Association between factor XIII single nucleotide polymorphisms and aneurysmal subarachnoid hemorrhage

Clinical article

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Object. Family studies have suggested a role of genetic factors in susceptibility to aneurysmal subarachnoid hemorrhage (aSAH), but the underlying genetic risk factors remain poorly defined. There is an activation of the fibrinolytic system in aSAH, and fibrinolytic markers may be useful in predicting outcome. The authors investigate associations between putative functional variants in genes of importance for fibrinolysis and aSAH and/or outcome following aSAH.

Methods. One hundred eighty-three patients presenting with aSAH at a neurointensive care unit were consecutively recruited. Two healthy controls per case, matched for age, sex, and geographic region, were randomly recruited. Outcome was assessed after 1 year according to the extended Glasgow Outcome Scale. Single nucleotide polymorphisms (SNPs) in the tissue-type plasminogen activator (tPA), plasminogen activator inhibitor type 1 (PAI-1), thrombin activatable fibrinolysis inhibitor (TAFI), and factor XIII (FXIII) genes were investigated.

Results. Participants carrying the FXIII34Leu allele showed an increased risk of aSAH. When adjusting for smoking and hypertension, 2 haplotypes, differing on either the FXIII Val34Leu or the Pro564Leu position, showed an association to aSAH. No significant association was observed for the tPA-7351 C > T, PAI-1-675 4G > 5G, or TAFI Ala147Thr SNPs. No specific SNP or haplotype was associated with outcome after aSAH, whereas a weak association was observed for a tPA/PAI-1 genotype combination.

Conclusions. Polymorphisms in the FXIII gene showed association to aSAH. The finding of an increased risk of bleeding in FXIII34Leu carriers is biologically plausible. (DOI: 10.3171/2008.7.JNS08272)

Key Words • fibrinolysis • genetics • polymorphism • subarachnoid hemorrhage

Abbreviations used in this paper: aSAH = aneurysmal subarachnoid hemorrhage; FXIII = factor XIII; GOSE = extended Glasgow Outcome Scale; ICH = intracerebral hemorrhage; NICU = neurointensive care unit; PAI-I = plasminogen activator inhibitor type 1; SNP = single nucleotide polymorphism; TAFI = thrombin activatable fibrinolysis inhibitor; tPA = tissue-type plasminogen activator.

Subarachnoid hemorrhage accounts for a relatively small portion of all strokes, but the loss of productive life years is comparable to that caused by cerebral infarctions.9 The main reasons for the large impact of SAH are relatively young age at onset and poor outcome.9,30 The hemorrhage is caused by a ruptured aneurysm in 85% of cases and most deaths are due to the initial bleeding and its immediate complications.30 Subarachnoid hemorrhage is a complex disease in which both genetic and environmental factors contribute. Risk factors include smoking, hypertension, heavy drinking, and female sex.9 Familial occurrence of SAH suggests that genetic factors are involved in the development of this disease. First-degree relatives of patients with SAH have a 3- to 5-fold greater risk of suffering a ruptured aneurysm than the general population.10 However, little is known about what genes are involved.14,25

Most candidate gene studies of SAH have investigated proteins involved in connective tissue formation.14,25 However, no specific genes have so far been identified as showing a clear association with SAH, perhaps due to lack of power and locus heterogeneity. In contrast, few studies have investigated the roles of the coagulation and fibrinolytic systems, although these systems are activated after SAH,12,18,20 interact with key components in extra-
This study. Furthermore, only patients admitted within 2 days of the acute event were included. During the inclusion period, 253 patients were considered and 183 fulfilled the aforementioned diagnostic criteria and were willing to participate in the study. All patients were treated with intravenous administration of tranexamic acid and nimodipine. In most patients this treatment was initiated within 48 hours of admission. The aneurysm responsible for the hemorrhage was treated with neurosurgical clip placement or by endovascular coil insertion. One year after the aSAH, a structured interview developed by Wilson and coworkers was used to categorize outcome in patients according to the GOSE.

