Association of the α-Fibrinogen Thr312Ala Polymorphism With Poststroke Mortality in Subjects With Atrial Fibrillation

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Background—The α-fibrinogen Thr312Ala polymorphism occurs in close proximity to several sites important for factor XIIIa-dependent cross-linking, which raises the possibility that it affects fibrin clot stability.

Methods and Results—We determined the association of this polymorphism with ischemic stroke, stroke subtype, and poststroke mortality. There was no significant difference in the genotype distributions of patients with acute ischemic stroke (n=519) and healthy control subjects (n=423), nor was there any association of this polymorphism with stroke subtype. In a Cox regression model, a significant interaction between Thr312Ala and atrial fibrillation was identified in relation to poststroke mortality (P=0.002). In subjects in sinus rhythm (n=418), there was no difference according to genotype in the proportion of subjects who survived (≈60% in each group), whereas in subjects with atrial fibrillation (n=101), there was decreased survival in those possessing the A allele (TT=42.1%, TA=18%, AA=0%).

Conclusions—The Thr312Ala polymorphism may give rise to an increased susceptibility for embolization of intra-atrial clot, and these findings could have important implications for identifying subjects most at risk of developing thromboembolic complications. (Circulation. 1999;99:2423-2426.)

Key Words: stroke ■ mortality ■ atrial fibrillation

The conversion of fibrinogen to fibrin gives rise to the formation of fibrin protofibrils,1 and stabilization of the forming fibrin clot requires cross-linking of adjacent γ- and α-chains by factor (F) XIIIa.2 The γ-γ crosslinks contribute to 35% and α-α cross-links to 65% of clot stability,2 highlighting the relative importance of α-fibrinogen cross-linking in determining overall clot stability.

A polymorphism of the α-fibrinogen gene has been identified that codes for a threonine-to-alanine amino acid substitution at position 312 (Thr312Ala).3 This polymorphism lies close to the FXIIIa cross-linking site at position Aα 328.2 FXIIIa also cross-links α2-antiplasmin to fibrin at position Aα 303; this results in localization of α2-antiplasmin within the fibrin clot and helps to protect it from cleavage by plasmin.4 In addition, the region of fibrinogen encompassing α-fibrinogen amino acid residues 242 to 424 enhances the activation of FXIII by reducing the Ca2+ concentration required for the dissociation of the FXIII a and b subunit dimers to physiological levels.5 Thus, Thr312Ala could influence clot strength and elasticity by interfering with these FXIIIa-dependent cross-linking processes.

To investigate whether clinical studies supported these possibilities, we determined the association of the Thr312Ala polymorphism with ischemic stroke, stroke subtype, and poststroke mortality. In addition, we identified possible interactions of this polymorphism with classic risk factors and determined whether Thr312Ala influences the incorporation of α2-antiplasmin into fibrin.

Methods

Subjects
The recruitment and characteristics of patients and control subjects have been fully described.6 Briefly, 519 patients with ischemic stroke, confirmed by noncontrast cranial CT scan, were recruited. Patients were subclassified according to the Oxfordshire Community Stroke Project classification into those with probable small-vessel disease (lacunar infarction caused by microatheromatous occlusion of small perforating arteries) and those with probable large-vessel disease (total or partial anterior circulation infarction caused by either in situ thrombosis or cardiogenic embolism).7 The presence of atrial fibrillation was established on clinical examination at the time of the acute stroke and confirmed on a diagnostic 12-lead ECG. Age-matched healthy control subjects (n=423) residing in the same postal districts as the patients were recruited from local Family Health Services Authority general practice registers. All subjects gave informed consent according to a protocol approved by the United Leeds Teaching Hospitals NHS Trust.

DNA Analysis
The Thr312Ala genotype was determined by use of the following oligonucleotide primers, which gave a polymerase chain reaction (PCR) product of 190 bp: forward, 5'-CCTAGCAGTGCTGGAAGCTG-3' and reverse, 5'-GGCTCCCAGGGTTTTTGGG-3'. Fragments were am-
plified by standard PCR procedures using 25 pmol of each primer, 2.0 mmol/L magnesium, and 64°C annealing temperature. PCR products were digested overnight with 5 U 

\[ \text{Rsa} \] 

(EMBL/GenBank accession no. X89931) using pooled normal plasma as reference. The APs was adjusted for dilution, and the 2-antiplasmin incorporated into the fibrin clot was quantified as (AP p

\[ \text{calcium mix (1:1, 200 U/mL thrombin, 1 mol/L CaCl}_2 \] ) and incubated at 37°C for 2 hours. The clot was then removed by careful winding onto a wooden toothpick. \( \alpha \)-Antiplasmin in serum (AP s ) and plasma (AP p ) was then determined by chromogenic substrate assay (Coamatic Plasmin Inhibitor, Chromogenix) using pooled normal plasma as reference. The AP was adjusted for dilution, and the proportion of \( \alpha \)-antiplasmin incorporated into the fibrin clot was calculated as (AP s −AP p )/AP p .

