

# The -33T → C Polymorphism in Intron 7 of the TFPI Gene Influences the Risk of Venous Thromboembolism, Independently of the Factor V Leiden and Prothrombin Mutations

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## Keywords

TFPI, venous thrombosis, genetic risk factors

## Summary

We have previously identified, in intron 7 of the TFPI gene, a T to C single-base polymorphism (-33T→C) which is strongly associated with total circulating TFPI antigen levels. Here we examined the influence of this polymorphism on the risk of venous thromboembolism. The polymorphism was identified in the PATHROS study population (330 cases with venous thromboembolism and 826 controls). The CC genotype was found in 6.4% of cases and 10.2% of controls (age-adjusted odds ratio 0.6; 95% CI 0.3-0.9;  $p = 0.03$ ). This protective effect persisted after adjustment for oral contraception and the factor V Leiden and prothrombin gene polymorphisms. In 171 controls and 49 cases in whom blood was taken at least three months after the thrombotic event, the CC genotype was associated with significantly higher total TFPI levels than the TT genotype. These results suggest that the CC genotype of the TFPI intron 7 polymorphism is an independent protective factor for venous thromboembolism, an effect probably mediated by increased TFPI levels.

## Introduction

Venous thromboembolism (VTE) is often triggered by gene-environment and gene-gene interactions (1). The most common point mutations associated with thrombophilia are factor V Leiden G1691A and prothrombin G20210A. Tissue factor pathway inhibitor (TFPI), a Kunitz-type serine protease inhibitor, is the main inhibitor of the factor VIIa-tissue factor pathway of blood coagulation. Several studies have shown the therapeutic efficacy of recombinant TFPI (rTFPI) in animal models of venous thrombosis (2). Holst et al. (3) showed that rTFPI was

as effective as low-molecular-weight heparin in preventing thrombosis of the rabbit jugular vein. Recombinant TFPI also prevents thrombotic reocclusion after fibrinolysis in rabbits (4). Huang et al. (5) reported that mouse embryos that were homozygous for an inactive TFPI gene never survived the neonatal period; the presence of intravascular thrombi suggested that consumptive coagulopathy was responsible for the bleeding diathesis in the oldest embryos. More recently, Ariens et al. showed that TFPI activity measured in the circulation after a heparin injection was lower in young subjects with venous thrombosis than in healthy controls (6). Finally, a study of total TFPI antigen in 122 patients with deep venous thrombosis (DVT) and 126 controls showed significantly lower TFPI levels in the patients (7). This last study pointed to a link between DVT and total TFPI levels, but showed no relationship between DVT and mutations in the coding sequence of the TFPI gene.

The human TFPI gene consists of 9 exons separated by 8 introns. Several polymorphisms have been described in the promoter, intron 7, and the coding sequence of the gene. Recently, Kleesiek et al. reported results suggesting a relationship between the Pro151Leu polymorphism and venous thrombosis in a German population (8). A C to T point mutation at position -399, which has been described in the Japanese population, was not associated with DVT (9). We recently described three polymorphisms, located in the promoter (T-287C), intron 7 (-33T→C), and exon IX (Val264Met) of the TFPI gene (10, 11). The -33T→C polymorphism of intron 7 is located 33 nucleotides upstream of the beginning of exon VIII (Fig. 1). The CC homozygous genotype is common (10%) and is strongly associated with an increase in total TFPI levels (12).

The aim of this study was to search for an association between the -33T→C polymorphism of intron 7 and VTE, and to confirm the impact of the polymorphism on plasma TFPI levels.

## Methods

### Subjects

The Paris thrombosis case-control study (PATHROS) started in November 1995 and is designed to identify genetic risk factors for VTE. All the patients and controls gave their written, informed consent to the study, which was approved by the local ethics committee. The inclusion criteria have been described in detail elsewhere (13, 14). Briefly, we enrolled 150 men and

