Fibrinolytic Gene Polymorphism and Ischemic Stroke

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Background and Purpose—The tissue-type plasminogen activator (tPA) −7351C>T and the plasminogen activator inhibitor type 1 (PAI-1) -675 4G >5G polymorphisms influence transcriptional activity. Both variants have been associated with myocardial infarction, with increased risk for the T and 4G allele, respectively. In this study we investigated the possible association between these polymorphisms, the respective plasma protein levels, and ischemic stroke.

Methods—In the Sahlgrenska Academy Study on Ischemic Stroke (SAHLSIS), 600 patients with acute ischemic stroke aged 18 to 69 years and 600 matched community controls were recruited. Stroke subtype was determined using Trial of Org 10172 in Acute Treatment criteria.

Results—There were no associations between individual genetic variants and ischemic stroke. The multivariate-adjusted odds ratio for overall ischemic stroke was 1.11 (95% CI 0.87 to 1.43) for tPA T allele carriers, and 0.84 (95% CI, 0.64 to 1.11) for subjects homozygous for the PAI-1 4G allele. When genotypes were combined, a protective effect for the tPA CC/PAI-1 4G4G genotype combination was observed (odds ratio 0.65, 95% CI 0.43 to 0.98; P<0.05). Plasma levels of tPA and PAI-1 antigen at follow-up were independently associated with overall ischemic stroke. tPA-antigen differed by stroke subtype and was highest among those with large-vessel disease and cardioembolic stroke.

Conclusions—Neither the tPA −7351C>T nor the PAI-1 675 4G >5G polymorphism showed significant association with ischemic stroke. For the tPA CC/PAI-1 4G4G genotype combination, a protective effect was observed. Collectively, these results are consistent with a more complex role for tPA and PAI-1 in the brain as compared with the heart. (Stroke. 2005;36:2077-2081.)

Key Words: ischemic stroke subtypes ■ plasminogen activator inhibitor type 1 ■ polymorphism ■ tissue-type plasminogen activator

The majority of ischemic strokes occur because of thrombotic or thromboembolic occlusions. This forms the rationale for use of thrombolytic drugs, eg, recombinant tissue-type plasminogen activator (tPA). As demonstrated by spontaneous reperusions, tPA also operates endogenously and its release from endothelial stores is triggered during thrombus formation.1 Consistent with this, tPA knockout mice challenged by a thrombotic insult display reduced patency and enhanced brain injury as compared with wild-type mice.2,3 Endothelial tPA release is not reflected at the systemic level (ie, plasma levels), and can thus only be assessed through invasive procedures that cannot be performed in large clinical studies.4-5 We have therefore screened the human tPA locus for genetic variants associated with invasively determined tPA release rates.3 A single nucleotide polymorphism (SNP) within the tPA enhancer (−7351C>T) was identified.5 The mutant T allele confers a reduced transcriptional activity in vitro, and in vivo this allele is associated with reduced tPA release rates as well as increased risk of myocardial infarction (MI).5-7 A recent study also reported an increased risk of ischemic stroke for the TT genotype.8

The main inhibitor of tPA in plasma is plasminogen activator inhibitor type 1 (PAI-1), and experimental studies demonstrate that PAI-1 delays clot lysis.2 Among genetic variants at the PAI-1 locus, the −675 4G >5G SNP in the promoter has been demonstrated to be functional with a higher transcriptional activity for the 4G allele.9 The 4G4G genotype has been associated with increased risk of MI.5,10 The possible connection to stroke has received less attention, and published studies do not reveal a clear association.11-16

Given the close interplay between tPA and PAI-1, the aim of the present study was to simultaneously test the possible association between the tPA −7351C>T and the PAI-1 to −675 4G >5G SNP, as well as different genotype combinations, and ischemic stroke. Because previous studies show that plasma tPA antigen and the tPA SNP are unrelated and may carry different prognostic information,7,17 plasma levels of tPA and PAI-1 were also investigated.
TABLE 1. Genotype Frequencies for Cases and Controls

