Complexity of the genetic contribution to factor VII deficiency in two Spanish families: clinical and biological implications

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Background and Objectives. Although many F7 DNA variants have been described to be associated with alterations in factor VII (FVII) levels, the correlation of functional levels of FVII with disease (i.e. bleeding) is highly variable indicating that other factors are likely involved in producing this phenotype.

Design and Methods. We studied two unrelated Spanish families, identified from two asymptomatic propositi with FVII:C levels lower than 1% and 3%. Family members showed a wide range of FVII:C levels. Amplification and direct DNA sequencing of the F7 (promoter, exons, 3'-UTR and a large proportion of introns) identified the genetic variants involved.

Results. We characterized 3 mutations in the F7 coding region (homozygous Q100R in one patient, and double heterozygosity for M298I and G331S in another patient). We also found 16 new DNA polymorphisms. The high variability of FVII levels in family members with the same mutation shows that the inheritance of FVII phenotypes is extremely complex and suggests that polymorphisms might play an important role in modulating FVII levels, and ensuring hemostatic balance under pathologic conditions.

Interpretation and Conclusions. These results highlight the importance of a concerted effect of multiple genetic factors in determining FVII levels. Since there is evidence that FVII levels constitute a risk factor for coronary heart disease and considering the importance of F7 DNA polymorphisms in determining FVII levels, further analyses of these polymorphisms should yield information to aid the understanding of the quantitative variation in FVII levels and the relative genetic risk for cardiovascular disease in the general population.

Key words: factor VII deficiency, bleeding diathesis cardiovascular disease, FVII polymorphisms.

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Coagulation factor VII (FVII) is a vitamin K-dependent glycoprotein that is synthesized in the liver and secreted into the blood as an inactive zymogen.[1] After endothelial damage, tissue factor (TF) is exposed and binds to FVII, activating it (FVIIa) by proteolytic cleavage at Arg152-Ile153. The FVIIa-TF complex initiates the extrinsic pathway of the coagulation cascade.[2,3]

The complete sequence of the human FVII gene (*F7*) was determined by O'Hara *et al.*[4] This gene contains 9 exons and 8 introns spanning 12.8 Kb in the 13q34-q.ter,[5,6] coding for a 406 amino acid protein, although its mRNA can undergo differential splicing depending on the skipping of exon 1b.

The coagulation activity of FVII has been identified as a potential risk factor for cardiovascular disease, particularly for fatal coronary events.[7] Recent studies have provided evidence for an association between common polymorphic markers in the *F7* gene and FVII plasma levels.[8] In addition, it is important to note that FVII levels play a key role in bleeding disorders. Thus, congenital deficiencies of this protein predispose to spontaneous and post-operative bleeding. However, FVII deficiency displays considerable phenotypic and molecular heterogeneity and does not necessarily correlate with plasma FVII activity levels.[9]

Although a large number of *F7* mutations have been described (*http://europium.csc.mrc.ac.uk/usr*) as causes of pathologic levels of FVII, these mutations are too rare to constitute the primary genetic influences on FVII variability in the normal population. This means that there are other genetic factors influencing the phenotype. Recently, as part of the GAIT (*Genetic Analysis of Idiopathic Thrombophilia*) Project, we reported that 52% of the variation in FVII levels in a Spanish population can be explained by genetic factors.[10] In agreement with these data, Bernardi *et al.*[8] reported that about 30-40% of the FVII level variability is due to polymorphisms in the *F7* gene. Given the implication of FVII levels in cardiovascular disease, there has been growing interest in studying other genetic factors that may affect cardiovascular risk. However, currently, only six polymorphisms in the *F7* gene have been reported to have a functional effect on FVII levels. These are: -402, -401, -323 0/10, 73, IVS7 and R353Q.[11-16]

In this report, we describe the molecular basis of a disease in two patients with severe FVII deficiency and their relatives. We believe that the lack of correlation between FVII phenotype and genotype indicates that FVII defi-

