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Three novel variants in the coagulation factor V gene associated with deep venous thrombosis in Chilean patients with Amerindian ethnic background

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ABSTRACT

Background: The activated protein C (APC) resistance is the most common prothrombotic defect in thrombosis patients, mainly related with alterations in the *F5* gene. In this work, we evaluated the presence of variants in the FV gene in Amerindian patients with deep venous thrombosis and APC resistance.
Methods: A total of 87 patients with deep venous thrombosis (DVT) confirmed by Doppler ultrasonography, and Amerindian genetic background, were included in this study. APC resistance was assayed by clotting methods and polymorphism *F5*1691G>A was genotyped by molecular methods. In Amerindian patients with APC resistance, the promoter region, exon 7 and exon 10 of the *F5* gene were screened by PCR-SSCP and DNA sequencing. The prediction of functional effect of novel mutations was analyzed using Polyphen-2 software.
Results: In DVT patients, 14.9% showed functional APC resistance in the absence of *F5*1691G>A polymorphism. Interestingly, three novel missense mutations in exon 10 of *F5* gene (M443L, E461Q and G493E) were identified. These genetic variants were absent in 100 healthy subjects. According to *in silico* analysis, the sequence variants G493E and E461Q are potentially deleterious.
Conclusions: Our data shows that the APC resistance phenotype is not associated with the presence of the *F5*1691G>A variant. We described, for the first time, the presence of three novel variants in F5 gene in Chilean patients with APC resistance. Further studies are required to investigate the real contribution of these novel mutations to the APC resistance phenotype.

1. Introduction

Thromboembolic disease constitutes an important health problem in occidental countries and results from the interaction of acquired and genetic factors [1]. The activated protein C (APC) resistance is recognized as the most important cause of venous thrombosis [2,3]. This phenotype is characterized as a reduced anticoagulant response of coagulation factor V for APC activity. In Caucasian population, it is related with a single-nucleotide polymorphism in the factor V gene, denominated factor V Leiden (*F5* G1691A, rs6025) [4].

A previous study suggests that the *F5*1691G>A variant presents a founder effect in Caucasian individuals [5]. This evidence explains the higher frequency in ancient Middle Eastern and Caucasian populations in contrast with populations with other genetic backgrounds. Previously, we demonstrate a total absence of *F5* G1691A polymorphism in Amerindian patients with deep venous thrombosis [6]. This low frequency is concordant with other studies in Chilean and Latin American populations [7,8].

Considering the virtual absence of *F5*1691G>A polymorphism observed in previous studies and the Amerindian genetic background of Chilean population, the aim of the present study was to evaluate the possible presence of sequence variants in the *F5* gene in Amerindian patients with deep venous thrombosis (DVT) and APC resistance.

2. Material and methods

2.1. Subjects

A total of 87 unrelated patients, with diagnosis of DVT confirmed by Doppler ultrasonography, were included in this study. None of the subjects had chronic hepatic diseases or thrombosis secondary to malignant diseases. Demographic data and history of hypertension, diabetes mellitus, hypercholesterolemia, and familial history of thrombosis

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Table 1
Primer sequence used for amplification of the *F5* gene.

Region		Oligonucleotide sequence	Product (bp)	*Annealing tempeture* (°C)
Promoter	FVpF	5′-ATCTCTGCCCCTTCTTCA CC-3′	247	61
	FVpR	5′-CGAGCTGCTAACCACACT CC-3′		
Exon 7	FV7F	5′-TCCTAACTCAGCTGGGAT GC-3′	213	52
	FV7R	5′-AACCTTTGCCCAGTGGTA TG-3′		
Exon 10	FV10aF	5′-TGCAAATGAAAACAATTT TGAA-3′	202	58
	FV10aR	5′-CTATTAGCCCAGAGGCGA TG-3′		
	FV10bF	5′-GAAAATGATGCCCAGTGC TT-3′	227	56
	FV10bR	5′-TTGAAGGAAATGCCCCAT TA-3′		

were assessed in each subject. We calculated the body mass index (body weight [kg] divided by square of height [m]) to assess obesity. The study protocol was approved by the local Ethics Committee, and all subjects gave written informed consent according to the basic principle of biomedical investigation enumerated in the Helsinki declaration.