**Statistics**

The 1-sample Kolmogorov-Smirnov goodness-of-fit test was used to determine whether the distributions of continuous variables deviated significantly from normal; nonnormally distributed variables were log-transformed to allow analysis by parametric tests. Differences in levels between 2 unrelated groups were compared by unpaired Student’s t tests and between >2 groups by 1-way ANOVA with Scheffé post hoc analysis. Results are expressed as mean or geometric mean and 95% CI. Differences in categorical variables between groups were assessed by the Kaplan-Meier log-rank statistic. The association of genotype with survival after acute stroke, taking into account above-mentioned factors to identify significant interactions. The only interaction term significantly associated with post-stroke mortality was Thr312Ala \times\text{atrial fibrillation} \quad (P=0.002). Other factors independently associated with post-stroke mortality in this model were age (RR for an increase of 10 years, 1.62 [1.42 to 1.85]; \( P<0.00001 \)), subtype (RR for large- compared with small-vessel disease, 2.37 [1.70 to 3.30]; \( P<0.00001 \)), and previous stroke (RR for previous compared with first-ever stroke, 1.41 [1.08 to 1.85]; \( P=0.01 \)).

The characteristics of patients in sinus rhythm \( (n=418) \) and those with atrial fibrillation \( (n=101) \) are presented in Table 1. A greater proportion of subjects with atrial fibrillation had died after the acute event, with 30-day and total mortality rates of 15.8% and 69.3%, respectively, in those with atrial fibrillation compared with 5.0% and 38.8%, respectively, in those in sinus rhythm \( (P<0.0001) \) for 30-day and total mortality).

On further investigation of the interaction of Thr312Ala with atrial fibrillation, in subjects in sinus rhythm \( (n=418) \), there was no significant difference in the genotype distributions of those who subsequently died \( (n=162, \text{TT}=59.3\%, \text{TA}=34.6\%, \text{AA}=6.2\% \) compared with those still alive \( (n=256, \text{TT}=57.6\%, \text{TA}=35.7\%, \text{AA}=6.7\% \) ). In subjects with atrial fibrillation \( (n=101) \), however, there was a significant difference in the genotype distributions of those who had died \( (n=70, \text{TT}=47.1\%, \text{TA}=45.7\%, \text{AA}=7.5\% ) \) compared with those still alive \( (n=31, \text{TT}=77.4\%, \text{TA}=22.6\%, \text{AA}=0\%, \text{P}=0.01 \). This association was confirmed by use of the Kaplan-Meier log-rank statistic, as shown in Figure 2, which indicated that in subjects with atrial fibrillation, possession of the A allele was associated with poor outcome. The proportions of subjects in sinus rhythm and with atrial fibrillation who were still alive at the end of March 1998 classified by Thr312Ala genotype are presented in Figure 3. Approximately 60% of subjects in sinus rhythm survived in each genotype group, whereas in those with atrial fibrillation, 24 of the 57 subjects with TT genotype \( (42.1\% ) \) survived, compared with 7 of the 39 with TA genotype \( (18\% ) \) and none of the 5 with AA genotype.

There was no association of Thr312Ala genotype with the proportion of \( \alpha \)-antiplasmin incorporated into the fibrin clot: \( \text{TT}=39.0\% (33.3\% \text{ to } 44.8\% ), \text{TA}=42.7\% (37.8\% \text{ to } 47.7\% ), \text{AA}=42.0\% (38.5\% \text{ to } 45.6\% ). \) \( \alpha \)-Antiplasmin in-

**Results**

The genotype distributions of the patients with ischemic stroke and healthy control subjects did not differ significantly from Hardy-Weinberg equilibrium. There was no significant difference in the genotype distributions of patients \( (\text{TT}=58.0\%, \text{TA}=35.8\%, \text{AA}=6.2\% ) \) and control subjects \( (\text{TT}=60.6\%, \text{TA}=32.8\%, \text{AA}=6.6\% ) \), nor was there any association of Thr312Ala with stroke subtype (data not shown).

