stroke in Iceland but only SNP 42 in SOF. The LD in cases showed similar differences (data not shown).

Discussion

Age, diabetes, and hypertension were associated with an increased risk of stroke in this nested case-control study. Smoking was not associated with stroke, perhaps because of the low prevalence (~8%) in the sample. SNP 9 and SNP 222 did not conform to HWE proportions. Extensive sequencing of the SNP regions and the observation that 2 other white studies using the same genotyping reagents were in HWE for both SNPs (data not shown) suggest that the modest departures from HWE observed here are likely attributable to chance (type 1 error).

Associations with the microsatellite and 4 SNPs were seen in the SOF stroke population when stratified on hypertension, mainly in the nonhypertensive subjects. Tests for interaction

### TABLE 3. Microsatellite AC008818-1 Allele Frequencies in the SOF Stroke Population

<table>
<thead>
<tr>
<th>Allele</th>
<th>Number of Repeats</th>
<th>Control Frequency</th>
<th>Case Frequency</th>
<th>RR</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Unstratified</td>
<td>Without Hypertension</td>
<td></td>
<td>Hypertension</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Frequency</td>
<td>Case</td>
<td>Frequency</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Frequency</td>
<td>Case</td>
<td>Frequency</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Frequency</td>
<td>Case</td>
<td>Frequency</td>
</tr>
<tr>
<td>−8</td>
<td>8</td>
<td>0.164</td>
<td>0.174</td>
<td>1.06</td>
<td>0.627</td>
</tr>
<tr>
<td>−4</td>
<td>9</td>
<td>0.197</td>
<td>0.203</td>
<td>1.03</td>
<td>0.805</td>
</tr>
<tr>
<td>0</td>
<td>10</td>
<td>0.270</td>
<td>0.234</td>
<td>0.86</td>
<td>0.120</td>
</tr>
<tr>
<td>4</td>
<td>11</td>
<td>0.202</td>
<td>0.223</td>
<td>1.11</td>
<td>0.333</td>
</tr>
<tr>
<td>8</td>
<td>12</td>
<td>0.158</td>
<td>0.165</td>
<td>1.04</td>
<td>0.725</td>
</tr>
<tr>
<td>12</td>
<td>13</td>
<td>0.009</td>
<td>0.002</td>
<td>0.23</td>
<td>0.086</td>
</tr>
</tbody>
</table>

Case n=242; control n=553.

Allele 12 was not considered significant because of low frequency in the population.

*Reported as RR; Iceland Cases=988; controls=652.

### TABLE 4. Stroke-Associated Estimated Haplotypes in the SOF and Iceland Populations

<table>
<thead>
<tr>
<th>Region</th>
<th>Stratification</th>
<th>Haplotype</th>
<th>Population</th>
<th>Control Frequency</th>
<th>Case Frequency</th>
<th>OR</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>None</td>
<td>AATAA</td>
<td>Iceland</td>
<td>0.12</td>
<td>0.19</td>
<td>1.8*</td>
<td>0.00002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GATAA</td>
<td>SOF</td>
<td>0.095</td>
<td>0.066</td>
<td>0.67</td>
<td>0.048</td>
</tr>
<tr>
<td></td>
<td>−htn</td>
<td>GATAA</td>
<td>SOF</td>
<td>0.097</td>
<td>0.044</td>
<td>0.43</td>
<td>0.015</td>
</tr>
<tr>
<td>D</td>
<td>None</td>
<td>GTACCA</td>
<td>Iceland</td>
<td>0.126</td>
<td>0.170</td>
<td>1.42*</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>−htn</td>
<td>GTATAG</td>
<td>SOF</td>
<td>0.016</td>
<td>0.046</td>
<td>2.96</td>
<td>0.0006</td>
</tr>
<tr>
<td></td>
<td>+htn</td>
<td>GTATAG</td>
<td>SOF</td>
<td>0.019</td>
<td>0.049</td>
<td>2.63</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GTATAG</td>
<td>SOF</td>
<td>0.022</td>
<td>0.050</td>
<td>2.29</td>
<td>0.033</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATATCG</td>
<td>SOF</td>
<td>0.044</td>
<td>0.015</td>
<td>0.32</td>
<td>0.048</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GTATAG</td>
<td>SOF</td>
<td>0.014</td>
<td>0.053</td>
<td>3.86</td>
<td>0.006</td>
</tr>
</tbody>
</table>

Haplotypes with P values <0.05 and allele frequency of at least 1% in both groups are shown.

A region haplotype SNPs, in order: SNP 9-26-32-34-42; D region haplotype SNPs, in order: SNP 148-175-199-219-220-222.

*Reported as RR; Iceland Cases=988; controls=652.
between the genotypes and hypertension were significant for SNP 9. In the D region, 1 uncommon haplotype was significant in both nonhypertensives and hypertensives and may have a higher OR in hypertensives. A limitation of the study is that only thiazide diuretic data were collected, and other hypertension medication information is not available. However, based on the BP values and proportion of hypertensives, we do not believe misclassification to be a concern. Furthermore, Zee et al also observed a stronger association between stroke and PDE4D SNPs in normotensive men. The inconsistency between the SOF results and those from Iceland may have several explanations. PDE4D is a very large gene, spanning 1.5 Mb, with several hundred SNPs. The investigated polymorphisms span large distances (98 kb in the A region and 169 kb in the D region), and haplotypes may differ between populations. Discordant association data in different populations can reflect different patterns of LD for noncausal markers.

Another difference is that subtype information is limited in SOF, and the Icelandic associations were strongest for carotid and cardiogenic stroke. Recently, several groups reported association studies with PDE4D, most examining only the A region of the gene. Some reported an association between the SNPs or microsatellite and ischemic stroke in unstratified analyses, whereas some did not. All studies that examined stratification by stroke type found significant P values, although the findings were not always consistent.

The hypertension status of the Icelandic controls is unknown (personal communication), and a difference in the proportion of hypertensives may also have contributed to the inconsistent results. In the absence of hypertension, the effect of genetic variation in PDE4D may be easier to discern. (We note that the risk estimate for diabetes, another established stroke risk factor, was higher for normotensives than for hypertensives.) Alternatively, different stroke types may occur in the absence of hypertension, and stratification may result in a more homogeneous population.

Four of the SNPs and the microsatellite showed association with stroke in SOF after stratification by hypertension, although the direction of association was opposite relative to the Iceland results. Similarly, the SNP haplotypes significantly associated in the 2 populations were not identical. Although the results are consistent with the PDE4D gene being associated with ischemic stroke in a white population outside of Iceland, more work is necessary to find potentially functional or consistently informative polymorphisms.

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