<table>
<thead>
<tr>
<th></th>
<th>Control, n=600</th>
<th>Ischemic Stroke, n=600</th>
<th>LVD, n=73</th>
<th>SVD, n=124</th>
<th>CE, n=98</th>
<th>Cryptogenic Stroke, n=162</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>tPA −7351C&gt;T</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CC, no. (%)</td>
<td>278 (46)</td>
<td>259 (43)</td>
<td>27 (37)</td>
<td>55 (44)</td>
<td>47 (48)</td>
<td>73 (45)</td>
</tr>
<tr>
<td>CT, no. (%)</td>
<td>266 (44)</td>
<td>277 (46)</td>
<td>37 (51)</td>
<td>60 (48)</td>
<td>40 (41)</td>
<td>72 (44)</td>
</tr>
<tr>
<td>TT, no. (%)</td>
<td>56 (9)</td>
<td>64 (11)</td>
<td>9 (12)</td>
<td>9 (7)</td>
<td>11 (11)</td>
<td>17 (11)</td>
</tr>
<tr>
<td><strong>PAI-1−675 4G&gt;5G</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4G4G, no. (%)</td>
<td>186 (31)</td>
<td>162 (27)</td>
<td>15 (21)</td>
<td>38 (31)</td>
<td>27 (28)</td>
<td>46 (28)</td>
</tr>
<tr>
<td>4G5G, no. (%)</td>
<td>280 (47)</td>
<td>307 (51)</td>
<td>39 (53)</td>
<td>66 (53)</td>
<td>48 (49)</td>
<td>80 (49)</td>
</tr>
<tr>
<td>5G5G, no. (%)</td>
<td>134 (22)</td>
<td>131 (22)</td>
<td>19 (26)</td>
<td>20 (16)</td>
<td>23 (23)</td>
<td>36 (22)</td>
</tr>
</tbody>
</table>

LVD indicates large vessel disease; SVD, small vessel disease; CE, cardioembolic stroke.

Subjects and Methods

Study Population
The study population includes the participants in the Sahlgrenska Academy Study of Ischemic Stroke (SAHLSIS). In short, white patients presenting with ischemic stroke before the age of 70 years were consecutively recruited from four Stroke Units in Western Sweden. Because the mortality related to ischemic stroke is low in this age group, patients were included regardless of previous cerebrovascular events. For each case, one white control without clinical atherothrombotic disease, matched for age, sex, and geographical residence area, was randomly selected. The study was approved by the Ethics Committee of Göteborg University. All participants gave their written informed consent. Next-of-kin consented for those participants who were unable to communicate.

Data Collection, Risk Factor Definition, and Stroke Subtyping
Information on risk factors was collected and defined as described previously. All patients were examined both at the acute stage and at a follow-up visit after 3 months by a physician trained in stroke medicine. All cases had an ECG and a computed tomography of the brain, and 62% also had an MRL. Additional diagnostic work-up was performed when clinically indicated as described. Stroke subtype was assessed according to Trial of Org 10172 in Acute Treatment. Adjudication of subtype was performed by two neurologists (K.J., C.B.) who were blinded to genotype.

Genotyping
Genotyping was performed by 5′ nucleate (TaqMan) assays as described with the modification that tPA −7351C and T probes were labeled in their 5′ ends with VIC and FAM, respectively. Genotyping was performed by those blinded to case and control status.

Blood Sampling and Laboratory Procedures
In cases, blood sampling was performed within 10 days of the stroke event and at 3-month follow-up. On both occasions, as well as in controls, venous blood samples were drawn between 8:30 and 10:30 AM after an overnight fast. Handling of blood samples, isolation of plasma, and determination of tPA antigen, tPA activity, and PAI-1 antigen were performed as described. The two samples from each case, and the corresponding matched control sample, were assayed on the same microtiter plate. Intra-assay coefficients of variation were on the average 2.2% for both tPA antigen and activity and 2.9% for PAI-1 antigen.