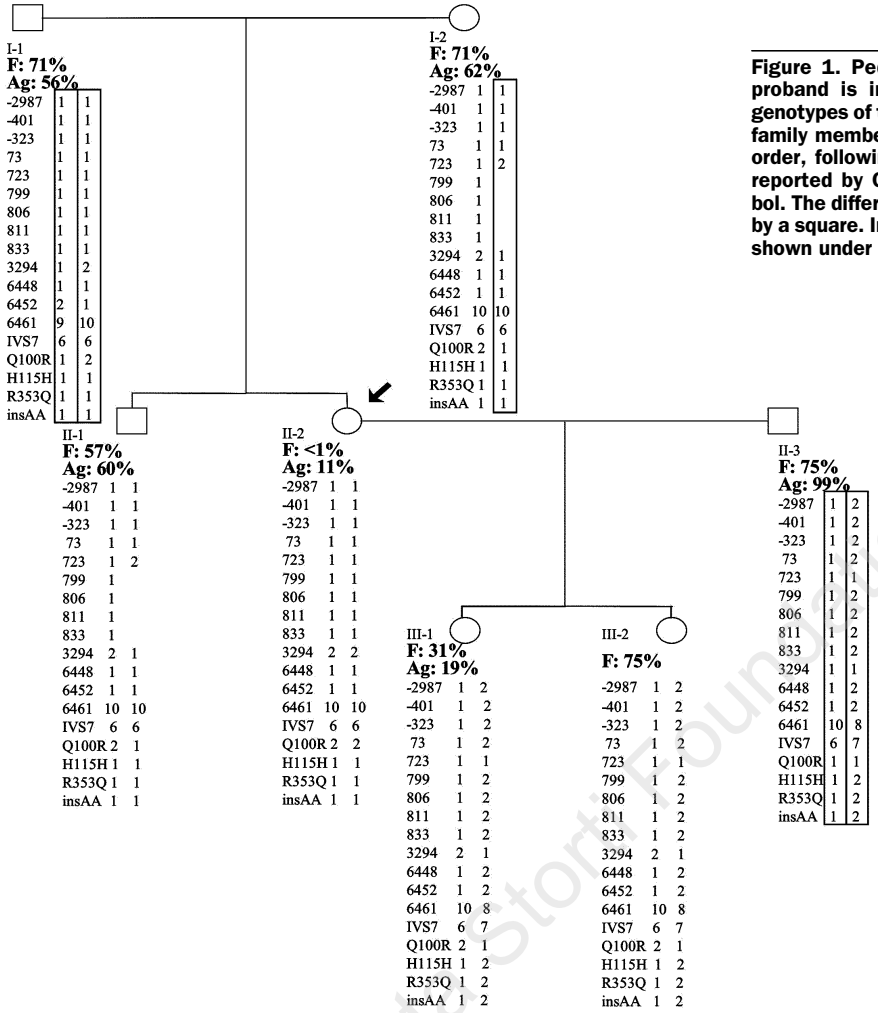

Figure 1. Pedigree of family FVII-1: the proband is indicated by an arrow. The genotypes of the identified DNA variants in family members are shown in descending order, following the sequence numbering reported by O'Hara,[4] under his/her symbol. The different haplotypes are indicated by a square. In addition, the FVII levels are shown under each symbol.

ciency is a complex disease. The new F7 polymorphisms reported here are also interesting because they are likely to be useful genetic markers for assessing the relationship between FVII levels and the risk of cardiovascular disease in the general population.

Design and Methods

Patients

Two unrelated individuals with FVII deficiency from two different families were studied. Propositus one: individual II-2 from family FVII-1 (Figure 1) was a 39-year old female without any history of hemorrhagic diathesis after dental extractions or surgical operations (amygdalectomy at the age of 10; extraction of cervical polyp at 17; and appendicectomy at 30). FVII antigen and FVII activity levels were 10% and <1%, respectively.

Propositus two: individual II-2 from family FVII-2 (Figure 2) was a 31-year-old female, also without any history of hemorrhagic episodes and with FVII activity levels of only 3%.

Both patients were investigated after incidental observation of prolonged coagulation times. All family members of both propositi were recruited and studied, with the exception of the husbands and a sister of the propositus from family FVII-2, in order to obtain extensive family studies. Their FVII antigen and functional levels are shown in Figures 1 and 2. No consanguinity was reported among the relatives in either family.

