ALOX5AP Gene and the PDE4D Gene in a Central European Population of Stroke Patients

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Background and Purpose—Recent evidence has implicated the genes for 5-lipoxygenase activating protein (ALOX5AP) and phosphodiesterase 4D (PDE4D) as susceptibility genes for stroke in the Icelandic population. The aim of the present study was to explore the role of these genes in a central European population of stroke patients.

Methods—A total of 639 consecutive stroke patients and 736 unrelated population-based controls that had been matched for age and sex were examined using a case-control design. Twenty-two single-nucleotide polymorphisms (SNPs) covering ALOX5AP were genotyped. For PDE4D, microsatellite AC008818-1 and 12 SNPs, which tag all common haplotypes in previously identified linkage disequilibrium (LD) blocks, were analyzed.

Results—A nominally significant association with stroke was observed with several SNPs from ALOX5AP, including SNP SG13S114, which had been part of the Icelandic at-risk haplotype. Associations were stronger in males than in females, with SG13S114 (odds ratio, 1.24; 95% CI, 1.04 to 1.55; \( P = 0.017 \)) and SG13S100 (odds ratio, 1.26; 95% CI 1.03 to 1.54; \( P = 0.024 \)) showing the strongest associations. No significant associations were detected with single markers and haplotypes in PDE4D. The frequencies of single-marker alleles and haplotypes differed largely from those in the Icelandic population.

Conclusions—The present study suggests that sequence variants in the ALOX5AP gene are significantly associated with stroke, particularly in males. Variants in the PDE4D gene are not a major risk factor for stroke in individuals from central Europe. Population differences in allele and haplotype frequencies as well as LD structure may contribute to the observed differences between populations. (Stroke. 2005;36:731-736.)

Key Words: genetics ■ stroke

Recently, the 5-lipoxygenase activating protein gene (ALOX5AP) on chromosome 13q and the phosphodiesterase 4D gene (PDE4D) on chromosome 5q have been reported to confer risk of stroke independently of conventional risk factors.\(^1\)\(^-\)\(^3\) The candidacy of these genes was identified through genome-wide scans and subsequent case-control association studies in individuals from Iceland. Both genes have been implicated in atherosclerosis.

ALOX5AP encodes 5-lipoxygenase–activating protein, which is required for the synthesis of leukotrienes.\(^4\) Leukotrienes are secreted by various types of inflammatory cells that cluster at the injured sites in blood vessels and are implicated in the progression of atherosclerosis.\(^5\)\(^-\)\(^7\) In the study from DeCode, a single ALOX5AP haplotype (termed HapA) was found to double the risk of myocardial infarction (MI) and stroke in patients from Iceland. Another haplotype (termed HapB) was associated with MI in individuals from the United Kingdom, but patients with stroke were not investigated.\(^5\)

PDE4D encodes phosphodiesterase 4D, which is a member of a large super-family of cyclic nucleotide phosphodiesterases. Phosphodiesterase 4D degrades second messenger cAMP, which is a key signal transduction molecule in different cell types, including inflammatory, vascular endothelial, and smooth muscle cells.\(^8\)\(^-\)\(^10\) Low levels of cAMP enhance cell migration and proliferation,\(^11\) processes that are proatherogenic. Gretarsdottir et al\(^2\) found that specific single markers and haplotypes at PDE4D are associated with carotid and cardiogenic stroke but not with other stroke subtypes.

Although of great potential importance, these findings need to be replicated in other populations. Therefore, we set up to evaluate the candidacy of ALOX5AP and PDE4D as susceptibility genes for stroke in a large population of stroke patients and matched controls from central Europe using single-marker and haplotype association tests and a case-control design.
Subjects and Methods

Study Sample
The study population consisted of 639 patients (mean age 65±18.2 years; 403 men) consecutively recruited from a single stroke unit (Department of Neurology, Klinikum Großhadern, University of Munich, Germany) that serves as a major referral center in the area covering a population of ~4 million people. Thus, for practical purposes, these patients may be considered unrelated. All patients underwent computed tomography of the head for initial imaging and an ECG immediately after hospital admission. Duplex sonography of the carotid arteries was performed in all patients. A total of 492 (77%) patients received an MRI including diffusion-weighted images, which was usually performed within 3 days after admission. Magnetic resonance angiography was obtained in 251 (39%) cases. Transcranial Doppler sonography and transthoracic echocardiography were done in >90% and 60% of the patients, respectively. Holter monitoring and transesophageal echocardiography were done if appropriate.