2.2. Characterization of genetic background

Characterization of the population was realized using Amerindian mitochondrial DNA markers according to a protocol previously described [9]. In the mitochondrial genome, this protocol amplifies fragments containing distinctive restriction sites characteristic for Amerindian mtDNA haplogroups A, C and D, and 9 bp deletion for haplogroup B.

2.3. Functional analysis

Blood samples were obtained by venipuncture using 3.2% citrate as the anticoagulant to functional assays and ethylenediaminetetraacetic acid for molecular genotyping. Factor V levels were assayed using clotting methods based on factor V-deficient plasma (Dade Behring, Marburg, Germany). APC resistance functional analysis was assayed using a clotting method according to a protocol previously described [10]. APC resistance normalized ratios less than 0.8 were considered abnormal. The polymorphism *F5* G1691A (*F5*, rs6025) was identified according to a protocol described elsewhere [4].

Table 2
Clinical and demographic characteristics of Chilean patients with diagnosis of deep venous thrombosis.

	Men $n = 40$	Women $n = 47$	*p*-Value[a]
Age, years	18–70	18–65	–
Mean ± SD	44.8 ± 12.3	44.6 ± 12.3	0.739
Diabetes mellitus	4 (10.0%)	5 (10.6%)	0.920
Dyslipidemia	3 (7.5%)	3 (6.4%)	0.838
Hypertension	6 (15.0%)	6 (12.8%)	0.763
Body mass index, kg/m^2			
<25	9 (22.5%)	19 (40.4%)	0.075
25–30	20 (50.0%)	17 (36.1%)	0.194
>30	9 (22.5%)	6 (12.8%)	0.231
Familial history	9 (22.5%)	16 (34.0%)	0.235
Previous thrombosis	11 (27.5%)	13 (27.7%)	0.987

[a] *p*-Values from Student's *t*-test or χ^2 test.

Table 3
Frequency of Amerindian mtDNA haplogroups in DVT Chilean patients.

Haplogroup	DVT patients *n*	%
A	3	3.5
B	11	12.6
C	29	33.3
D	34	39.1
NA	10	11.5
Total	87	100.0

NA, non-Amerindian.

2.4. PCR-SSCP analysis

Genomic DNA was purified from blood leukocytes by a procedure previously described by Salazar et al. [11]. Polymerase chain reaction (PCR) was performed using oligonucleotide sequences designed by