Blood collection and plasma determinations

Blood samples were collected from an antecubital vein and immediately anticoagulated with a 1/10 volume of 0.129-M sodium citrate. Platelet-poor

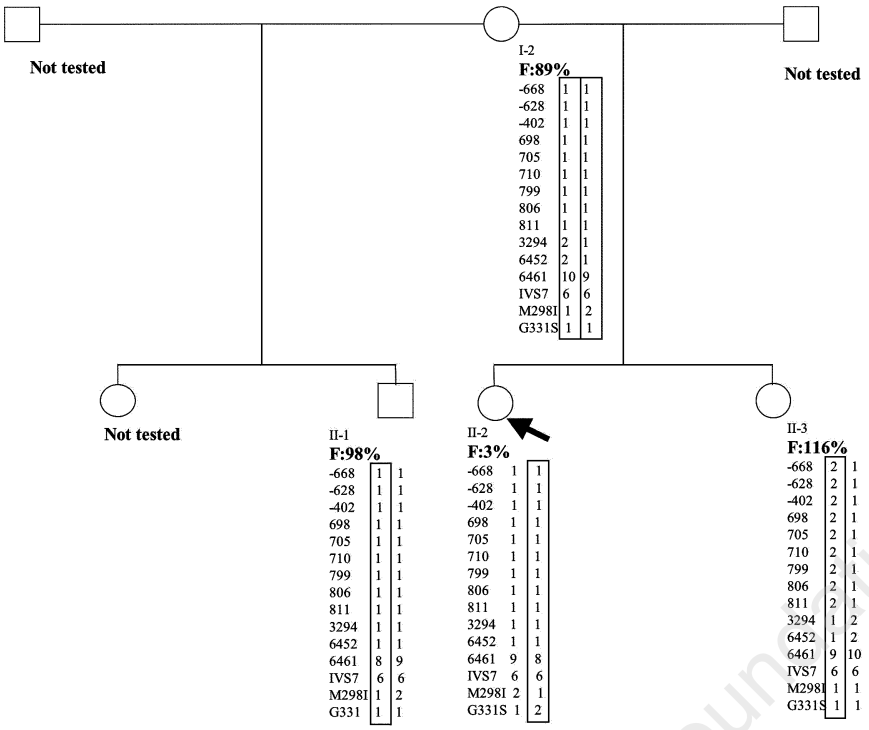

Figure 2. Pedigree of family FVII-2; the proband is indicated by an arrow. The genotypes of the DNA variants in the family members are shown in descending order, following the sequence numbering reported by O'Hara,[4] under his/her symbol. The different haplotypes are indicated by a square. In addition, the FVII levels are shown under each symbol.

plasma was obtained by centrifugation at 2000 g for 20 min. and was frozen and stored at -40°C until analyzed.

The FVII antigen was quantified by an ELISA technique using Asserachrom® VII:Ag (Diagnostica Stago, Asnieres, France). FVII activity was measured in a clotting assay with the STA instrument, using Thromborel S (Behring ref. OUHP), Stago deficient VII (Stago Ref 5000743), Unicalibrator (Stago Ref. 00625), and STA Preciclot Plus II (Boehringer Manheim ref 1776894).[17]

DNA isolation and amplification

DNA was purified from peripheral blood using a standard technique.[18] We analyzed a large region of the *F7* gene, including the promoter region, exons, introns and 3'-untranslated region. Our strategy was to amplify 11 overload fragments using a polymerase chain reaction (PCR) ranging from 403 to 1,830 base pairs (bp). Fragment 1 (403 bp) was amplified using primers 71 (from position –3355 to position –3337) and 71.9 (from –2952 to -2971); fragment 2 (536bp) was amplified with primers 71.4 (from –772 to -753) and 72 (from –237 to –256); fragment 3 (1830 bp) was amplified with primers 73 (from –402 to -382) and 74 (from 1588 to 1567); fragment 4 (1468 bp) was amplified with primers 77 (from 2899 to 2917) and 78 (from 4367 to 4349); fragment 5 (1000 bp) was amplified with primers 79B (from 5317 to 5338) and 710B (from 6317 to 6297); fragment 6 (705 bp) was amplified with primers 79C (from 6138 to 6157) and 710.1 (from 6862 to 6843); fragment 7 (1099 bp) was amplified with primers 711.3 (from 6784 to 6803) and 712.1 (from 7883 to 7864); fragment 8 (702 bp) was amplified with primers 711.1 (from 7737 to 7757) and 712.2 (from 8439 to 8420); fragment 9 (1165 bp) was amplified with primers 711.2 (from 8313 to 8331) and 712 (from 9478 to 9450); fragment 10 (1701 bp) was amplified with primers 713 (from 9310 to 9328) and 714 (from 9706 to 9725); and, finally, fragment 11 (1510 bp) was amplified with primers 715 (from 10809 to 10827) and 716 (from 12319 to 12298). The position of the initial and final nucleotides of all primers used refer to the *F7* GenBank sequence (accession number J02933) from O'Hara *et al.*[4]