For each case, 2 healthy controls matched for age (± 2 years), sex, and geographic residence area were randomly selected from participants in GOT-MONICA, a population-based health survey, or from the Swedish Population Register. A venous blood sample was drawn in both patients and controls at inclusion.

The Medical Ethics Committee at the University of Gothenburg approved the study. All participants gave informed consent. When a patient was unable to communicate, his or her next of kin gave informed consent.

Clinical Characteristics

Smoking history was coded as current versus never or former (smoking cessation at least 1 year before inclusion in the study), and hypertension was defined by pharmacological treatment for hypertension prior to the event. The GOSE was dichotomized into favorable (GOSE Scores 5–8) and unfavorable (GOSE Scores 1–4) outcomes.

Genotyping of DNA From Blood Samples

The DNA was extracted from venous blood samples with a QIAamp 96DNA Blood Kit (QIAGEN), using the manufacturer’s reagents as directed.

The DNA concentration was measured using an ND-1000 spectrophotometer (NanoDrop) and diluted in Tris–ethylenediaminetetraacetic acid buffer to 10 ng/µl; 20 ng of DNA was used in each assay. The tPA -7351 C > T (dbSNP reference ID rs2020918), PAI-1 -675 4G > 5G (rs1799889), TAFI Ala147Thr (rs3742264), and FXIII Val34Leu (rs5985) SNPs were analyzed using 5’ nuclease (TuqMan) assays as described. The assay designs of FXIII Tyr204Phe (rs3024477) and FXIII Pro564Leu (rs5982) are presented in Table 1. Amplifications were carried out on a Dual 96-Well GeneAmp PCR System 9700 (Applied Biosystems) and fluorescence was read on an ABI PRISM 7900HT Sequence Detector System (Applied Biosystems). Genotyping was performed by researchers who were blinded to case/control status.

Statistics and Risk Factor Definition

Differences in characteristics between cases and controls were examined using the chi-square test. The association between specific SNPs and case/control status was investigated using uni- and multivariate binary logistic regression, adjusted for smoking status and hypertension. Similar models were used to test the association between specific SNPs and outcome. However, in these

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**Table 1: Designs of 5’ nuclease assays**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Concentration (nM)</th>
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<tbody>
<tr>
<td>FXIII Tyr204Phe (rs3024477)</td>
<td>300</td>
</tr>
<tr>
<td>FP204: 5’-GATGATGCTGTGATCTGGACAATG-3’</td>
<td>300</td>
</tr>
<tr>
<td>RP204: 5’-CCACGTTCTGGTCTGATGCA-3’</td>
<td>300</td>
</tr>
<tr>
<td>Tyr204: (VIC)-AAGAGTATGCTCTGAATG-(MGB)</td>
<td>100</td>
</tr>
<tr>
<td>Phe204: (FAM)-AGAGTTTGTCCTGAATG-(MGB)</td>
<td>100</td>
</tr>
<tr>
<td>FXIII Pro564Leu (rs5982)</td>
<td>300</td>
</tr>
<tr>
<td>FP564: 5’-TACAGTCGAACGTCTCCTTCT-3’</td>
<td>300</td>
</tr>
<tr>
<td>RP564: 5’-AACCCTTACACCTACACGCTATC-3’</td>
<td>300</td>
</tr>
<tr>
<td>Pro564: (VIC)-ATTCTGCCTCGGGAC-(MGB)</td>
<td>100</td>
</tr>
<tr>
<td>Leu564: (FAM)-TCTCCTCAAGACC-(MGB)</td>
<td>100</td>
</tr>
</tbody>
</table>

* Thermal cycling conditions were as follows: 2 initial holds (50°C for 2 minutes and 95°C for 10 minutes) followed by a 40-cycle 2-step polymerase chain reaction (95°C for 15 seconds and 60°C for 1 minute). Abbreviations: FP = forward primer; MGB = minor groove binder; RP = reverse primer.