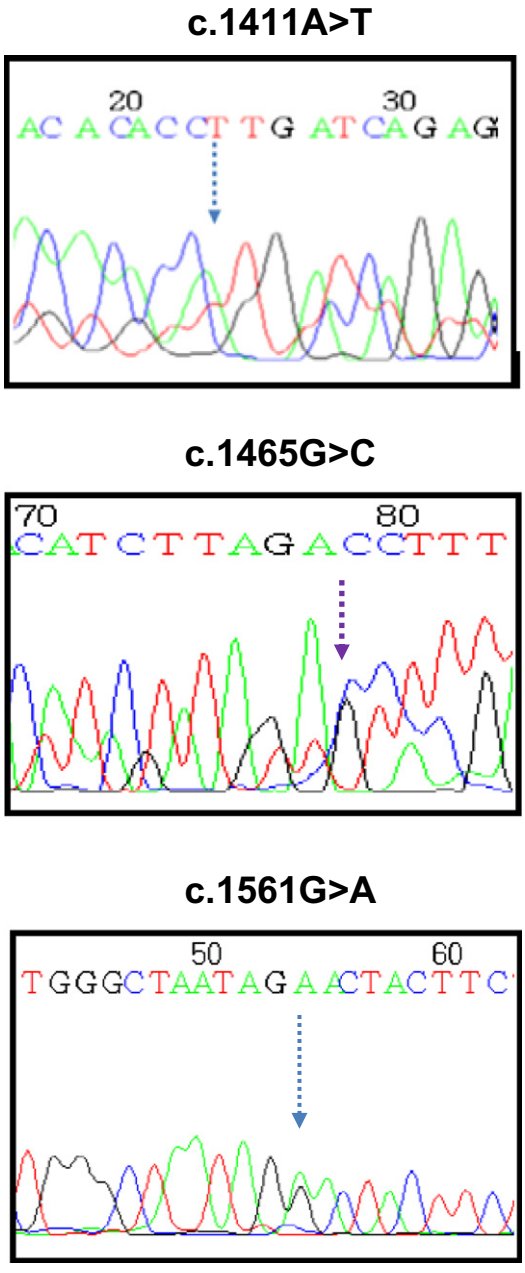

Fig. 1. Novel *F5* gene mutations identified in Chilean DVT patients with Amerindian genetic background.

Table 4
F5 gene mutations in Amerindian Chilean DVT patients with APC resistance.

Location	Nucleotide change[a]	Type	Effect on protein	Name
Exon 10	c.1411A>T	*Missense*	Met → Leu	M443L
Exon 10	c.1465G>C	*Missense*	Glu → Gln	E461Q
Exon 10	c.1561G>A	*Missense*	Gly → Glu	G493E

[a] Sequence variants are identified as suggested by Den Dunnen and Antonarakis [15]. The novel mutations are not described in http://www.ncbi.nlm.nih.gov/sites/varvu?gene=2153, and http://www.hgmd.cf.ac.uk/ac/gene.php?gene=F5.

a bioinformatic software and the *F5* sequence GenBank (accession AY364535, NCBI) (Table 1) on the promoter region, exons 7 and 10 of the *F5* gene that encode for cleavage regions for APC in the protein. Exon 10 was analyzed in two separate reactions (10a and 10b). Each amplification reaction was performed in a final volume of 25 mL containing 50 ng of genomic DNA, 100 nM of each primer, 200 mM of each deoxynucleotide triphosphate, 1 unit of *Taq* DNA polymerase, and PCR buffer (50 mM KCl, 2 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 75 mM Tris–HCl, pH 9.0).

Single-Strand Conformational Polymorphism (SSCP) analysis was used to identify possible sequence variations. Briefly, 1 μL of the PCR product was added to 20 μL of loading buffer (950 mL/L formamide, 10 mmol/L EDTA, 0.5 g/L bromophenol blue and 0.5 g/L xylene cyanol FF), denatured by heating at 95 °C for 8 min, and thereafter directly placed on ice to prevent reannealing of the single-strand products. Five microliters of this mixture was applied to the gel GeneGel Excel 12.5/24 Kit (Amersham Biosciences, Uppsala, Sweden). The SSCP electrophoresis was carried out using the semiautomated GenePhor™ system. The optimized SSCP electrophoretic conditions included the use of two different temperatures (5 °C and 15 °C) for each region of the *F5* gene, described by Salazar et al. [12]. Gels were stained with silver nitrate. Purified PCR products from DNA samples with abnormal SSCP patterns were directly sequenced in two directions using the amplification primers and the results were analyzed with Geneious Pro software, v.5.1.4. All mutations were detected on electropherograms of sequencing reactions, and each sequence was read in both directions. Mutations were confirmed using a second, independent amplification of the affected genomic region and re-sequenced on another day. The nucleotide and amino acid sequences for each exon were compared with the sequences previously reported [13], according to protocols described by Sedano-Balbás et al. [14]. The mutations were identified as suggested by Den Dunnen and Antonarakis [15].

2.5. In silico functional analysis

The prediction of functional effect of the novel mutations was analyzed using the Polyphen-2 software (http://genetics.bwh.harvard.edu/pph2/). For a given mutation, this algorithm uses a Naïve Bayes classifier to score position-specific independent counts and structural features to predict the probability of damage. Computations are based on databases and counting identical alignments in a multiple alignment framework summarized in a score [16]. The value means that the higher the score the most probable the effect on the functionality, separating results among three cases: benign, possibly damaging, and probably damaging [17].