Ischemic stroke was diagnosed in 601 patients and was further classified into stroke subtypes using the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification. A total of 186 patients had large vessel disease (or “carotid stroke,” as referred to by the local ethics committee.

EDTA blood using standard protocols. The study was approved by the ethics committee.

The control group consisted of 736 unrelated individuals (mean age 62±11.7 years; 447 men), who were selected from the stroke. The control group was sampled to match stroke patients for age and sex, which was usually performed within 3 days after admission.

Magnetic resonance angiography was obtained in 251 (39%) cases. Transcranial Doppler sonography and transthoracic echocardiography were done in >90% and 60% of the patients, respectively. Holter monitoring and transesophageal echocardiography were done if appropriate.

Marker Selection and Genotyping
The nomenclature of markers used in the original studies was determined etiology was diagnosed, and in 141, the etiology re-

determined. For ALOX5AP, we selected all 22 single-nucleotide polymorphisms (SNPs) that had been used for haplotype analysis in the original study by the DeCode group. They cover the entire ALOX5AP gene by a mean distance of 1.9 kb between SNPs. For PDE4D, we selected 2 SNPs (SNP45 and SNP41) and 1 microsatellite marker (AC008818-1), which had shown the most significant association with stroke in the original study. We further identified an optimal set of haplotype tagging SNPs (htSNPs) that distinguished 95% of all chromosomes within linkage disequilibrium (LD) blocks “B” and “C” using a nested algorithm implemented in the program SNPtagger. Eight htSNPs were from block B (SNP73, SNP69, SNP67, SNP57, SNP59, SNP48, SNP45, and SNP41) and 4 htSNPs from block C (SNP34, SNP30, SNP20, and SNP6). Genotyping of SNPs was achieved by primer extension of multiplex polymerase chain reaction products with detection of allele-specific extension products by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF; Sequenom) mass spectroscopy. Microsatellite AC008818-1 was genotyped using fluorescently labeled oligonucleotide probes by ABI 3730 sequencer (Applera Corporation). Amplification of AC008818-1 marker alleles was obtained by using the sense primer 5′ TGCTTGGTGAAAGAATAACC 3′ and antisense primer 5′ GAGCCTGGGGTCTCCAGGAAT 3′. Marker sequences were obtained from the Genome database, and SNPs were selected from the initial publications. There were no deviations from Hardy–Weinberg equilibrium (tested by a χ² test), and all markers were genotyped with a high call rate (average 99%).

Statistical Methods
Allele and genotype frequencies were calculated for each locus and tested for Hardy–Weinberg equilibrium. Pairwise LD values were plotted using the software package Haploviz (Barrett; Whitehead Institute, 2003). The program PHASE version 2.0 was used for haplotype frequency estimation, which uses a Bayesian method accounting for the evolutionary relationship of estimated haplotypes.

Single-marker associations between phenotype and genotype were tested by logistic regression analyses (STATA statistical software package). The association of haplotypes was inferred by the haplotype trend regression method proposed by Zaykin et al. Individuals’ haplotype probabilities estimated by the phase estimation procedure are regressed against the phenotype state. We tested 2-, 3-, 4- and all marker haplotypes from adjacent SNPs in a sliding-window fashion, as well as using all different 2-marker combinations.

Markers and haplotypes significantly associated in the original studies, experiment-wise significance levels were evaluated by a permutation procedure, which corrects for the number of tests performed in a given marker set.