A total of 232 deaths in the patient group had been notified by March 31, 1998, representing a median follow-up of 2.8 (0.7 to 4.0) years. There was no significant difference in the genotype distributions of those who died \( (\text{TT}=55.6\%, \text{TA}=37.9\%, \text{AA}=6.5\% ) \) compared with those still alive \( (\text{TT}=59.8\%, \text{TA}=34.3\%, \text{AA}=5.9\% ). \) A Cox regression model using backward stepwise selection with the probability for removal from the model of \( P=0.1 \) was used to identify independent predictors of poststroke mortality, including Thr312Ala and factors associated with mortality in univariate analyses (age, sex, atrial fibrillation, stroke subtype, smoking history, previous stroke, and previous MI) as covariates, and interaction terms were created between Thr312Ala and other risk factors (introduced into the model in addition to the above-mentioned factors) to identify significant interactions.
corporation was significantly correlated with fibrinogen level ($r=0.66$, $P<0.0001$); after adjustment for fibrinogen level, no association of Thr312Ala with $\alpha_2$-antiplasmin incorporation remained (data not shown).

### Discussion

The $\alpha$-fibrinogen Thr312Ala polymorphism occurs in a region of fibrinogen that is important for FXIIIa-dependent cross-linking, suggesting that this polymorphism may influence clot stability. In the present study, there was no association of Thr312Ala with stroke or stroke subtype. However, a significant interaction between Thr312Ala and atrial fibrillation was identified in relation to poststroke mortality.

Atrial fibrillation is the most common sustained cardiac arrhythmia, and the incidence of atrial fibrillation increases with advancing age, with a prevalence of 4% in subjects >60 years old and >10% in those >80 years old. Atrial fibrillation is associated with a 5-fold increased risk of developing stroke, probably as a result of embolization of left atrial thrombus. In the present study, 20% of subjects with ischemic stroke had atrial fibrillation, which is in keeping with the proportion of subjects with atrial fibrillation and stroke in the prospective Framingham study. In addition to an increased risk of stroke, subjects with atrial fibrillation have an increased mortality rate after stroke compared with...
those in sinus rhythm. In keeping with these findings, in the present study, a greater proportion of subjects with atrial fibrillation died after acute stroke compared with those in sinus rhythm. In the patients in sinus rhythm, there was no association of Thr312Ala genotype with survival. In the patients with atrial fibrillation, however, only 18% of subjects with TA genotype survived, and none of the subjects with AA genotype survived, compared with 42% of those with TT genotype.

Because no association was observed in those in sinus rhythm, these findings suggest that Thr312Ala contributes to the pathogenesis of thromboembolic complications associated with the presence of atrial fibrillation. Whether this is related to an increased susceptibility to form intra-atrial thrombus or an increased susceptibility for embolization of intra-atrial clot is unclear from the present study. However, we have also found a significant difference in the Thr312Ala genotype distributions of 218 patients with venous thromboembolism compared with 250 age- and sex-matched healthy control subjects (Carter et al, unpublished observations, 1998). When patients with venous thromboembolism were categorized into those with deep vein thrombosis (DVT, n=120) and those with pulmonary embolism (PE, n=98), only the genotype distribution of those with PE differed significantly (P=0.02) from that of healthy control subjects (PE: TT=0.49, TA=0.36, AA=0.15; DVT: TT=0.50, TA=0.42, AA=0.08; controls: TT=0.60, TA=0.34, AA=0.06), related to an increased incidence of the AA genotype in those with PE. These findings, therefore, suggest that Ala312 may give rise to an increased susceptibility for embolization of thrombus, possibly due to defective FXIII-dependent cross-linking. We did not find any difference in the amount of α2-antiplasmin incorporated into fibrin clots by Thr312Ala genotype; however, it is possible that this polymorphism interferes either with the FXIII-dependent α-fibrin/α-fibrin cross-linking or with the ability of α-fibrinogen residues 242 to 424 to enhance the dissociation of FXIII a and b subunit dimers. In both of these situations, it might be expected that this would result in a less tightly cross-linked fibrin clot, which would reduce its mechanical strength and lead to an increased tendency to embolization. A recent report by Curran et al13 suggested that Thr312Ala is associated with differences in vitro fibrin gel structure parameters, e.g., gel porosity and density, lending further support to our findings. Further studies are currently being undertaken to determine the effect of this polymorphism on α-fibrin cross-linking and FXIII a and b subunit dimer dissociation.

Further prospective studies will be necessary to confirm these observations, in particular to determine whether possession of the A allele is related to an increased incidence of stroke in subjects with atrial fibrillation. The present study suggests that possession of Ala312 may prove to be an additional factor to be considered when stratifying risk of stroke in subjects with atrial fibrillation and may help to target subjects most at risk of thromboembolic complications in whom more aggressive therapy could be considered.

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References
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