Functional consequences of the prothrombotic *SERPINC1* rs2227589 polymorphism on antithrombin levels

Ana I. Antón, Raúl Teruel, Javier Corral, Antonia Miñano, Irene Martínez-Martínez, Adriana Ordóñez, Vicente Vicente, and Beatriz Sánchez-Vega

Servicio de Hematología y Oncología Médica H.U. Morales Meseguer, Centro Regional de Hemodonación, Universidad de Murcia, Spain

ABSTRACT

Genetic factors involved in the interindividual variability of antithrombin have not been identified. We studied two polymorphisms of the gene coding for antithrombin (*SERPINC1*) in 298 Spanish Caucasian blood donors: rs3138521, a DNA length polymorphism located on the promoter region and rs2227589, a SNP located on intron 1 that has been described as a mild thrombotic risk factor. We detected a complete linkage disequilibrium between these polymorphisms (D'=0.999). The rs3138521 polymorphism has no functional consequences. However, the rs2227589 SNP significantly associated with plasma anti-FXa activity and antithrombin levels: carriers of the A allele had slightly but significantly lower anticoagulant activity and levels than GG subjects (97.0±7.3% *vs.* 94.6±8.4%; p=0.032; 99.5±5.8% *vs.* 94.8±5.6%; p=0.001; respectively). Our results identified a functional effect of the rs2227589 polymorphism not explained by its linkage with the promoter polymorphism that support the moderate thrombotic risk associated with the A allele.

Key words: antithrombin, polymorphism, thrombotic risk.

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Introduction

Antithrombin is a serpin that efficiently inhibits multiple serine proteases of the coagulation, mainly factor X activated (FXa) and thrombin, by a suicide mechanism.[1] Heterozygous deficiency of this anticoagulant significantly increases the risk of venous thrombosis[1] and its absence causes embryonic lethality.[2] Antithrombin activity is significantly heterogeneous among the general population. The study of this parameter in 9,669 healthy blood donors (5,525 male and 4,144 female) revealed a distribution approximately *normal* with mean 105.6 IU/dL and standard deviation 11.2.[3] This variation might have clinical relevance, as the thrombotic risk of subjects with lower levels of anticoagulant activity is potentially higher. Antithrombin levels are known to be affected by factors such as sex, body mass index, oral contraceptives or race,[4] but it may also be influenced by genetic factors, as revealed by the high heredability (h=0.486).[5] Polymorphisms on the gene encoding antithrombin (*SERPINC1*) may be involved in the interindividual variability of antithrombin. There are no missense polymorphisms,[6] which reflect the structural susceptibility of this molecule to even minor changes on the primary structure.[7] Therefore, our search for genetic elements associated with differential levels of this potent anticoagulant was driven to noncoding regions of the gene. In this framework, two polymorphisms deserved our attention:

(i) rs3138521, a DNA length polymorphism located on the *SERPINC1* promoter region located -345 basepairs (bp) upstream the first codon. Two alleles nonhomologous in sequence have been described, one of 32 bp (called F), and one of 108 bp long (called S).[8] This polymorphism affects sequences with potential transcriptional relevance, as revealed by using different programs for predicting transcription factor binding sites (Alibaba 2.1, Match 1.0, p-Match 1.0, MatrixCatch 2.5, and SignalScan) (Figure 1).

(ii) rs2227589 (786 G>A), on intron 1 located 140 bp downstream exon 1 (Figure 1). This SNP associated with the risk of venous thrombosis in a recent study that evaluated 19,682 gene-centric SNPs located in 10,887 genes in three independent case-control studies enrolling 3,155 patients with venous thrombosis and 5,087 controls.[9]

The aim of our study was to test the functional relevance of these polymorphisms on the plasma antithrombin activity and levels in healthy subjects.

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Correspondence: Javier Corral, Centro Regional de Hemodonación. C/ Ronda de Garay s/n. Murcia 30003. Spain. E-mail: javier.corral@carm.es

Design and Methods

Subjects and blood sampling

Our study was performed on 298 Spanish Caucasian blood donors (133 male/165 female), with a mean age of 42.4 years. All subjects gave their informed consent to enter the study, which was approved by the ethics committee of the Centro Regional de Hemodonación and performed according to the declaration of Helsinki, as amended in Edinburgh in 2000.