3. Results

The clinical and demographic characteristics of patients enrolled in the study are summarized in Table 2. The frequency of mtDNA Amerindian haplogroups is shown in Table 3. In DVT patients, 88.5% present Amerindian background, defined by the presence of Amerindian mtDNA haplogroups. We determined that 14.9% of DVT patients present APC resistance based on functional assays, and abnormal levels of factor V were not observed in the DVT group. In addition, a total absence of *F5*1691G>A variant was observed in subjects included in this study.

Three novel mutations were identified in *F5* gene (exon 10) in Chilean DVT patients with Amerindian genetic background (Fig. 1). Table 4 summarizes the characteristics of these novel mutations. Each of the mutations in the factor V gene was found in 1 of the 87 (1.1%) DVT patients with APC resistance. In addition, in 100 healthy subjects matched by age and gender in relation to the group of DVT patients, these genetic variants were absent. For promoter region and exon 7 of the F5 gene, we did not observe abnormal SSCP patterns.

The clinical information, laboratory results and *in silico* functional analyses of DVT patients with APC resistance carrying the novel mutations are summarized in Table 5. Selecting HumVar training database, the prediction in Polyphen-2 shows that mutation M443L is benign, meanwhile mutations E461Q and G493E are probably damaging with scores of 0.922 and 0.999, respectively (Fig. 2).

4. Discussion

Venous thrombosis is a multicausal disease where the pathogenesis often includes several hereditary factors, including APC resistance. In the current study, we have examined the potential presence of sequence variants in the *F5* gene in Amerindian patients with deep venous thrombosis (DVT) and APC resistance.

Based on the presence of mtDNA haplogroups, the results showed that the population included in this study presents a predominant Amerindian background, consistent with prior studies conducted in our country. In Chilean population, the frequency of Amerindian haplogroups presents a north–south flow, A and B decrease from North to South, while the haplogroups C and D increase [9,18,19].

Interestingly, our data demonstrate that in these patients, the APC resistance phenotype occurs in the absence of the *F5* G1691A polymorphism. In Caucasoid population, this variant accounts for 95% of APC resistance cases [4,20]. These apparently contradictory results may be explained by the genetic background of the Chilean population. In Amerindian, African and Asiatic population, the FV Leiden polymorphism is exceedingly rare [21,22].

Evidence demonstrates that the downregulation of procoagulant activity of factor V activated (FVa) is mediated by APC-proteolysis at positions Arg306, Arg506, and Arg679 [23]. These regions of the protein are encoded by exons 7 and 10 of the *F5* gene. Previous studies described the presence of variants in exon 7 associated with APC resistance phenotype and thrombosis risk [24–26]. Factor V (*F5*1090A>G) results in an Arg306 to Gly substitution, with a high prevalence (4.5%) in Chinese population [27]. However, in patients with APC resistance, we did not observe sequence variants in the promoter region and exon 7 of the analyzed gene.

Three novel missense mutations (1411A>T, 1465G>C and 1561G>A) were identified in exon 10, in Amerindian patients. These variants

Table 5
Demographic characteristics, laboratory results and *in silico* analysis of DVT patients with APC resistance carrying the novel mutations.

DVT patient	Mutation	Age (years)	Gender	APCR (normalized ratio)	FV levels (%)	F5 rs6025 polymorphism	*In silico* analysis (score)
1	M443L	58	Women	0.56	100	Absent	Benign (0.001)
2	E461Q	40	Men	0.52	95	Absent	Probably damaging (0.922)
3	G493E	37	Women	0.47	84	Absent	Probably damaging (0.999)

APCR, activated protein C resistance.