The first two authors contributed equally to this work

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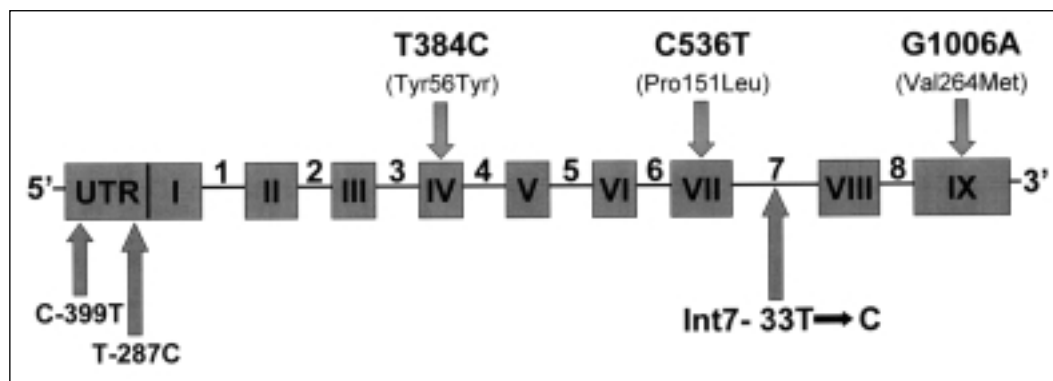


Fig. 1 Tissue factor pathway inhibitor polymorphisms. Exons are denoted by boxes, numbered I-IX. Introns are numbered 1-8. Polymorphisms located in exons are indicated above the gene; those located in the untranslated 5' region and the -33T→C polymorphism located in intron 7, 33 nucleotides upstream the beginning of exon VIII, are indicated below the gene. UTR: untranslated region

180 women younger than 61 years who had been admitted to Broussais university hospital vascular medicine department (Paris, France), and who had had at least one episode of objectively diagnosed deep venous thrombosis (DVT) (compression ultrasonography or venography) and/or pulmonary embolism (perfusion and ventilation lung scan, conventional pulmonary angiography, or computed tomographic angiography). The patients were evaluated with a detailed medical questionnaire covering age, personal and family history of thromboembolic disease, and acquired risk factors including pregnancy, postpartum, use of oral contraceptives, surgery within the past three months, lengthy immobilization (>72 h), and known malignancy.

Eight hundred and twenty-six unrelated healthy subjects (407 men and 419 women), matched with the patients for age and sex, were recruited between May and September 1996 from a health care center specializing in cardiovascular prevention, to which they had been referred for a routine check-up. None of these subjects had a personal history of arterial disease (stroke, myocardial infarction, angina, or peripheral vascular disease) or of venous thrombosis (pulmonary embolism, DVT).

#### Genotyping Study

##### Factor V Arg506Gln and prothrombin G20210A mutations

DNA was extracted from blood leukocytes by standard methods and stored at 4° C until analysis. The factor V Arg506Gln and prothrombin G20210A gene mutations were identified by polymerase chain reaction (PCR) amplification followed by restriction enzyme analysis (15).

##### Intron 7 polymorphism identification using molecular beacons

To identify the intron 7 polymorphism in the TFPI gene, two differently colored molecular beacons, synthesized by Eurogentec, were used in a single reaction as described elsewhere (16). One probe, labeled with 6-carboxyfluorescein (FAM), was specific for the mutated allele, while the other, labeled with 6-carboxy-tetramethyl-rhodamine (TAMRA), was specific for the wild-type allele. Both molecular beacons were covalently linked to DABCYL at their 3' end. Their nucleotide sequences were: 5'-FAM-GCG-ACC-TAT-CAC-ACA-TGG-CTT-ACC-GTC-GC-DABCYL-3' and 5'-TAMRA-GCG-ACC-TAT-CAC-ATA-TGG-CTT-ACC-GTC-GC-DABCYL-3', respectively. The PCR primers, purchased from Eurogentec, were 5'-TAC-AAT-ACG-AAA-ACC-TGA-AAT-CCA-C-3' (forward primer) and 5'-ACC-ATG-AGG-GAC-CGT-GAA-ATT-3' (reverse primer). Each 25- $\mu$ l PCR reaction mixture contained 1  $\mu$ g of genomic DNA, 0.4  $\mu$ M forward primer, 0.4  $\mu$ M reverse primer, 0.2  $\mu$ M each molecular beacon, 200  $\mu$ M dNTPs (Life Technologies, Cergy-Pontoise, France), 1 $\times$  PCR buffer (50 mM KCl, 10 mM Tris HCl, pH 8.3; Life Technologies) and 1.25 U of Taq DNA polymerase (Taq Platinum-Life Technologies). The 96-well plate containing the PCR mixture was placed in a PTC100AGVHB thermal cycler (MJ Research, Watertown, MA). The reaction