Blood samples were obtained by venopuncture collection into 1:10 volume of trisodium citrate. Platelet poor plasma fractions were obtained (within 5 min after blood collection) by centrifugation at 4°C for 20 minutes at 2,200 g and stored at -70°C. Genomic DNA was purified by the salting out procedure and stored at -20°C.

Antithrombin activity and levels

FXa-inhibiting activity was measured using a chromogenic method in presence of heparin (Instrumentation Laboratory, Italy), and antithrombin antigen levels determined by immunodiffusion, as previously reported.[10] Values were expressed as a percentage of the result observed in a control pool of citrated plasma from 100 healthy subjects (100%).

Genetic analysis

Genotyping of the *SERPINC1* rs3138521 polymorphism was assessed by PCR-based method. The promoter region of SERPINC1 was amplified by primers: AT3-PF2 (6FAM-GCCTGAAGGTAGCAGCTTGT); and AT3-PR2 (CCCACACTCCCTCACTCTTC). Thermal cycling conditions were 94°C for 2 min, followed by 30 cycles at 94°C for 15 s, 57°C for 30 s and 72°C for 30 s and a final step of 5 min at 72°C. Amplified fragments were resolved by capillary electrophoresis on a 3130xl Genetic Analyzer (Applied Biosystems) and analyzed by GeneMapper® software (Applied Biosystems).

Genotyping of the *SERPINC1* rs2227589 polymorphism was determined by the validated TaqMan SNP Genotyping Assay C__16180170_20 (Applied Biosystems) following the manufacturer's instructions. SNP genotyping reactions were performed on a LC480 Real Time PCR (Roche) using standard conditions for end-point genotyping assays on this machine.

Genotypes were verified by sequencing. Briefly, 6 subjects with each haplotype were selected. A PCR covering both polymorphisms was performed with AT3-PIF (GGACACCTTGGCACTCAGAT) and AT3-PIR (ACCCAAGGGGTAGCTTAGGA) primers. Thermal cycling conditions were 94°C for 2 min, followed by 30 cycles at 94°C for 15 s, 57°C for 30 s and 72°C for 45 s and a final step of 5 min at 72°C. The sequence reaction was performed with the same primers and BigDye® Terminator v3.1 Cycle Sequencing chemistry, and resolved on a 3130xl Genetic Analyzer (Applied Biosystems).

Statistical analysis

Allele and genotype frequencies, deviations from Hardy-Weinberg expectations, haplotype analysis, and measure of the D' and r^2 values for assessment of linkage disequilibrium was performed with the SNPstats software.[11]

Statistical analysis was performed by Statistical Package for Social Science (version 15.0; SPSS, Chicago, IL, USA). Data are presented as mean ± standard deviation. Differences of plasma anti-FXa activity and antithrombin antigen levels between genotypes was analyzed by means of Student's *t* test using a dominant model. Linear regression analysis was used to study the contribution of the *SERPINC1* polymorphisms to antithrombin activity and levels. Statistical significance was taken as $p<0.05$.

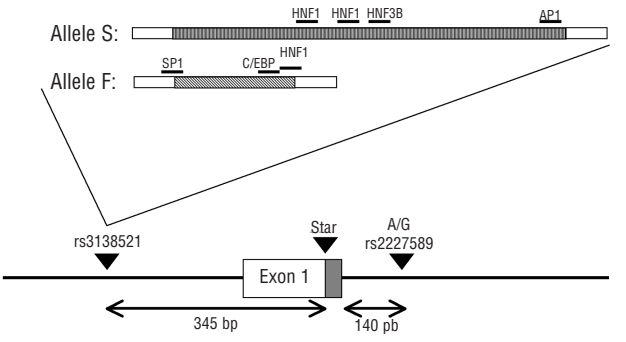


Figure 1. Representation of a fragment of the *SERPINC1* gene, indicating the localization of the studied polymorphisms. The possible binding sites of transcription factors that may be affected by the rs3138521 polymorphism at the promoter region are indicated. The figure shows only the binding sites predicted by at least three of the five prediction programs used.