Briefly, PCR products were generated in 50 mL reaction mixtures that contained 200 ng of genomic DNA, 0.5U of Taq DNA polymerase (Biotaq DNA Polymerase, Bioline), oligonucleotide primers corresponding to each fragment at a concentration of 0.5 mM each, dNTPs at a concentration of 0.05 µM, 1.5 mM $MgCl_2$ for fragments 3, 4, 5, 7, 8, 9, 10 and 11, and 1mM $MgCl_2$ for fragments 1, 2 and 6 in 1X Bioline PCR buffer. The PCR reaction mixtures for fragments 2, 3, 4, 7, 8, 9 and 11 also contained 5% DMSO.

The PCR program started with 5 min initial denaturation at 94° C followed by 30 cycles of amplifica-

tion (35 cycles for fragment 3) consisting of 1 min at 94°C, 1 min at annealing temperature (57°C for fragments 2, 3, and 7; 59°C for fragments 1, 4, 5, 6, 8, 9 and 11; and 61°C for fragment 10) and 2 min at 72°C, followed by a final extension of 10 min at 72°C. The amplified fragments were electrophoresed on 1% normal agarose gel with controls.

DNA sequencing and DNA variation detection

PCR products were purified with the Quiagen *QIAquick PCR Purification Kit* to prepare them for sequencing. The sequencing reaction was performed in a 10 μL reaction volume containing 3 μL of the purified DNA, 4 μL of the DNA Sequencing Kit *BigDye Terminator Cycle Sequencing Ready Reaction* (Applied Biosystems), 5% DMSO vol/vol and 0.32 mM of the sequencing primer. To sequence, we used the same primers as those used in the PCR amplification and nested primers in the longest fragments to perform gene walking: primers 73.1 (from 86 to 101), 73.2 (from 415 to 434), 74.1 (from 1101 to 1080) and 74.2 (from 1080 to 1101) in fragment 3; primers 77.1 (from 3213 to 3233), 77.2 (from 3116 to 3136), 77.3 (from 3830 to 3849) and 78.1 (from 3988 to 3969) in fragment 4; primer 710G (from 6295 to 6314) in fragment 6; primer 711.5 (from 7195 to 7214) in fragment 7; primer 711.4 (from 8716 to 8736) in fragment 9; primers 714.1 (from 10522 to 10503), 714.2 (from 10201 to 10181), 713.2 (from 10503 to 10522) and 713.3 (from 9706 to 9725) in fragment 10; and, finally, primers 715.1 (from 11259 to 11278) and 716.1 (from 12808 to 12789) in fragment 11. The position of the initial and final nucleotides of these primers refer to the *F7* GenBank sequence (accession number J02933) from O'Hara *et al.*[4]

The sequencing program started with 3 min at 94°C, followed by 25 cycles consisting of 10 sec at 96°C, 5 sec at annealing temperature (50°C for primers 71, 71.4, 71.9A, 77, 77.1, 77.2, 77.3, 78, 79B, 79C, 710.1, 710B, 710G, 711.1, 711.2, 711.3, 711.4, 711.5, 712, 712.1, 712.2, 713, 714, 714.1, 715, 715.1, 716 and 716.1; and 52°C for primers 72, 73, 73.1, 73.2, 74, 74.1, 74.2, 78.1, 713.2, 713.3 and 714.2) and finally 4 min at 60°. Sequences were analyzed using the ABI PRISM 310 Genetic Analyzer according to the manufacturer's instructions.

Screening strategy for mutations and polymorphisms

We carried out an exhaustive genetic analysis of the *F7* gene in order not to miss any DNA variant that might affect the phenotypic variability. The sequence of the *F7* gene was analyzed for each subject. Every alteration in nucleotide sequence was confirmed on at least one independent PCR product by either restriction enzyme digestion, when the DNA variant modified a restriction site (e.g. Apo I for polymorphism –2987C→A), or another independent sequencing analysis with forward or reverse primers. If the DNA variant did not modify a restriction site, one was created by a mutagenesis primer. This was the case for the primer 7insAA (ccctcagtctct ctcagtct) from 11374 to 11355, used to create a new restriction site for *AlwN* I to genotype the 11293-11295insAA insertion.

