Polymorphisms in Inflammatory Genes and the Risk of Ischemic Stroke and Transient Ischemic Attack: Results of a Multilocus Genotyping Assay

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BACKGROUND: Single-nucleotide polymorphisms (SNPs) in inflammation-related genes have been linked to an increased risk of ischemic stroke. Most of these SNP results have not been replicated, however, and meta-analyses of the effects of inflammation-related genes are rare. We investigated 49 SNPs in 34 genes previously reported to be related to inflammation in our study. We tested 459 patients with acute ischemic stroke or transient ischemic attack and 459 controls individually matched by sex and age.

METHODS: We studied genetic variation by PCR analysis and subsequent hybridization to linear arrays of sequence-specific oligonucleotides. We used univariate conditional logistic regression analysis to test for associations of conventional vascular risk factors and the SNPs with stroke. Variables showing significant differences (P < 0.05) between cases and controls were included in a multivariate model. ROC curves were plotted to assess the contribution of genetic variation to stroke risk in addition to that of conventional risk factors.

RESULTS: Univariate regression analysis revealed 3 SNPs with significant allelic differences between patients and controls, which fulfilled the criteria for further analysis. Only one of these SNPs, the C5 (complement component 5) 2416A>G variant (rs17611), remained significant after the multivariate analysis (odds ratio, 0.585; P = 0.0037). ROC curve analysis revealed no contribution of this genetic variation to stroke risk.

CONCLUSIONS: We found evidence for an association of the 2416A>G polymorphism in the C5 gene with the risk for ischemic stroke. Our data suggest that the C5 gene particularly influences the risk for patients with microangiopathy.

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Inflammatory mechanisms are implicated at all stages of the pathogenesis of ischemic stroke. The inflammatory response has been linked to the development of atherosclerotic lesions (1) in the carotid arteries or the aorta, which are causative in 30% of ischemic strokes (2). Both humoral inflammation and cellular inflammatory processes are also involved in the response to injury in acute ischemic stroke (3). Furthermore, markers of inflammation are associated with many classic vascular risk factors, such as smoking, hypertension, diabetes mellitus, and hyperlipidemia (4).

Some studies have linked single-nucleotide polymorphisms (SNPs)6 in inflammation-related genes to an increased risk of ischemic stroke. In most cases, however, these results have not been replicated, and metaanalyses of the relationship of inflammation-related genes to stroke risk are rare (5).

We investigated 49 SNPs in 34 genes that had previously been associated with inflammatory diseases and evaluated their contribution to the risk of acute ischemic stroke and transient ischemic attack in a large cohort of consecutive patients and controls individually matched by sex and age.

Patients, Materials, and Methods

PATIENTS AND CONTROL INDIVIDUALS

The study included randomly selected patients who had experienced acute ischemic stroke or transient ischemic attack and had been documented in the Vienna...
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Stroke Registry (6, 7). The diagnosis had been established clinically (8), and all patients had undergone cranial computed tomography or magnetic resonance imaging examinations. The patients were carefully documented according to a standardized protocol (6) with respect to conventional risk factors, medical history, laboratory and technical investigations, and classification of the cerebrovascular event, including classification of the subtype according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria (6) and measurement of stroke severity according to validated scales. We excluded both patients with hemorrhagic stroke, sinus thrombosis, or rare causes of stroke (e.g., arterial dissection) and patients from whom written informed consent could not be obtained.

Patients were individually matched to controls by sex and age (± 3 years; the difference was 4 years in 3 cases and 5 years in 1 case). The control individuals were participants in an official health service program of the City of Vienna and came from the same geographic region. They were free of clinically manifested vascular disease and reported an absence of vascular disease in first-degree relatives.

Written informed consent to participate in the study was obtained from all individuals. The study complied with the Declaration of Helsinki and was approved by the local ethics committee.

DEFINITIONS
Arterial hypertension was defined as a history of arterial hypertension, blood pressure values >140/90 mmHg (measured in patients 1 week after the qualifying event), or use of antihypertensive medication. Presence of diabetes mellitus was defined by fasting blood glucose concentrations >6.94 mmol/L, a history of diabetes, or treatment with antidiabetes medication. Initial blood glucose concentrations were measured at the time of admission, and the fasting blood glucose concentrations were generally first measured on the first workday after admission. Hyperlipidemia was defined as a fasting total serum cholesterol concentration >5.18 mmol/L, a history of hyperlipidemia, or use of lipid-lowering medication.