The power of our case-control sample to detect a similarly sized effect as in the original studies was determined by using the reported

| TABLE 1. Single-Marker Allelic Association in the ALOX5AP Gene With Markers Constituting HapA Reported Previously to be Associated With Stroke |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Individuals     | SNP             | Public Name     | Allele | Cases | Controls | OR   | 95% CI             | P Value |
| (No. of cases/controls) |                |                |        |       |          |      |                   |         |
| All (639/736)   | SG13S25        | G              |       | 89.4  | 90.1     | 0.93 | 0.73–1.19          | NS      |
|                 | SG13S114       | T              |       | 68.5  | 65.0     | 1.17 | 1.00–1.37          | 0.025   |
|                 | SG13S89        | G              |       | 94.7  | 96.0     | 0.75 | 0.53–1.07          | NS      |
| Males (408/447) | SG13S25        | G              |       | 89.8  | 89.7     | 1.00 | 0.74–1.37          | NS      |
|                 | SG13S114       | T              |       | 69.2  | 63.8     | 1.24 | 1.04–1.55          | 0.017   |
|                 | SG13S89        | G              |       | 94.9  | 95.4     | 0.91 | 0.59–1.41          | NS      |
| Females (241/289)| SG13S25        | G              |       | 88.8  | 90.1     | 0.82 | 0.54–1.23          | NS      |
|                 | SG13S114       | T              |       | 69.2  | 67.0     | 0.94 | 0.74–1.23          | NS      |
|                 | SG13S89        | G              |       | 94.3  | 96.9     | 0.52 | 0.28–0.97          | 0.040   |
|                 | SG13S32        | A              |       | 46.5  | 47.9     | 1.25 | 1.03–1.5           | 0.022   |

OR indicates odds ratio.
relative risks and risk allele frequencies at a 5% type I error rate under a multiplicative model.

Results

**ALOX5AP**

We first performed single-marker association tests with the 4 SNPs (SG13S25, SG13S114, SG13S89, and SG13S32) constituting HapA (ie, the haplotype that was associated with stroke and MI in patients from Iceland). As shown in Table 1, the T allele of marker SG13S114 was more frequent in our stroke cases compared with controls (P=0.025). This effect was most significant in males and not significant in women. Subgroup analysis for stroke subtypes revealed no significant association with stroke subtypes (data not shown).

Next, we looked at the HapA haplotype. As shown in Table 2, there was no significant association with HapA in our continental population of stroke patients. Also, association tests with males or females separately and with different stroke subtypes revealed no significant associations. Contrasting with the Icelandic study, HapA occurred more often in controls than in cases (P=0.025). This effect was most significant in males and not significant in women. Subgroup analysis for stroke subtypes revealed no significant association with stroke subtypes (data not shown).

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Because we could not replicate associations with HapA, we expected that there might be other unidentified haplotypes in ALOX5AP that confer risk to stroke in our population. Therefore, we performed different haplotype combination tests constructed from 2, 4, and all SNPs. Several haplotypes were significantly associated with stroke (minimum P value=0.004; supplemental Table I, available online at http://www.strokeaha.org; data not shown). However, after correction for multiple testing, associations were no longer significant. Single-marker analysis with the remaining SNPs (not included in HapA) revealed nominally significant associations between several markers (SG13S100, SG13S34, SG13S86, and SG13S96) and overall stroke (Table II, available online at http://www.strokeaha.org). However, after correction for multiple testing, associations were no longer significant.

To check for possible differences in LD structure between our German and the Icelandic sample, we determined the LD structure and haplotype diversity of ALOX5AP in our population (Table I). Most SNPs were in strong LD, and the LD block structure was similar to that in the Icelandic population (Figure 1).3

**PDE4D**

We started by performing single-marker association tests with SNP45, SNP41, and AC008818-1, which had shown the most significant associations with the combined carotid and cardiogenic stroke subtypes in the Icelandic population.2 No association was found with the overall population of stroke patients, the combined phenotype, or any of the stroke subtypes (Table 3 and data not shown). Again, the frequency of the original at-risk alleles was considerably higher in our controls than in the Icelandic control sample.2

Next, we constructed haplotypes using the same combination of markers (AC008818-1 and SNP45), which had shown