The *F7* polymorphisms were determined by endonuclease digestion or by sequencing. The following polymorphisms were typed according to published protocols: -402 G→A; -401 G→T and –(323-333)ins10bp in the promoter region, 73, IVS7, H115H, and R353Q.[11-16,19]

Allele frequencies

Two hundred unrelated Spanish individuals were genotyped to calculate the allelic frequencies of the polymorphisms that had been previously reported,[11-16,19] as well as the new polymorphisms identified in this study (Table 1).

Nucleotide position and nomenclature

The position of the mutations, polymorphisms and primers are based on the original *F7* gene sequence reported by O'Hara.[4] For the nomenclature of gene mutations, we followed the recommendations of den Dunnen and Antonarakis,[20] and use the gene symbols recommended by the HUGO Gene Nomenclature Committee.[21]

Results

F7 gene analysis

We analyzed a large proportion of the *F7* gene, including the promoter region, exons, introns and 3'-untranslated region, in order to characterize the genetic basis of FVII deficiency and to identify DNA variants that might influence FVII levels. We did not analyze a highly repetitive GC-rich region in intron 1b because of the complexity of the necessary amplifications. The *F7* gene is 12.8 kb long;[4] with the exception of this repeat in intron 1b, we sequenced 11.6 kb.

F7 mutations in patients with FVII deficiency

We investigated the genetic mutations in two asymptomatic patients belonging to two unrelated Spanish families with FVII deficiency. The levels of FVII activity (FVII:C) in the plasma of these two patients were 1% and 3%; their relatives presented a broad range of FVII levels.

In the first propositus (II-2 from family FVII-1) we found a homozygous A to G transition at nucleotide 7834, which changed glutamine to arginine at codon 100 (Q100R). We were able to demonstrate that individuals I:1, I:2, II:1, III:1 and III:2 were heterozygous for this mutation, exhibiting FVII func-

tional levels ranging from 31% to 71%. Moreover, patient II-2 was also homozygous for a new G to A transition at nucleotide 3294 in intron 1b, which co-segregated with the Q100R in individuals I:1, I:2, II:1, III:1 and III:2 (Figure 1).

Patient II-2 (from family FVII-2), was a double heterozygote for two mutations in exon 8. One mutation was a G to A transition at nucleotide 10811, resulting in the substitution of methionine by isoleucine at codon 298 (M298I), and the other mutation was a G to A transition at nucleotide 10908, resulting in the substitution of glycine by serine at codon 331 (G331S). Individuals I:2 and II:1 were also heterozygous for the M298I mutation. However, individual II-2 was the only carrier of the G331S, which presumably came from her father although his DNA was not available (Figure 2). Only the propositus (II-2) had reduced FVII functional levels.

None of 200 unrelated Spanish controls was a carrier of these three mutations, indicating that these are rare mutations rather than polymorphisms in this Spanish population.

F7 polymorphisms

Our extensive analyses of the *F7* gene in these two families allowed us to detect a number of sequence variations. Seven have already been published as common polymorphisms,[11-16,19] whereas 16 had not been previously described (Table 1). We considered all these DNA variants as polymorphisms because their allelic frequency was equal to or higher than 1% in a sample of 200 unrelated Spanish individuals (Table 1).

In family FVII-1, in addition to the mutations described, we identified a new C to T transition at nucleotide –2987 in the promoter region (in individuals II-3, III-1 and III-2); a 102 bp deletion in intron 1a which covered from 723 to 825 nucleotides, called 723-825IVS1del (individuals I-2, II-1), a T to C transition at nucleotide 799 (in individuals II-3 III-1 and III-2), a G to A transition at nucleotide 806 (in individuals II-3 III-1 and III-2), a C to G transversion at nucleotide 811 (individuals II-3 III-1 and III-2) and a T to C transition at nucleotide 833 (in individuals II-3 III-1 and III-2), all of them in intron 1a. In intron 4, we found a G to T transversion at nucleotide 6448 (in individuals II-3, III-1 and III-2), a G to T transversion at nucleotide 6452 (in individuals I-1, II-3, III-1 and III-2) and a variable number tandem repeat (VNTR) starting at nucleotide 6461 for which individual I-1 had 9/10 repeats, individuals I-2, II-1 and II-2 were homozygous for the 10 allele and individuals II-3, III-1 and III-2 were heterozygous for alleles 8/10 (Figure 1 and Table 1). Finally, we also found an (AA) insertion in the 3'UTR region (in individuals II-3, III-1, and III-2), at nucleotide 11293, called 11293-11295insAA. All of these polymorphisms were present in a heterozygous state.