GENOTYPING
Blood was collected by venipuncture into citrate-containing tubes and frozen at −20 °C within 3–5 h after sampling. DNA was extracted with the Puregene® DNA-isolation kit (Qiagen/Gentra Systems). We used a PCR-based inflammatory-marker panel developed by Roche Molecular Systems to genotype DNA samples (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol55/issue1; for prevalence, see Table 2 in the online Data Supplement), essentially as previously described (9). The SNPs on the strip system represent a selection of genetic variants, essentially all of which had been associated with inflammatory diseases before the development of the strips. In brief, a multiplex pool of biotinylated primer pairs was used to amplify genomic DNA. The amplified fragments in each PCR-product pool were hybridized to sequencesspecific oligonucleotide probes that had been immobilized in a linear array on nylon membranes. Hybridization was detected colorimetrically. The linear arrays were then scanned on a flatbed imager, and proprietary software from Roche Molecular Systems was used to analyze the images and assign genotypes. Images were evaluated independently by 2 individuals who had been blinded to the clinical data.

STATISTICAL METHODS
We used the program at http://kursus.kvl.dk/shares/vetgen/Popgen/genetik/applets/kitest.htm to test genotype frequencies for conformity to the Hardy–Weinberg equilibrium.

To investigate the influence of each polymorphism (dominant and recessive models) and conventional risk factors (hypertension, hyperlipidemia, diabetes, and smoking), we carried out univariate conditional logistic regression analyses after controlling for the matching variable of age. In this analysis, the test statistics conform to a χ² distribution with 1 degree of freedom. Odds ratios (ORs), 95% CIs, and P values were calculated.

Variables showing statistically significant (P < 0.05) differences between patients and controls were further analyzed in a stepwise multiple conditional logistic regression model. Additional criteria for the inclusion of SNPs in the multivariate analysis were no deviation from the Hardy–Weinberg equilibrium, a minor-allele frequency of >20%, or a variant homozygote frequency of >5%.

The threshold to enter or stay in the model was set to P = 0.05. We chose this “liberal” approach to minimize false-negative associations, because the estimated effect of a single SNP on polygenic stroke is expected to be modest.

We graphed ROC curves to explore the relationship between the sensitivity and the specificity of a clinical test for all possible cutoff points.

We plotted 2 ROC curves, one for risk factors only and one for the resulting model of risk factors and genetic variants, and used a nonparametric approach to compare the areas under the 2 ROC curves (10).

To estimate power, we assumed that the case and control groups were independent (χ² test with equal group sizes). A χ² test with a 0.05 2-sided significance level would have an 85% power to distinguish a genotype occurring at a frequency of 0.1 from one occurring
at 0.17 (patients vs controls) for a group sample size of 460 each (OR, 1.8). With a frequency of 0.3 for any genotype in the patient group and 0.4 in the control group, we reached 87% power with a sample size of 460 in each group (OR, 1.6).

The data were analyzed with SAS, version 9.1 (SAS Institute), and R, version 2.6.

Results

We genotyped 49 inflammation-related SNPs for 459 stroke patients and 459 healthy control individuals (see Table 1 in the online Data Supplement). The baseline characteristics of the patients and controls are summarized in Table 1. Of the 49 SNPs, 29 featured minor-allele frequencies of <20%. Five SNPs located in the IL6\(^2\) [interleukin 6 (interferon, beta 2)], C5 (complement component 5), ADRB2 (adrenergic, beta-2-, receptor, surface), IL9 (interleukin 9), and CXCL12 [chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1); formerly SDF1] genes showed allelic differences between patients and controls that were statistically significant (see Table 3 in the online Data Supplement) in the recessive univariate model, in addition to the risk factors of smoking, hyperlipidemia, hypertension, and diabetes mellitus. Three SNPs located in the ADRB2, TGF\(\beta\)1 (transforming growth factor, beta 1), and NOS2A (nitric oxide synthase 2, inducible A) genes showed a statistically significant frequency difference in the dominant model.

The IL9 4244C>T and CXCL12 880G>A SNPs were excluded from further analysis because the genotype frequencies deviated from the Hardy–Weinburg equilibrium in the controls (\(P < 0.001\)). Two SNPs (in NOS2A and TGF\(\beta\)1) were statistically significant (\(P < 0.05\)) in a dominant model, but the recessive and dominant models for the SNPs for these 2 genes showed incongruent associations with stroke: In the dominant model, both the NOS2A and TGF\(\beta\)1 variants appeared to confer a reduction in risk, whereas they seemed to confer an increased risk in the recessive model (see Table 3 in the online Data Supplement). Therefore, these 2 SNPs were excluded from further analysis.