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analyses, sex and age were also included as covariates. For all specific SNP analyses, additive and dominant or recessive models were considered. In addition, multivariate logistic regression models with combinations of 2 SNPs were tested as described. Differences in risk estimates between women and men for FXIII SNPs and aSAH were evaluated using sex by genotype interaction in a general linear model. Allele frequencies were derived from genotype data, and deviations from the Hardy-Weinberg equilibrium were tested. The FXIII haplotype frequencies were estimated using PHASE version 2.1.28 The individual likelihood for each haplotype was used to estimate ORs for each haplotype by comparison with a reference haplotype, represented by the most frequent allele for each SNP. It was estimated that the study had 80% power to detect ORs > 1.7 at the 5% level for SNPs with minor allele frequency > 0.3. Data were analyzed using SPSS version 15.0, and statistical analyses were performed in a two-tailed fashion; a probability value < 0.05 was considered significant and no corrections for multiple comparisons were done, because the aim of the study was exploratory.

**Missing Values**

Information on smoking status was missing in 6 cases due to intervening death, and information on hypertension was missing in 1 case. The GOSE was not determined in 2 patients, and genotype information was missing in 2 controls for both the tPA -7351C>T and the FXIII Val34Leu.

**Results**

**Characteristics of the Study Population**

The mean age of the patients with aSAH was 55 years, and 74% were female (Table 2). As expected, smokers were more frequent in cases compared with controls. The OR for aSAH in smokers compared with nonsmokers was 4.9 (95% CI 3.3–7.2, p < 0.001). There was no statistically significant difference in the number of participants receiving treatment for hypertensive disease between patients and controls. After 1 year, 126 (70%) of the patients had a favorable outcome (GOSE Scores 5–8), whereas 23 (13%) had died. Those with a favorable outcome were younger (54 vs 57 years, p < 0.05) and were less likely to have had hypertensive treatment prior to the aSAH (p < 0.05). The participants’ sex and smoking status were not related to outcome.

**Association Between SNPs and aSAH**

Initially we investigated 1 putative regulatory SNP in each of the 4 fibrinolytic genes that have been reported to show an association with thrombotic and/or hemorrhagic disease.32 Regulatory promoter SNPs were chosen for tPA and PAI-1, and coding SNPs for TAFI and FXIII. Genotype frequencies are presented in Table 3. All SNPs were in Hardy-Weinberg equilibrium in both cases and controls. There was a significant association between...
aSAH and the FXIII Val34Leu SNP. Carriers of the Leu34 allele showed an increased risk of aSAH compared with participants who were homozygous for the Val34 allele (OR 1.48, 95% CI 1.03–2.12, p = 0.03). The strength of the association remained, but was no longer statistically significant after inclusion of smoking and hypertension in a multivariate model (OR 1.44, 95% CI 0.98–2.12, p = 0.07). No significant association was detected between aSAH and SNPs in the tPA, PAI-1, or TAFI genes.

Because 1 study has reported an association between hemorrhagic stroke, including SAH, and 2 other FXIII variants (that is, the FXIII Tyr204Phe and Pro564Leu SNPs), we went on to type these SNPs. We did not detect any association between aSAH and the FXIII Tyr204Phe SNP, but there was a trend for an association between participants homozygous for the FXIII Leu564 allele, compared with those homozygous for the Pro564 allele (multivariate OR 2.65, 95% CI 0.91–7.74, p = 0.07).

Because the study by Reiner et al. was based on young women, we performed a gender-specific subanalysis for the FXIII SNPs. In support of these investigators’ findings, women homozygous for the Leu564 allele showed an increased risk of aSAH compared with those who were homozygous for the Pro564 allele (multivariate OR 4.87, 95% CI 1.35–17.5, p = 0.02). Furthermore, the association between carriers of the Leu34 allele and aSAH was statistically significant in women (multivariate OR 1.59, 95% CI 1.01–2.50, p = 0.05), but not in men (multivariate OR 1.06, 95% CI 0.49–2.29, p = 0.88). However, this difference was not statistically significant when testing for sex by genotype interaction in a general linear model. There is also a study reporting a gene-environment interaction between the FXIII Val34Leu SNP and smoking in ischemic stroke. However, we were not able to detect any such interaction between FXIII SNPs and smoking in aSAH.