mixture was subjected to 35 cycles of PCR with denaturation at 94° C for 20 sec, annealing at 51° C for 30 sec and extension at 70° C for 45 sec. The reaction mixture was then held at 70° C for 7 min. Fluorescence was measured with a Fluostar-BMG spectrofluorometer using maximal excitation and emission wavelengths (520 and 590 nm, for FAM and TAMRA fluorescence, respectively). The fluorescence intensity ratios of the two molecular beacons (TAMRA/FAM) were calculated according to the genotype. The means and standard deviations of the intensity ratios obtained from twelve 96-well microplates were  $1.60 \pm 0.10$ ,  $0.52 \pm 0.03$  and  $0.24 \pm 0.02$  for the TT, TC and CC genotypes, respectively. Initially, the results obtained using this method were compared with those obtained using RFLP (10) on 20 DNA samples of each genotype. Perfect concordance was obtained.

#### TFPI Antigen Assays

Total and free TFPI were assayed with specific monoclonal antibody-based ELISAs (Asserachrom Total or Free TFPI, Diagnostica Stago, Asnières, France). Venous blood was collected in fasting subjects into tubes containing 0.11 M trisodium citrate (1:10) and centrifuged within 2 h. Plasma was obtained by two centrifugation steps at 12° C for 15 min at 2500  $\times$  g, then distributed into aliquots and stored at -40° C until analysis. TFPI was measured in a subset of 49 cases from whom plasma taken at least 3 months after the thrombotic event was available, and in 171 age-matched controls. None of these subjects were using oral contraception (OC).

#### Statistical Analysis

The Hardy-Weinberg equilibrium was tested using a Chi-square test. Logistic regression analysis (Systat statistical software) was used to identify links between the three genotypes and venous thromboembolic disease, after accounting for age, sex, oral contraception and other genetic risk factors (factor V Leiden and prothrombin G20210A). The genotype was included in the equation as a three-class variable corresponding to the three genotypes, coded 1, 2 and 3. Interactions between risk factors and genotypes were also tested for by logistic regression. TFPI levels were compared between genotypes by using the Kruskal-Wallis test (performed between the groups carrying the TT, TC and CC genotypes) followed, when significant, by the Mann-Whitney test. Results are expressed as means  $\pm$  sem.

## Results

### Characteristics of the Study Population

The cases and controls did not differ significantly according to age or sex (Table 1). Women on oral contraception were significantly more

**Table 1** Characteristics of the study population

	Cases (n=330)		Controls (n=826)		p‡
Age, y					
mean ± SD†	42.7 ± 11.1		42.0 ± 10.8		
Females	180	(54%)	419	(51%)	ns
Oral contraception in females	60	(33%)	97/419	(23%)	< 0.01
First thrombotic event < 45 y	220	(66%)	-		
Pulmonary embolism	107/327	(33%)	-		
Recurrence	102/328	(32%)	-		
Spontaneous onset*	129	(39%)	-		
Family history of thrombosis	123/327	(38%)	-		
Heterozygous FV Leiden	67/330	(20%)	28/802	(3.5%)	< 0.001
Heterozygous FII polymorphism	39/328	(12%)	31/789	(4%)	< 0.001
Intron 7 CC genotype	21	(6.4%)	84	(10.2)	< 0.05

\* events occurring in the absence of any identified acquired risk factor (pregnancy, oral contraception, cancer, surgery or immobilization).  
†SD : standard deviation  
‡p values (Chi-square test)

frequent among the cases (33%) than the controls (23%,  $p < 0.01$ ). Age at the first thrombotic event was below 45 years in 66% of the cases. Recurrent thrombosis occurred in 32% of the cases. Pulmonary embolism was diagnosed in 33% of the cases. The frequencies of two most common genetic risk factors for venous thrombosis, factor V Arg506Gln and prothrombin G20210A, were within the expected ranges for a Caucasian population. As expected, their frequency was significantly higher in the cases than in the controls.