Statistical Methods
Plasma levels of tPA and PAI-1 are presented as medians and inter-quartile ranges. Interindividual differences were compared using Wilcoxon Match-Pairs Signed Ranks and between-group differences by Mann–Whitney U test and Kruskal–Wallis test. Allele frequencies were calculated by gene counting, and deviations from those estimated by the Hardy-Wienberg equilibrium were tested using the χ² test. Logistic regression was performed to estimate odds ratios (OR) and 95% confidence intervals (CI). Because subjects homozygous for the −7351C allele had twice the tPA release rate compared with both heterozygotes and subjects homozygous for the T allele, ORs for this SNP were computed assuming a dominant model for the T allele. In line with this, carriers of the rare PAI-1 5G allele were compared with subjects homozygous for the 4G allele. Our analyses concerned the whole study group and were subsequently stratified by the four main ischemic stroke subtypes; in each stratum, cases were compared with the entire control group. In the whole group, it was estimated that for a statistical power of 80%, ORs >1.4 will be significant at the 5% level for both SNPs. For subtypes, corresponding ORs were 1.7 to 2.3. The multivariate logistic regression model included the two SNPs, tPA antigen level, age, sex, and major risk factors for ischemic stroke: eg, hypertension, diabetes, and smoking. There was a strong positive correlation between tPA and PAI-1 antigen and both were inversely correlated with tPA activity. Consequently, only tPA antigen was included in the model because it showed the highest approximated R-square in univariate logistic regression. A separate prespecified multivariate logistic regression model with the combined tPA and PAI-1 genotypes was also tested. The tPA CC/PAI-1 5G carrier group was chosen as reference, as this genotype combination in theory should lead to highest fibrinolytic activity. Data were analyzed with the SPSS 12.0 package (SPSS Inc), and results were considered statistically significant at P<0.05 using a 2-tailed test.

Missing Values
There were no missing genotype data. Information about hypertension was missing in 9 participants, diabetes in 2, smoking habits in 3, and plasma tPA/PAI-1 in the acute stage in 23. Five cases received thrombolytic therapy with alteplase and were excluded from the analysis of acute plasma tPA/PAI-1. In 43 patients, plasma levels at follow-up were missing because of intervening death (7), technical difficulties (12), or the patient was unwilling to take part in the follow-up examination or provide a blood sample (24). In the regression analyses, missing values for tPA antigen were replaced by the mean value, and for categorized variables dummy variables were introduced.

Results
Genotype frequencies for controls, ischemic stroke, and the main etiological subtypes are shown in Table 1. Undetermined stroke (n=92) and strokes of other determined etiology (n=51) were not included in the subtype analysis. Genotype distributions were consistent with those predicted by Hardy-Weinberg equilibrium.

Logistic regression analysis showed that hypertension (OR 2.32, 95% CI 1.78 to 3.01), diabetes (OR 3.31, 95% CI 1.15 to
and smoking (OR 2.75, 95% CI 2.07 to 3.66) were independently associated with overall ischemic stroke. In contrast, no significant associations were observed for the genetic variants (Table 2). In a second step, combined genotypes were investigated and the tPA CC/PAI-1 4G4G genotype combination was protective (Figure 1).

Among cases, plasma tPA antigen and activity were significantly higher during the acute phase compared with 3-month follow-up (P<0.05 and P<0.01, respectively), whereas PAI-1 was lower in the acute phase (P<0.001). These data are presented in Table 3. For each plasma measure, there was a strong correlation between levels at the 2 time points (Spearman r, >0.54). Plasma levels of tPA antigen at both time points, and tPA activity during the acute phase, differed significantly among subtypes (P<0.001 and P<0.05, respectively), whereas no significant subtype differences were observed for PAI-1 or tPA activity at follow-up.

At both time points, tPA and PAI-1 antigen were higher in cases than in controls. With the exception of small vessel disease (SVD), tPA activity was higher in cases during the acute phase than in controls. With the exception of small vessel disease. With the exception of small vessel disease (SVD), tPA activity was higher in cases during the acute phase than in controls. With the exception of small vessel disease (SVD), tPA activity was higher in cases during the acute phase than in controls. With the exception of small vessel disease.

To our knowledge, this is the first study on ischemic stroke in which associations with both the tPA −7351C>T and the PAI-1 4G>5G SNP have been investigated. When analyzed as independent covariates, we failed to demonstrate significant associations for the genetic variants. However, there was a tendency for a protective effect for the PAI-1 4G4G genotype and when analyzing the effect of combined genotypes a significantly lower risk was observed in subjects homozygous for both the tPA C and the PAI-1 4G allele.