Table 1. Position, nucleotide variant and type of DNA variant found, together with allelic frequencies of these DNA variants in 200 unrelated Spanish individuals. Published polymorphisms are in bold.

Nucleotide (ref)	*Position*	*DNA variant*	*Type#*	*Allelic frequencies*
-2987	Promoter	C→A	SNP	0.82 - 0.18
-668	Promoter	A→C	SNP	0.82 - 0.18
-628	Promoter	A→G	SNP	0.82 - 0.18
-402(*14*)	**Promoter**	**G→A**	**SNP**	**0.81 - 0.19**
-401 (*11*)	**Promoter**	**G→T**	**SNP**	**0.82 - 0.18**
-323 (*13*)	**Promoter**	**0/10 bp**	**Insertion**	**0.81 - 0.19**
73 (*15*)	**Intron 1a**	**G→A**	**SNP**	**0.81 - 0.19**
698	Intron 1a	T→C	SNP	0.81 - 0.19
705	Intron 1a	G→A	SNP	0.81 - 0.19
710	Intron 1a	C→G	SNP	0.81 - 0.19
723	Intron 1a	VNTR	Deletion	0.99 - 0.01
799	Intron 1a	T→C	SNP	0.61 - 0.39
806	Intron 1a	G->A	SNP	0.61 - 0.39
811	Intron 1a	C→G	SNP	0.61 - 0.39
833	Intron 1a	T→C	SNP	0.81 - 0.19
3294	Intron 1b	G→A	SNP	0.99 - 0.01
6448	Intron 4	G→T	SNP	0.79 - 0.21
6452	Intron 4	G→T	SNP	0.44 - 0.56
6461	Intron 4	VNTR	VNTR	0.03 - 0.26 - 0.31 - 0.40
IVS7 (*12*)	**Intron 7**	**VNTR**	**VNTR**	**0.02 - 0.62 - 0.35 - 0.01**
7880 (*19*)	**Exon 5**	**C→T***	**SNP**	**0.82 - 0.18**
10976 (*16*)	**Exon 8**	**G→A°**	**SNP**	**0.82 - 0.18**
11293insAA	3'UTR	AA	Insertion	0.85 - 0.15

**amino acid substitution H115H ; °amino acid substitution R353Q; #type of polymorphism: SNP: single nucleotide polymorphism; VNTR: variable number tandem repeat.*

When we genotyped all of the family members for previously reported polymorphisms in *F7*, we found that individuals II-3, III-1 and III-2 were heterozygous for the –401G→T and –(323-333)ins10bp in the promoter region, and for the 73G/A in intron 1a, H115H in exon 5 and the R353Q in exon 8. Moreover, although patient II-2 and her relatives I-1, I-2 and II-1 were homozygous for the 6 allele of the IVS7 VNTR in intron 7, her husband and daughters (II-3, III-1 and III-2) were heterozygous (6/7) for this functional polymorphism (Figure 1).

In family FVII-2, the analysis of the *F7* gene revealed several new DNA variants. Individual II-3

was heterozygous for an A to T transversion at nucleotide –668 and an A to G transition at nucleotide –628 in the promoter; a T to C transition at nucleotide 698, a G to A substitution at nucleotide 705, a C to G transversion at nucleotide 710, a T to C transition at nucleotide 799, a G to A transition at nucleotide 806 and a C to G transversion at nucleotide 811, all of them in intron 1a. These DNA variants presumably co-segregated from her father since her mother was not a carrier. The remaining two DNA variants in this individual were a G to A transition at nucleotide 3294 into intron 1b and a G to T transversion at nucleotide 6452 in intron 4, which was also present in her mother, I-2 (Figure 2). The VNTR starting at nucleotide 6461 was also present in this family; individuals I-2 and II-3 were heterozygous for 9/10 individuals II-1 and II-2 were heterozygous for 8/9. Regarding the functional polymorphisms previously reported in *F7*,[11-16] individual II-3 was the only heterozygote for the –402 G→A transition. All individuals were homozygous for the common genotype with the most frequent allelic forms of the polymorphisms, including the VNTR IVS7 polymorphism (Figure 2).