The remaining 3 SNPs (in the IL6, C5, and ADRB2 genes) were included in a stepwise conditional logistic regression analysis along with the vascular risk factors. Besides smoking, hypertension, diabetes mellitus, and hyperlipidemia, only the C5 2416A>G variant remained significant (OR, 0.585; 95% CI, 0.41–0.84; \(P = 0.0037\)) (Table 2). Allowing interaction between the C5 variant and the vascular risk factors did not change the results (data not shown). The C5 2416A>G variant showed no clear contribution to risk in addition to that of the conventional vascular risk factors, and the difference in the areas under the ROC curves was not significant (0.760 vs 0.766; \(P = 0.300\)) (Fig. 1).

A subgroup analysis of patients with macroangiopathy (n = 86), cardioembolic stroke (n = 75), microangiopathy (n = 96), and stroke of unknown etiology (n = 200) showed that the G allele of the C5 2416A>G SNP conferred an OR of 0.274 (95% CI, 0.10–0.96; \(P = 0.0426\)) for lacunar stroke (see Table 4C in the online Data Supplement).

We identified statistically significant associations with SNPs in the macroangiopathy and microangiopathy groups, as well as in the group of patients with unknown etiology: in the macroangiopathy group, CCR5 [chemokine (C-C motif) receptor 5] SNP 59029G>A (OR, 0.221; 95% CI, 0.07–0.7; \(P = 0.0105\)) and CCL11 [chemokine (C-C motif) ligand 11; formerly SLYA11] SNP 361G>A (OR, 6.19; 95% CI, 1.81–20.8; \(P = 0.0001\)) (Table 3).

### Table 1. Baseline characteristics of the patients and control individuals.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients (n = 459)</th>
<th>Controls (n = 459)</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age, years</td>
<td>59</td>
<td>59</td>
<td>NS*</td>
</tr>
<tr>
<td>Female sex, n (%)</td>
<td>198 (43)</td>
<td>198 (43)</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>330 (72)</td>
<td>224 (49)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hyperlipidemia, n (%)</td>
<td>342 (75)</td>
<td>380 (83)</td>
<td>0.002</td>
</tr>
<tr>
<td>Diabetes n (%)</td>
<td>131 (29)</td>
<td>21 (5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Current cigarette smoking</td>
<td>182 (40)</td>
<td>89 (19)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* NS, not statistically significant.

### Table 2. Stepwise conditional logistic regression with the C5 2416A>G SNP and vascular risk factors.

<table>
<thead>
<tr>
<th>Variable</th>
<th>(P)</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.1319</td>
<td>1.202</td>
<td>0.95–1.53</td>
</tr>
<tr>
<td>Smoking</td>
<td>&lt;0.0001</td>
<td>3.424</td>
<td>2.32–5.02</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>0.002</td>
<td>0.506</td>
<td>0.33–0.77</td>
</tr>
<tr>
<td>Hypertension</td>
<td>&lt;0.0001</td>
<td>2.270</td>
<td>1.60–3.22</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>&lt;0.0001</td>
<td>9.961</td>
<td>5.24–18.9</td>
</tr>
<tr>
<td>C5 2416A&gt;G</td>
<td>0.004</td>
<td>0.585</td>
<td>0.41–0.84</td>
</tr>
</tbody>
</table>

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\(^2\) Human genes: IL6, interleukin 6 (interferon, beta 2); C5, complement component 5, ADRB2, adrenergic, beta-2-, receptor, surface; IL9, interleukin 9; CXCL12, chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1); TGF\(\beta\)1, transforming growth factor, beta 1; NOS2A, nitric oxide synthase 2, inducible A; CCR5, chemokine (C-C motif) receptor 5; CCL11, chemokine (C-C motif) ligand 11; IL10, interleukin 10; SELE, selectin E; PRKCH, protein kinase C eta.
0.0032); in the microangiopathy group, IL6 SNP 987G>C (OR, 13.42; 95% CI, 2.00–90.0;  \( P = 0.0075 \)) and IL10 (interleukin 10) SNP 8700C>G (OR, 13.42; 95% CI, 2.00–90.0;  \( P = 0.0048 \)); in the stroke of unknown etiology group, ADRB2 SNP 1666C>G polymorphism in the C5 gene was not associated with the risk of ischemic stroke. \( 2416A \) >G SNP to stroke risk in addition to the classic risk factors. Importantly, the presence of the G allele as a risk factor for lacunar stroke showed an OR similar to what we have also observed for the total stroke population. So far, only a few replicable associations of SNPs with the risk of ischemic stroke have been reported: associations with genes involved in endothelial function, including those regulating the renin–angiotensin system and endothelial nitric oxide (15). Recently, a large-scale genetic epidemiologic study showed that SNPs in the PRKCH (protein kinase C eta) gene were significantly associated with lacunar infarction (16). One small study investigated the influence of one candidate inflammatory-gene variant (the IL6 987G>C polymorphism) on stroke risk and found an association with lacunar stroke (17). Our study is the first to report an association between the C5 2416A>G variant and lacunar stroke. We also detected associations of other SNPs with risk in other subgroups of stroke; however, because