The FXIII Haplotypes and aSAH

The FXIII haplotype frequencies are reported in Table 3. In statistical analysis, the H1 haplotype was used as a reference, and both the H2 and H3 haplotypes were significantly more frequent in aSAH compared with controls (Fig. 1). The association between aSAH and haplotype H2 remained statistically significant after adjustment for smoking and hypertension.

A gender-stratified subanalysis revealed that the associations between aSAH and the H2 and H3 haplotypes were statistically significant in women (multivariate OR 1.58, 95% CI 1.03–2.43, p = 0.04 and OR 1.95, 95% CI 1.18–3.40, p = 0.02, respectively), but not in men (multivariate OR 1.28, 95% CI 0.59–2.81, p = 0.54 and OR 0.75, 95% CI 0.28–2.03, p = 0.57, respectively).

Genetic Variation and Outcome Following aSAH

Neither specific SNPs nor FXIII haplotypes showed an association with outcome 1 year after the event (data not shown). However, the tPA -7351 CC and PAI-1 -675 4G4G genotype combination was significantly more common in patients with an unfavorable outcome compared with those who had a favorable outcome (Table 4). The multivariate OR for an unfavorable outcome (GOSE Scores 1–4) for this genotype combination compared with the reference (that is, tPA -7351 CC and PAI-1 -675 5G-carriers) was 3.88; 95% CI 1.34–11.26, p = 0.013.

Discussion

To the best of our knowledge, this is the first study in which a combination of gene polymorphisms affecting fibrinolysis in a relatively large case-control study on aSAH has been investigated. We found an association between common FXIII genetic variants and aSAH. When
Association between FXIII SNPs and aSAH

| TABLE 4: The tPA -7351 C>T and PAI-1 4G>5G genotype combinations and outcome* |
|-----------------------------------------------|----------------|----------------|----------------|----------------|
| Outcome                                      | tPA CC & PAI-1 5G & PAI-1 4G4G | tPA CC & PAI-1 5G & PAI-1 4G4G | tPA T-Carrier & PAI-1 5G & PAI-1 4G4G | tPA T-Carrier & PAI-1 5G & PAI-1 4G4G |
| no. w/ favorable outcome                      | 46             | 11             | 48             | 21             |
| no. w/ unfavorable outcome                   | 16             | 14             | 19             | 6              |

* p = 0.025 (chi-square test).

stratified for sex, this association was detected in women but not in men.

The FXIII genetic variation has previously been studied in relation to hemorrhagic stroke.4,5,7,11,23 One study investigated the Pro564Leu SNP in young Caucasian women and, in line with our findings, reported an increased proportion of individuals who were homozygous for the FXIII Leu564 allele in patients with aSAH compared with controls.23 The results of our study are also in line with the early hypothesis of Catto et al.4 that the 34Leu allele is associated with increased risk of primary ICH. Furthermore, in a larger Italian study,11 Leu34 carriers were at an increased risk of suffering a primary ICH. In contrast, Reiner et al.23 found no association between the Val34Leu SNP and nonfatal hemorrhagic stroke in a small sample of 42 young women, and 3 other studies5,7,27 could not detect any association for primary ICH. Only Corral et al.5 published separate data on a subgroup of 60 patients with SAH. In this subgroup the prevalence of the Leu34 allele was higher than in patients with primary ICH, although this difference did not reach statistical significance. Consequently, the divergent results on the Val34Leu SNP in primary ICH may be due to the fact that different phenotypes have been studied and that the sample sizes have been relatively small. In contrast to earlier studies, only patients with SAH were included in the present study. Furthermore, only SAH with a confirmed aneurysm was studied, and the sample size is larger than in earlier studies.