#### *Distribution of the Intron 7 Polymorphism in the Cases and Controls*

The frequency of the -33T→C polymorphism showed no deviation from Hardy-Weinberg equilibrium. However, the genotype frequencies in the cases differed significantly from that in the controls. As shown in Table 2, the age-adjusted odds ratio (OR) associated with the CC versus the TT genotype was 0.6 [95% confidence interval (CI), 0.3-0.9;

$p = 0.03$ ]. Thus, the CC genotype was associated with a lower risk of VTE. The OR associated with the TC genotype was not significantly different from one (OR = 0.9, 95% CI, 0.7-1.2,  $p = 0.5$ ). The protective effect of the CC genotype was not modified when sex, and the factor V Arg506Gln and prothrombin G20210A polymorphisms were added to the logistic equation. The ORs adjusted for age, sex and the two mutations were 0.5 (95% CI, 0.3-0.9,  $p = 0.02$ ) and 0.9 (95% CI, 0.7-1.2), for the CC and TC genotypes versus the TT genotype, respectively. The ORs adjusted for both age and oral contraception were 0.4 (95% CI, 0.2-0.9,  $p = 0.03$ ) and 0.9 (95% CI, 0.6-1.3,  $p = 0.5$ ) for the CC and TC genotypes versus the TT genotype, respectively. The intron 7 polymorphism did not influence the risk of spontaneous DVT, pulmonary embolism, recurrence, or age at first thrombosis in the overall population. No significant interaction was found between the intron 7 genotype and any of the adjustment variables (age, sex, oral contraception, factor V Leiden and prothrombin G20210A) when adding interaction terms in the logistic model.

**Table 2** Genotype frequencies of the intron 7 -33T→C polymorphism in cases and controls

Genotype	Cases	Controls	OR* (95% CI)	p
	n (%)	n (%)		
	n=330	n=826		
TT	167 (50.1)	384 (46.5)	1	
TC	142 (43.0)	358 (43.3)	0.9*	0.7-1.2 ns
CC	21 (6.4)	84 (10.2)	0.6*	0.3-0.9 0.03

\* Age-adjusted ORs associated with the TC and CC genotypes versus the TT genotype

Table 3 Total TFPI levels in cases and controls according to genotype and gender

	Controls		Cases	
	Males (n=90)	Females (n=81)	Males (n=14)	Females (n=35)
<b>Age, y</b> (mean±SD)	45.6±12.1	43.0±9.9	49.5±9.9	43.0±9.4
<b>Genotype</b>	<b>Total TFPI ng/ml</b>			
<b>TT</b>	70.2±1.9 (46)	65.3±2.1 (35)	74.1±4.7 (4)	62.4±3.8 (18)
<b>TC</b>	74.2±2.2* (37)	70.4±2.7 (39)	76.5±4.9 (8)	63.2±2.5* (13)
<b>CC</b>	94.2±8.6* (7)	80.6±5.6* (7)	79.9±6.5 (2)	81.1±3.7† (4)

\*p<0.01: CC vs TT, TC vs CC  
†p<0.05: CC vs TT  
(): number of subjects carrying each genotype

#### TFPI Levels According to the Intron 7 Polymorphism

The relationship between the intron 7 polymorphism and plasma TFPI levels was analyzed in detail in 49 cases and 171 controls (Table 3). These subjects were matched by age, and none were using OC. As TFPI levels are higher in men than in women (17), the comparisons were made separately for each sex. Among the controls, the CC genotype was associated with significantly higher total TFPI levels than the TT genotype, in both men and women; among the cases, only women with the CC genotype had significantly increased total TFPI levels (CC vs TT:  $p < 0.05$ , CC vs TC:  $p < 0.01$ ). Free TFPI antigen levels, by contrast, did not differ significantly according to the genotype (data not shown).

#### Discussion

These results suggest that the CC polymorphism of TFPI gene intron 7 is associated with a reduced risk of VTE, and that this effect is independent of established risk factors, including oral contraception and the factor V Leiden and prothrombin gene polymorphisms. The CC polymorphism was also associated with elevated plasma total TFPI antigen levels.

We have previously reported that the intron 7 polymorphism of the TFPI gene, which is located 33 nucleotides upstream of the beginning of exon VIII, is not associated with the risk of acute coronary events (10) or with restenosis after angioplasty (12); however, this polymorphism did influence levels of total TFPI antigen in a group of control subjects (12). The primary aim of the present study was to seek an association between the intron 7 genotype and the risk of VTE. For this

purpose, we used a specific and rapid method to determine the intron 7 genotype, based on multicolor molecular beacons (16, 18, 19). This method has two main advantages, namely a considerable time saving, and a low risk of contamination by PCR products, as fluorescence is measured without opening the tubes. Accuracy is also improved, as problems of incomplete digestion are avoided. The frequency of the CC genotype in the controls (10.2%) was very similar to that in two previous reports (10.2 and 10%) (10, 12). The frequency of the CC genotype was lower in the cases than in the controls.