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**TABLE 2. Association of HapA (ALOX5AP) to Stroke**

<table>
<thead>
<tr>
<th></th>
<th>German Sample</th>
<th></th>
<th>Icelandic Sample2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Cases</td>
<td>% Controls</td>
<td>P value</td>
</tr>
<tr>
<td>HapA*</td>
<td>14.5</td>
<td>15.2</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Constituted by SNPs SG13S25 (G allele), SG13S114 (T allele), SG13S89 (G allele), and SG13S32 (A allele).3

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**Figure 1.** Pairwise LD between SNPs in the ALOX5AP gene region. A color-coded scale for D’ (a measure of LD strength) is provided.
TABLE 3. Single-Marker Allelic Association in the PDE4D Gene With Markers Reported Previously to be Associated With Combined Carotid and Cardiogenic Stroke

<table>
<thead>
<tr>
<th>Stroke phenotype (No. of cases/controls)</th>
<th>Marker</th>
<th>At-Risk Allele</th>
<th>Frequency (%)</th>
<th>OR 95% CI</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients (639/736)</td>
<td>SNP45</td>
<td>G</td>
<td>86.1/86.3</td>
<td>0.96</td>
<td>0.78–1.18 NS</td>
</tr>
<tr>
<td></td>
<td>SNP41</td>
<td>A</td>
<td>89.8/89.4</td>
<td>0.97</td>
<td>0.76–1.24 NS</td>
</tr>
<tr>
<td></td>
<td>AC008818</td>
<td>0*</td>
<td>29.2/29.0</td>
<td>0.95</td>
<td>0.82–1.12 NS</td>
</tr>
<tr>
<td>Combined carotid and cardiogenic stroke (n=329/736)</td>
<td>SNP45</td>
<td>G</td>
<td>87.5/86.3</td>
<td>1.11</td>
<td>0.85–1.45 NS</td>
</tr>
<tr>
<td></td>
<td>SNP41</td>
<td>A</td>
<td>90.2/89.4</td>
<td>0.91</td>
<td>0.66–1.24 NS</td>
</tr>
<tr>
<td></td>
<td>AC008818</td>
<td>0</td>
<td>26.0/29.0</td>
<td>1.02</td>
<td>0.84–1.25 NS</td>
</tr>
</tbody>
</table>

OR indicates odds ratio.
*At-risk allele in the Icelandic population.3

Discussion

In this study, we applied a 2-step replication approach. We first examined the most significant single markers and haplotypes that were associated with stroke in the Icelandic population. Analysis was then extended to additional markers and haplotypes.

We found no association with the Icelandic at-risk haplotype (HapA) of ALOX5AP. However, we found a significant association with 1 of the single markers (SG13S114) constituting this haplotype. Also, association between SG13S114 and stroke was stronger in males than in females, which is similar to the findings from Iceland. Finally, several other markers not contained in HapA showed a nominally significant association with stroke. Together, these findings suggest a weak but significant association between sequence variants in ALOX5AP and stroke in the present sample from central Europe.

The observed lack of association with HapA may relate in part to population differences in allele and haplotype frequencies. In support of this, the frequency of HapA was much higher in our continental sample of control individuals than in the Icelandic controls. Another explanation are different patterns of association of the high-risk allele with marker alleles and haplotypes.17–19 In fact, in the original study, haplotypes associated with MI were found to differ between the Icelandic and British population.1 However, interestingly, all haplotypes that had shown significant association contained either the T allele of SG13S114 or the A allele of SG13S100, both of which showed nominally significant P values in the current study. Further studies are needed to identify the causative sequence variant or variants in ALOX5AP.

The initial study provided data on an overall sample of stroke patients without detailed specification of stroke phenotypes. In the current study, we also looked at stroke subtypes but found no association with particular stroke etiologies. This observation suggests that the associations in the overall group are not related to associations with a single stroke subtype. Future studies with larger subgroups may specify the role of ALOX5AP in different stroke subtypes.