The analysis of FXIII haplotypes in aSAH shows that the Leu34 and Leu564 allele associations represent 2 different genetic effects, because they are present on different haplotypes. The H2 haplotype corresponds to a change only at Val34Leu, whereas the H3 haplotype corresponds to a change at Pro564Leu. It could thus be speculated that the H4 haplotype, with a change at both of these positions, might correspond to an even higher risk. However, the estimated frequency of this haplotype was only 4%, and a larger study is required to estimate its effect.

When stratified for sex, the associations for all FXIII genetic variants were only detected in women. However, the power to detect an association was smaller in the male subgroup, and there was no significant sex-by-genotype interaction. Still, one might speculate that a lack of or a weaker association in men may have contributed to the discrepancy in data from studies on FXIII gene variation and hemorrhagic stroke. Therefore, studies on possible gender-specific effects are warranted.

The present results on FXIII are biologically plausible. Both fibronectin and collagen types I, II, III, and V are substrates for FXIII and can be cross-linked to fibrin.19,21 The cross-linking of both fibronectin and collagen to fibrin suggests that these reactions could stabilize the extracellular matrix that forms at sites of aneurysm formation. The properties of FXIII have been shown to be influenced by its gene variants.1 In particular, the effects of the Val34Leu SNP have been investigated. This SNP is located close to a thrombin activation site, and in vitro studies have demonstrated that Leu34 FXIII is activated more rapidly than the Val34 variant.1 The Leu34 allele also decreases clot stability and produces clots with thinner cross-linked fibrin fibers and smaller pores.1 Clot formation time has also been shown to be significantly shortened in FXIII Leu34 samples.26

No specific SNP was significantly associated with 1-year outcome, nor were FXIII haplotypes. We have previously observed an association between ischemic stroke and a tPA -7351 C>T and PAI-1 -675 4G > 5G genotype combination.13 Both these SNPs influence the expression of the gene product by affecting the rate of transcription.8,36 This genotype combination was therefore investigated in our study, both in relation to aSAH and outcome. Our results indicate that a combination suggesting high tPA (-7351 CC) and high PAI-1 (-675 4G4G) gene expression is associated with an increased risk of unfavorable outcome after aSAH. The finding for PAI-1 is in line with earlier studies showing increased plasma levels of PAI-1 in patients with an unfavorable outcome after aSAH.20 It has also been shown that treatment with nimodipine increases fibrinolytic activity by reducing PAI-1 levels in plasma.24 Furthermore, a tendency for an unfavorable outcome in patients with aSAH who have the 4G genotype has been reported.35 However, the role of tPA in the brain is complex,3,16 and the reason why an effect of the 4G allele was only observed in patients homozygous for the tPA C allele is unclear. It is of note that this subgroup analysis should be interpreted with great caution, because the power is low, and because multiple testing may contribute to false associations.

There are some possible shortcomings in the interpretation of our findings. The inclusion of patients was consecutive. However, the recruitment of patients was biased because the fatality rate in the acute phase is high and because patients with initial signs of an unfavorable prognosis are often treated outside the NICU. The fact that we only included patients with aSAH also introduced some bias, because some patients with a poor prognosis do not undergo angiography. In contrast, the effect of the 2-day limit for inclusion of patients most likely is random. The control group was recruited by random sampling from the general population in the same geographic areas as patients. This makes the possibility of spurious results due to population stratification less likely. The main advantage of our study is the relatively homogeneous and large patient group with confirmed aneurysms and follow-up after 1 year.
Conclusions

We show an increased frequency of carriers of either the FXIII Leu34 or the FXIII Leu564 allele in patients admitted to a NICU following aSAH. In multivariate analysis of FXIII haplotypes, this association was independent of smoking and hypertension. This finding is biologically plausible. However, given the modest evidence of association, the size of the study, and that multiple testing was performed, the associations should be interpreted as a preliminary finding that requires replication in independent data sets. So far there are very few genetic association studies on SAH. Therefore, there is a need to collect samples of well-characterized patients with SAH, preferably also including registration of both early and long-term outcome. In the future, knowledge about the impact of genetic variation on the risk of suffering an SAH and its relationship with complications after SAH may lead to new treatment strategies and a better understanding of the differences in recovery from SAH.

Disclosure

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