The complement system consists of a family of plasma proteins that mediate opsonization and induce inflammation. C5 has a central role in the complement cascade. Two cleavage products are generated after cleavage by C5 convertase. C5b interacts with other complement proteins to form the membrane attack complex, which consists of a lytic pore that leaks the intracellular contents. C5a, the smaller fragment, is a potent inflammatory molecule. Experimental studies have shown that C5a and the membrane attack complex directly stimulate adhesion molecule production and the generation of chemokines by endothelial cells, thereby promoting proinflammatory mechanisms at several cellular levels (11).

A previous study found increased serum concentrations of C5 in individuals with the 2416AA homozygous genotype (12). The same study showed higher stages of fibrosis in hepatitis C patients who were homozygous carriers of the A allele. In addition, increased C5 concentrations have recently been associated with increased cardiovascular risk in patients with atherosclerosis (13). Our findings of an apparently lower risk for ischemic cerebrovascular events among carriers of the GG genotype are consistent with these data. Through C5a and C5b, C5 may represent a modifier of chronic inflammatory diseases, and carriers of the AA genotype may be at higher risk.

Berger et al. recently carried out a study with the same genotyping system as ours and reported that the 2416A>G polymorphism in the C5 gene was not associated with the risk of stroke (14). These investigators analyzed the contribution of 106 SNPs in 63 candidate genes in a study of 2 large independent German cohorts, the smaller of which served as the replication cohort. The differences in findings between our study and the study of the German patients could be due to clinical differences in the patient populations. Ethnic differences could also be responsible, even in central Europe. In addition, the German study did not include smoking status, and certain SNPs could represent significant risk factors, depending on the smoking status. We therefore searched for an interaction of the C5 2416A>G variant with smoking but found no such effect (data not shown). Visualization of our results with ROC curves showed little if any contribution of the single C5 SNP to stroke risk in addition to the classic risk factors.

In the subgroup analysis, the C5 variant was associated with lacunar stroke. Importantly, the presence of the G allele as a risk factor for lacunar stroke showed an OR similar to what we have also observed for the total stroke population. So far, only a few replicable associations of SNPs with the risk of cerebral microangiopathy have been reported: associations with genes involved in endothelial function, including those regulating the renin–angiotensin system and endothelial nitric oxide (15). Recently, a large-scale genetic epidemiologic study showed that SNPs in the PRKCH (protein kinase C eta) gene were significantly associated with lacunar infarction (16). One small study investigated the influence of one candidate inflammatory-gene variant (the IL6 987G>C polymorphism) on stroke risk and found an association with lacunar stroke (17). Our study is the first to report an association between the C5 2416A>G variant and lacunar stroke. We also detected associations of other SNPs with risk in other subgroups of stroke; however, because

Discussion

We analyzed the association of 49 SNPs in genes related to inflammatory diseases with stroke risk in a Caucasian population. After a multivariate analysis controlling for generally accepted vascular risk factors, we found a statistically significant association between the 2416A>G polymorphism in the C5 gene and the risk of ischemic stroke.

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**Fig. 1.** ROC curves of the model with vascular risk factors alone and after addition of the C5 2416A>G SNP.
of the smaller sample sizes of these subgroups, some of the results show very wide CIs. Importantly, only the C5 2416A>G SNP had a minor-allele frequency of 0.42 (A allele). Importantly, we did not perform a formal Bonferroni adjustment for multiplicity in the stepwise multiple regression analysis because we wanted to minimize false-negative results. After Bonferroni adjustment, C5 would not have remained significant.

In conclusion, our analysis of an association of 49 SNPs in 34 inflammation-related genes with the risk of ischemic stroke indicates a small but statistically significant effect of the 2416A>G SNP in the C5 gene. The GG genotype was associated with a lower risk for ischemic stroke and transient ischemic attack in our population, specifically in patients with lacunar stroke. This observation underlines the impact of inflammatory mechanisms related to the pathogenesis of ischemic stroke. We encourage larger studies and metaanalyses of similar patient cohorts to confirm our findings.