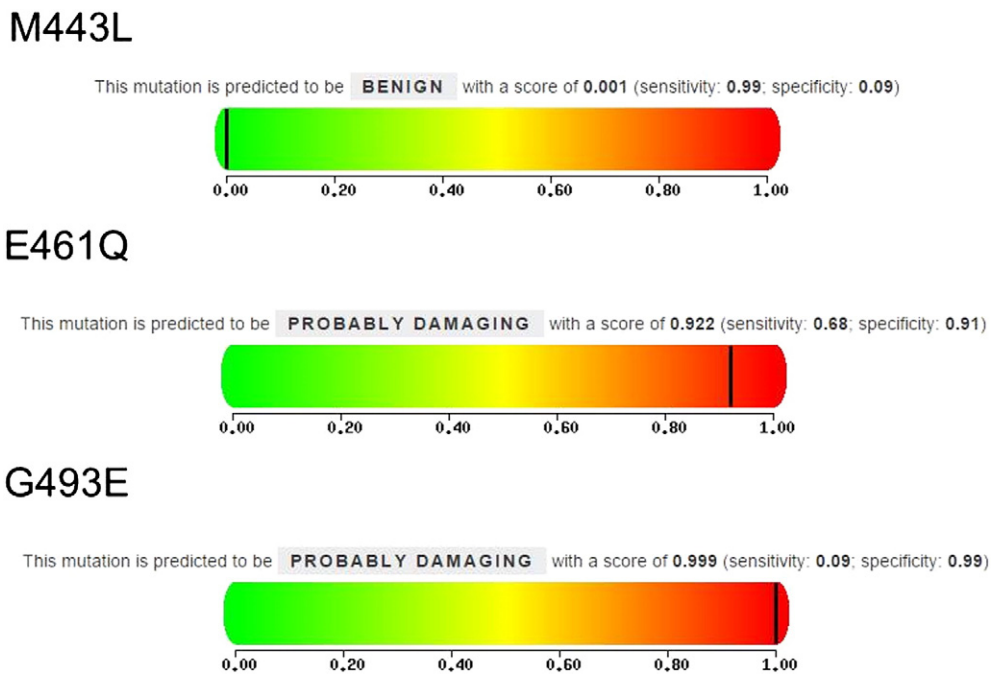

Fig. 2. *In silico* analysis of sequence variants in the *F5* gene identified in Chilean DVT patients with Amerindian genetic background.

result in amino acid substitution in factor V protein (M443L, E461Q and G493E).

Few variants have been described in this region, being the most important the SNP *F5*1691G>A, associated with an increase in the risk for venous thrombosis by 5–7 times in heterozygous individuals. In 1998, Hiyoshi et al. described the *F5* G1628A variant (rs6020), as a thrombosis risk factor in indigenous Thai population [28]. In addition, this polymorphism was associated with a poor response to APC and increased risk for coronary artery disease [29]. In the same region analyzed, Kovács et al. described the mutation p.G493R, associated with factor V deficiency and surgical bleeding [30]. However, the sequence variation described in our study shows no factor V deficiency or bleeding history.

A recent study showed that Polyphen-2 is a bioinformatics tool that provides an important range of information regarding biochemical, structural or functional effect of the substitution may be [31]. According to the functional analysis *in silico*, mutation M443L is considered benign in Polyphen-2 based on similarity between the residues methionine and leucine, which are both non-polar with similar structures. Second, mutation E461Q is considered probably damaging where glutamic acid is replaced by glutamine causing a loss of negative charge affecting non-covalent interactions in this position. Finally, mutation G493E is a replacement of a tiny non-charged polar residue for a larger negative charge inside a beta-sheet forming a beta barrel in the protein. These changes in size and charge are most likely to contribute to undermine the protein structure due to the newly formed electrostatic and steric interactions.

Several studies suggest that the regions near the Arg506 activated protein C cleavage site would have a functional role in the FV activity [32,33]. Interestingly, Heeb et al. demonstrated that residues 493–506 in FVa contribute to binding sites for both FXa and protein S, providing a rationale for the ability of protein S to negate the protective effect of FXa toward APC cleavage of FVa [34]. Based on this experimental evidence that demonstrates the potential role of FVa heavy chain residues 493–506 and *in silico* analysis performed in this study, the *F5* G493E mutation could be a candidate SNP to study by functional assays.

Limitations of the study should consider the need to assess the frequency of the mutations described in a larger cohort of individuals. In addition, functional studies are required to investigate the contribution of these novel variants to the APC resistance phenotype and thrombosis risk. In summary, our data show that the APC resistance phenotype is not associated with the presence of the *F5* G1691A variant. Additionally, in the *F5* gene we describe, for the first time, the presence of sequence variations in Amerindian patients with APC resistance.

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