Related to the tPA −7351C>T SNP, a recent study from Australia including 182 cases and 301 controls showed higher risk for the tPA TT as compared with the CC genotype. However, this association was confined to a subgroup of 44 patients with lacunar stroke as defined according to the Oxfordshire Community Stroke Project classification. The present study included 124 patients with SVD in whom the phenotype was more extensively characterized. In this group only 7% were homozygous for the T allele compared with 9% in the control group. The present findings are supported by preliminary data from a population-based prospective study within northern Sweden. In this study, including 322 first-ever ischemic stroke cases, no significant association to the tPA T SNP, the PAI-1 4G4G genotype and when analyzing the effect of combined genotypes a significantly lower risk was observed in subjects homozygous for both the tPA C and the PAI-1 4G allele.

An alternative hypothesis is that a deleterious effect of tPA in stroke counterbalances its thrombolytic effect. It has been suggested that tPA destabilizes atherosclerotic plaques, and the spontaneous reperfusion rate seems to be similar in cerebral compared with coronary vessels, making such an explanation unlikely.

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A more likely explanation is that the biology of tPA extends beyond the vascular system. tPA is expressed both by neurons and microglia, and experimental data suggest that TPA makes neurons more vulnerable to excitotoxic damage. Thus, provided that an allele-specific expression of tPA occurs in brain-
derived cells, the thromboprotective effect of the CC genotype might be offset by neurotoxic effects.

We observed a tendency for a protective effect of the PAI-1 4G4G genotype, which contrasts the increased risk of MI reported for this genotype as well as the observed association between stroke and high plasma PAI-1 levels. For overall ischemic stroke, the OR of the 4G4G genotype was 0.8 (95% CI 0.6 to 1.1), which is similar to the OR of 0.7 (0.5 to 1.0) earlier reported from the GÉNIC study.14 This finding is also consistent with two prospective studies in the elderly showing reduced risk of stroke and cerebrovascular mortality for the 4G4G genotype.15,16 Interestingly, PAI-1 released from astrocytes can rescue neurons from the deleterious effects of tPA.23 Given that the 4G4G genotype confers a high PAI-1 expression in astrocytes, this may tentatively explain a protective effect of the 4G4G genotype in stroke.

A novel finding was that subjects with the tPA CC/PAI-1 4G genotype combination displayed a significantly reduced risk for overall ischemic stroke, suggesting a gene-gene interaction. Although requiring confirmation, this finding is in line with what has been discussed above. Thus, the explanation for this interaction may be a complex interplay between these two pleiotropic proteins within the brain tissue and in plasma. For the tPA CC/PAI-1 4G4G genotype combination, the protective effect of increased endothelial tPA expression may be disclosed because the neurotoxic effects of an increased tPA expression may be neutralized by a simultaneous high PAI-1 expression in the central nervous system. This hypothesis assumes that the 4G4G genotype does not confer a simultaneous high PAI-1 expression in astrocytes. As discussed above, such an assumption is supported by the observed independence between PAI-1 plasma levels and the 4G4G SNP. Although highly speculative, and not withstanding the role of neuroserpin as an inhibitor of tPA in the brain, this provides a potential biological mechanism behind the present observations.

Related to plasma levels, tPA antigen and activity were higher in the acute phase compared with follow-up, indicating a general fibrinolytic activation in response to the ischemic event. Both tPA and PAI-1 antigen were increased in ischemic stroke compared with controls, which is consistent with previous data from both prospective and case-control studies.24,25,11,26,27 Despite this, tPA activity was not significantly reduced at follow-up, which is in contrast to one smaller study.28 Plasma tPA and PAI-1 levels by etiological subtype have previously only been investigated in one smaller study in the acute phase.29 In contrast to results from this study, we found significant differences between subtypes with respect to tPA antigen, and multivariate analysis showed independent associations for LVD and CE stroke. As tPA antigen is a marker of atherosclerotic disease, this may indicate that atherosclerosis is less frequent in the other subtypes. For tPA activity, a significant difference between subtypes was only noted in the acute phase, and the lack of an increase in tPA activity for the SVD group might be explained by the smaller infarct volume in this group.