Results and Discussion

Allele and genotype frequencies, and linkage analysis

The allele frequencies observed in the Spanish population for both polymorphisms are in close agreement with values previously reported in other Caucasian populations: rs3138521 S allele: 0.23 (present study); 0.21 (Northern Ireland);[12] 0.25 (US);[8] 0.21 (The Netherlands).[13] rs2227589 A allele: 0.10 (present study); 0.12 (The Netherlands).[9]

Table 1 shows the genotype frequencies of rs2227589 and rs3138521. The genotypes of these two polymorphisms are in Hardy-Weinberg equilibrium ($p\geq0.01$).

Since these polymorphisms are close in the sequence of the *SERPINC1* gene (526 bp), we evaluated their linkage. We found a complete linkage disequilibrium between these two polymorphisms in the Spanish population (D'= 0.999; r^2= 0.396). The haplotype frequency was estimated via the EM Algorithm setting the threshold for rare haplotypes as 1%. The haplotype analysis revealed three haplotypes (Figure 2).

Effect of SERPINC1 polymorphisms and haplotypes on plasma anti-FXa activity and antithrombin levels

The association results of the studied *SERPINC1* polymorphisms on plasma anti-FXa activity and antithrom-

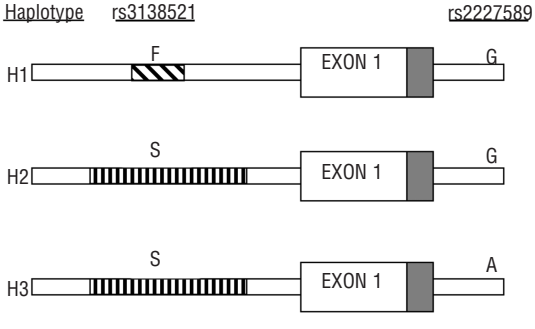
bin levels are shown in Table 1. According to our data, promoter rs3138521 polymorphism had no significant functional effects in the Spanish population. However, intron 1 rs2227589 polymorphism showed a significant association with both plasma anti-FXa activity and antithrombin levels. Thus, carriers of the A allele had slightly but significantly lower anti-FXa activity than GG subjects (Table 1). Homozygous AA subjects had similar anti-FXa and antithrombin levels than GG subjects, probably because we have only identified 4 blood donors carrying this genotype. Further studies including more subjects are required to clarify this issue.

Linear regression analysis suggested that the *SERPINC1* rs2227589 polymorphism explained up to 7.2% of the interindividual variation of antithrombin levels (Table 1). The analysis of the contribution of each haplotype to the plasma anti-FXa activity and antithrombin levels confirmed that haplotype H3, defined by the 786 A allele, associated with reduced antithrombin activity and levels (Figure 2).

The search for genetic risk factors involved in the development of venous thrombosis has been intense since 1965 when Egeberg identified the first genetic defect causing a heterozygous deficiency of antithrombin.[14] After the discovery and characterization of two important polymorphisms involved in thrombophilia, factor V Leiden and prothrombin G20210A,[15,16] this search was moved on to the identification of new prothrombotic polymorphisms with quite frustrating results.[17] A recent report analyzed the relevance of 19,682 gene-centric SNPs in 3 case-control studies of first deep venous thrombosis, finding only three polymorphisms consistently associated with deep venous thrombosis.[9] One of these polymorphisms (rs2227589) is located on the *SERPINC1* gene, the gene coding for antithrombin. Carriers of the low frequent A allele had a modest but significant thrombotic risk in all the 3 case-control studies evaluated: LETS (443 cases and 453 controls; OR: 1.42; 95%CI: 1.04-1.94); MEGA-1 (1,398 cases and 1,757 controls; OR: 1.24; 95%CI: 1.05-1.47); MEGA-2 (1,314 cases and 2,877 controls; OR: 1.29; 95%CI: 1.10-1.49).[9] The key role of antithrombin on hemostasis and thrombosis, and the high interindividual heterogeneity on the levels of this anticoagulant support this association, although authors did not perform functional studies to demonstrate it. Additionally, the thrombotic effect of this polymorphism may be caused directly by itself or by another linked polymorphism that may have functional effects on the levels or anticoagulant activity of antithrombin. With the aim of investigating the interactions between genotype and phenotype,[18] we demonstrate the strong linkage disequilibrium between this polymorphism and rs3138521, a DNA length polymorphism affecting the *SERPINC1* promoter. The thrombotic risk identified in the Dutch study associated with the intronic polymorphism might be explained by the functional effects of the linked promoter polymorphism. Indeed, the rs3138521 length polymorphism may influence the binding of relevant transcriptional factors as shown in Figure 1, which may have consequences on the transcriptional rate of this gene. However, available data concerning the functional role of the promoter rs3138521 polymorphism are conflicting. Transfection experiments reported that the 108 bp-sized DNA fragment (S allele) showed a promoter activity 1.7-fold higher than the 32 bp-sized DNA fragment (F allele).[19] Similar results were obtained by Winter *et al.*[12] Nevertheless, these experiments are not conclusive, as the difference in promoter activity