This lower relative risk persisted when conventional risk factors such as oral contraception and the factor V Arg506Gln and prothrombin gene polymorphisms were taken into account (adjusted OR = 0.52, 95% CI, 0.3-0.9;  $p = 0.02$ ). Interestingly, the protective effect of the CC genotype was also found in oral-contraceptive users and carriers of the mutations, as shown by the lack of statistical interaction when adding interaction terms in the logistic regression.

To date, five polymorphisms have been described in the TFPI gene (8-11), and three (C-399T, Pro151Leu and Val264Met) have been assessed for their effect on the risk of venous thrombosis in case-control studies. Pro151Leu was first reported to be associated with venous thrombosis (8), but this was not confirmed in three subsequent studies (20-22). However, in one of the latter studies (21) this polymorphism was associated with an increased risk of VTE in individuals with deficiencies in natural coagulation inhibitors. No association was found between the risk of venous thrombosis and either the Val264Met (6, 23) or the C-399T polymorphism (9).

We have previously shown a significant association between the intron 7 genotype and total TFPI antigen levels (12), total TFPI levels being higher in healthy subjects with the C allele (CC>TC>TT). In contrast, free TFPI antigen levels were not influenced by the genotype. In the present study, the association between total TFPI antigen levels and the intron 7 genotype was clearly confirmed in the control population, in both males and females (Table 3). Interestingly, a similar relationship was found in female cases. Only two male cases bore the CC genotype, which probably explains why the association was not found in this subgroup. These results strongly suggest that increased total TFPI levels mediate the protective effect on VTE in subjects carrying the CC genotype. They are also in keeping with recent data showing a relationship between the risk of venous thrombosis and low total TFPI levels (7). In the present study, TFPI levels were only slightly lower in the cases ( $68.1 \pm 2.0$  ng/ml) than in the controls ( $70.4 \pm 1.2$  ng/ml). The relationship between TFPI levels and thrombosis may have been masked by differences in LDL-cholesterol levels, as LDL-cholesterol levels correlate strongly with TFPI levels (17). Cholesterol levels were not measured in this study, and TFPI levels could not therefore be adjusted for this parameter. It should be noted that the method used to assay TFPI was the same in the two studies, and measures both lipoprotein-bound and free TFPI. The anticoagulant effect of TFPI is generally thought to be mediated by the full-length unbound molecule (24), while the lipoprotein-bound form, which is truncated at its C-terminal end, is thought to be less active. In this study, as in a previous report (12), the -33T→C polymorphism had no effect on free TFPI levels, suggesting that the protective effect may be mediated by an increase in the LDL-bound, truncated form of TFPI. Our results, like those of Amini-Nekoo et al., thus suggest that the lipoprotein-bound, truncated form of TFPI also has a role in preventing thrombosis. This is keeping with the reported protective effect of truncated TFPI in an animal model of venous thrombosis (3).

The mechanism by which this intronic polymorphism influences TFPI expression is not clear, although in a previous study we ruled out

the involvement of altered mRNA splicing (12). The mechanism could be direct but is more probably related to a linkage disequilibrium with another, unknown mutation.

In conclusion, this study, which needs confirmation, suggests that the intron 7 polymorphism of the TFPI gene is an independent risk factor for venous thromboembolic disease, and that its effect is mediated by increased total TFPI levels.

## Addendum

### Role of each author

Nejma Ameziiane, Cendrine Seguin and Didier Moatti carried out the genetic study; Frédéric Fumeron carried out the statistical analysis of the results; Delphine Borgel and Martine Alhenc-Gelas carried out the collection of plasma and DNA samples and the TFPI assays; Bernard Grandchamp carried out the modelling of the molecular beacons; Joseph Emmerich conceived and carried out the PATHROS study; Nejma Ameziiane and Dominique de Prost prepared the first draft of the paper; Martine Aiach, Joseph Emmerich and Dominique de Prost carried an overall supervision of the work and contributed to the final version of the paper.

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