The PAI-1 5G SNP was not associated with plasma PAI-1, and earlier studies show contrasting results.3,11,16,30,31 The lack of genotype-phenotype relationship in the present study may explain the paradox of high plasma PAI-1 being associated with increased risk for ischemic stroke, whereas a reduced risk was related to the 4G4G genotype. Consistent with our previous studies,5,17 both plasma tPA antigen and activity were unrelated to genotype. This is in line with our earlier data reporting that plasma levels of tPA are unrelated to tPA release rates.5,7,17,20

This study has some limitations worth noting. First, the case-control design limits interpretation of results on plasma levels and on other factors that vary over time. In contrast, genetic markers are not biased in this way. Second, confounding effects of pharmological therapy in cases cannot be excluded. Third, case and control ascertainment may influence results via selection bias. However, in our study, patients were consecutively recruited when arriving at the hospital. The stroke admission rate in Sweden is high, and as the early case fatality rate in ischemic stroke is low, especially for the age group studied here, it is unlikely that this type of bias had any major influence on our results. The control group was recruited by random sampling from the general population in the same geographical areas as patients, which diminished the possibility of spurious results attributable to population stratification. Fourth, although as many as 1200 participants were recruited to the present study, power was not sufficient to detect small effects of the individual SNPs. Fifth, a potential remaining confounding bias cannot be excluded. We controlled for the major risk factors of ischemic stroke, but the influence of other potential risk factors, such as alcohol intake and hyperlipidemia, was not evaluated.

### TABLE 3. Median and Inter-Quartile Range for Plasma Levels of tPA Antigen, tPA Activity and PAI-1 Antigen

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Ischemic Stroke</th>
<th>LVD</th>
<th>SVD</th>
<th>CE</th>
<th>Cryptogenic Stroke</th>
</tr>
</thead>
<tbody>
<tr>
<td>tPA antigen, μg/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control/acute</td>
<td>9.8 (7.2–12.7)</td>
<td>11.9 (8.7–15.7)‡</td>
<td>12.6 (9.7–17.5)‡</td>
<td>11.8 (8.4–14.7)‡</td>
<td>14.1 (10.2–18.7)‡</td>
<td>11.0 (8.0–13.6)‡</td>
</tr>
<tr>
<td>3-month follow-up</td>
<td>11.6 (8.8–14.9)‡</td>
<td>12.5 (9.7–16.3)‡</td>
<td>11.2 (8.9–13.2)‡</td>
<td>13.2 (9.5–16.3)‡</td>
<td>10.1 (8.06–13.6)†</td>
<td></td>
</tr>
<tr>
<td>tPA activity, μg/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control/acute</td>
<td>0.85 (0.56–1.24)</td>
<td>1.06 (0.63–1.66)‡</td>
<td>1.03 (0.62–1.43)*</td>
<td>0.83 (0.50–1.54)</td>
<td>1.22 (0.69–1.91)‡</td>
<td>1.04 (0.64–1.64)‡</td>
</tr>
<tr>
<td>3-month follow-up</td>
<td>0.79 (0.48–1.25)</td>
<td>0.85 (0.50–1.36)</td>
<td>0.71 (0.41–1.19)</td>
<td>0.90 (0.53–1.54)</td>
<td>0.82 (0.52–1.21)</td>
<td></td>
</tr>
<tr>
<td>PAI-1 antigen, μg/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control/acute</td>
<td>39.1 (23.6–60.0)</td>
<td>51.4 (35.0–72.8)‡</td>
<td>56.7 (38.8–74.2)‡</td>
<td>51.2 (38.7–71.8)‡</td>
<td>52.7 (35.0–68.8)‡</td>
<td>50.9 (32.0–69.9‡</td>
</tr>
<tr>
<td>3-month follow-up</td>
<td>56.1 (36.6–77.6)‡</td>
<td>61.9 (44.1–78.8)‡</td>
<td>53.8 (34.4–77.1)‡</td>
<td>52.9 (34.2–75.7)‡</td>
<td>54.7 (37.1–72.9)‡</td>
<td></td>
</tr>
</tbody>
</table>

Control vs overall ischemic stroke (Mann–Whitney U test) and control vs subtypes (Kruskal-Wallis test and posthoc by Mann–Whitney U test): *P<0.05; †P<0.01; ‡P<0.001.
In conclusion, neither the tPA $-7351C>T$ nor the PAI-1 4G $>5G$ SNPs showed significant association with overall ischemic stroke or any of the four main etiological subtypes. However, there was a tendency for a protective effect of the PAI-1 4G4G genotype, and a reduced risk of ischemic stroke was observed for the tPA CC/PAI-1 4G4G genotype combination. With respect to plasma tPA antigen, there were significant differences between the etiological subtypes. Collectively, these results are consistent with a differentiated and more complex role for tPA and PAI-1 in the brain as compared with the heart.

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References


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