Table 1. Genotype frequencies of the studied *SERPINC1* polymorphisms and their association with plasma antithrombin anti-FXa activity and antithrombin levels.

	N.	Anti-FXa %	Antigen %
rs3138521			
FF	181 (60.7%)	96.6±7.2	98.8±6.3
FS	99 (33.2%)	96.4±8.1	97.1±6.2
SS	18 (6.1%)	96.3±8.7	98.2±5.5
S carrier	117 (39.3%)	96.4±8.1	97.7±7.7
p		0.818	0.346
R^2		2E-4	0.004
rs2227589			
GG	240 (80.5%)	97.0±7.3	99.5±5.8
AG	54 (18.1%)	94.3±8.2	94±5.7
AA	4 (1.3%)	99.1±10	99.2±2.5
A carrier	58 (19.5%)	94.6±8.4	94.8±5.6
p		0.032	0.001
R^2		0.014	0.072



Haplotype	Freq.	Anti-FXa*	Antigen*
H1	0.773	0 –reference-	0 –reference-
H2	0.123	1.17 (-0.7/3.0 p=0.22)	1.48 (-0.8/3.7 p=0.21)
H3	0.104	-1.65 (-3.6/0.3 p=0.10)	-2.55 (-4.8/-0.3 p=0.03)

*Difference respect to reference H1 (95% CI)

Figure 2. Haplotypes of the *SERPINC1* gene defined by the rs3138521 and rs2227589 polymorphisms and their association with anti-FXa and antithrombin levels.

between these two alleles was not observed when larger constructs were used.[19] Moreover, two studies including 155 and 148 healthy control individuals found that this polymorphism did not correlate with plasma antithrombin activity.[12,13] Our present study on a large sample of Spanish healthy blood donors confirms that this polymorphism has no functional effects *in vivo*.

In contrast, we observed a significant association of the intron 1 rs2227589 polymorphism with plasma anti-FXa activity and antithrombin levels. Carriers of the A allele associated with slightly but significantly lower levels of antithrombin and anti-FXa activity than subjects with GG genotype. Therefore, our study identified a functional effect of this polymorphism, not explained by its linkage with the promoter polymorphism, that clarify the moderate thrombotic risk associated with the A allele (OR: 1.3).[9] These results open three relevant questions that deserve further studies. First, a slightly reduced anticoagulant activity (the difference on anti-FXa activity and antithrombin levels between carriers of the rs2227589 A and G allele is only 4%) results in a mild but significant risk of venous thrombosis. In agreement with this result, individuals with low anticoagulant levels but within the normal range (70-90%) might have a significant risk of venous thrombosis. Second, the functional consequences observed in this study may be caused directly by the rs2227589 polymorphism or by any other genetic change linked to this SNP. Finally, it is important to note that this polymorphism only explains up to 7.2% of the interindividual variation of antithrombin. Therefore, the search for genetic factors associated with the interindividual variability of antithrombin, with potential relevance on the risk of thrombosis, must continue by evaluating other *SERPINC1* polymorphisms and other genes that indirectly may influence the plasma levels or the anticoagulant function of this key hemostatic molecule.

Authorship and Disclosures

JC, BS-V, IMM, VV: project planning, design of the study, analysis and interpretation of data; manuscript writing (JC), molecular and functional analysis (AIA, RT, AM, AO), statistical analysis (AIA).

The authors reported no potential conflicts of interest.

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