In the current study, no significant association was found between single markers or specific haplotypes of PDE4D and stroke. This applies to all subgroups including carotid stroke, cardiogenic stroke, and the combined carotid and cardiogenic stroke subtypes, which had shown the strongest associations in the Icelandic population. Assuming a multiplicative model and the relative risks of single markers and haplotypes reported by DeCode, the power of our sample to detect the

significant association with the combined carotid and cardiogenic stroke phenotypes in the Icelandic population. However, no associations were found (Table 4). In addition, we looked at all possible haplotypes constructed from 2, 3, 4, or all SNPs from blocks B and C using the full set of htSNPs. Again, no significant associations were found after correction for multiple testing (Table III, available online at http://www.strokeaha.org; data not shown). Single-marker analysis with the remaining markers (all but SNP45, SNP41, and AC008818-1) revealed nominally significant associations between SNP73 and cardiogenic stroke and between SNP57 and carotid stroke and the combined carotid and cardiogenic stroke subtype (Table IV, available online at http://www.strokeaha.org; data not shown). However, after correction for multiple comparisons, these associations were not significant.

To check for possible differences in LD structure between our German and the Icelandic sample, we determined the LD structure and haplotype diversity of PDE4D in our population (Table III). The LD between SNPs was less pronounced than in the Icelandic population, and the LD block structure slightly differed from that reported by DeCode (Figure 2).2

TABLE 4. Association of PDE4D Haplotypes Reported Previously to be Associated With Combined Carotid and Cardiogenic Stroke

<table>
<thead>
<tr>
<th>German Sample</th>
<th>Icelandic Sample3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haplotype</td>
<td>% Cases</td>
</tr>
<tr>
<td>AX*</td>
<td>14.3</td>
</tr>
<tr>
<td>GX*</td>
<td>56.8</td>
</tr>
<tr>
<td>GO</td>
<td>28.9</td>
</tr>
</tbody>
</table>

*X designates the joint set of alleles, excluding the original at-risk allele G of microsatellite AC008818-1.2 Therefore, GX haplotype is the composite of all haplotypes including the G allele of SNP 45, except for the GO haplotype and AX haplotype, including the A allele of SNP 45. Haplotype AG does not exist. In the study by Gretarsdottir et al, GX was wild-type, AX was protective, and GO was the at-risk haplotype.
same associations should be $>99\%$ (data not shown). Also, phenotyping of our patients was particularly careful because all cases were recruited from a single unit specialized in stroke care and because diagnostic classification was based on extensive investigations with a high proportion of Doppler sonography, ECG, and MRI performed. Thus, we conclude that the \textit{PDE4D} gene is probably not a major risk factor for overall stroke or stroke subtypes in our population.

Like the data from DeCode, our findings need to be interpreted cautiously. Our analysis of the \textit{PDE4D} gene was limited to single markers and htSNPs from the 5$'$ region of the gene. Thus, we cannot exclude the possibility of association with sequence variants in other regions of the \textit{PDE4D} gene. However, we consider this possibility unlikely because most markers that had shown significant associations in the Icelandic sample were located in the 5$'$ region of the gene.

Another possible limitation is the low number of markers used to characterize \textit{PDE4D}. Our set of htSNPs markers was designed to distinguish 95% of all haplotypes reported by DeCode. However, we found that the LD between single markers was less pronounced in our population, and the LD block structure differed slightly from that reported from Iceland. Thus, our set of htSNPs may have covered too little of the genetic variability in \textit{PDE4D} in our population. On the other hand, the higher LD reported by DeCode might well reflect a founder effect in the Icelandic population, which, as a relatively isolated population, has a restricted gene pool. In fact, a founder effect in Iceland could explain the initial association and the current lack of replication in an independent population.

In conclusion, the present study suggests that sequence variants in \textit{ALOX5AP} are in fact a significant risk factor for stroke, particularly in males. In contrast, the \textit{PDE4D} gene is no major risk factor for stroke in the central European population. Our findings emphasize differences in allele and haplotype frequencies as well as LD structure between populations which may account in part for the different association findings between studies.

Acknowledgments
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References


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