Haplotype analysis

Based on our results, we constructed 10 different haplotypes (Figures 1 and 2), exemplifying the extensive genetic variability found in the *F7* gene in these families. It is important to note that, although members of the FVII-1 family denied any history of consaguinity, evidence of a likely consanguineous union was suggested from the haplotype linked to the Q100R mutation and the low prevalence of FVII deficiency in all populations. Thus, it seems unlikely that the patient inherited two independent alleles each carrying the same rare mutation.

Discussion

During the last decade, evidence has accumulated indicating that high plasma FVII levels increase the risk for ischemic heart disease and arterial or venous thrombosis.[7] Recent reports suggest that the alleles associated with low levels of FVII might protect against myocardial infarction.[15,22] FVII also plays a key role in bleeding disorders. Thus, congenital deficiencies of this protein predispose to spontaneous and post-operative bleeding. However, FVII deficiency displays considerable phenotypic and molecular heterogeneity and does not necessarily correlate with plasma FVII activity levels.[9] Patients with FVII deficiency may have no clinical symptoms or a range of manifestations up to severe hemorrhagic disorders.[9]

To study this issue, we screened a large proportion of the *F7* gene (11.6 kb) for DNA variants. Our samples came from two unrelated asymptomatic patients with inherited FVII deficiency and their relatives, who had a wide range of FVII levels. Given the possibility that variation within non-coding regions may influence the regulation of gene transcription as it does other gene functions,[23,24] we considered that DNA sequencing should not be limited only to the protein-coding regions of the gene but also include the 5' and 3' regulatory regions, as well as introns. To our knowledge, our sequencing analysis is the most extensive applied to identification of genetic variability in FVII deficient families.

This strategy allowed us to identify three different mutations. In family FVII-1 homozygosity for Q100R in the propositus (II-2), was probably the result of a consanguineous marriage (see haplotype in Figure 1). In family FVII-2, the M298I and the G331S mutations are both found in heterozygous state in the propositus (II-2). These three mutations were not detected in any of 200 unrelated Spanish individuals who served as controls.

Curiously, although these mutations (Q100R, M298I and G331S) have been associated with bleeding diseases in several patients,[25-27] our patients with these mutations were asymptomatic and did not exhibit a bleeding tendency. Their lack of symptoms may be explained by the ability of very small amounts of activated FVII to initiate the coagulation cascade.[28] In fact, it has been reported that very small amounts of thrombin formed during the initial stage of the coagulation cascade, although insufficient to initiate significant fibrin polymerization alone, are able to back-activate the intrinsic pathway that finally generates sufficient amounts of thrombin to form a clot.[29]

Based on this evidence, it would appear that other environmental factors influence the bleeding tendency and, more importantly, that genetic factors might contribute to the FVII plasma levels. Our studies identified some *F7* polymorphims that were previously associated with FVII levels in plasma.[11-16] In addition, we identified 16 new polymorphisms (Table 1), indicating that genetic factors play a role in FVII levels and may also affect the risk of bleeding disorders. Of special interest are those polymorphisms located in the promoter region since the promoter contains major regulatory elements for transcription.[11] In addition, polymorphisms located in intron 1a are likely to affect the regulatory process, since FVII mRNA can undergo alternative splicing in this intron with the result that exon 1b is skipped.[30] Thus, it is reasonable to postulate that these polymorphisms might be involved in transcriptional regulatory mechanisms that could affect the synthesis, stability, and/or transport of corresponding FVII molecules (primary transcripts, mRNA, or protein), especially the deletion of 102 bp (723-825IVS1del) in intron 1a. Additional insight into the causative role of these polymorphisms may be gleaned from molecular modeling, comparative analyses, coupled with mutagenesis, expression and functional studies.

Based on our findings, we hypothesize that the differences in FVII levels in individuals carrying the Q100R, G331S and M298I mutations might be explained by the modulating effect of polymorphisms. For example, in FVII-1, although the propositus was homozygous for the Q100R mutation, she had a notable reduction of functional and antigen levels, and her heterozygous relatives showed marked variability. This could be partially explained by environmental factors, but most likely there are background genetic factors that modify the FVII levels. Interestingly, individuals with the same FVII:C levels have different genotypes for each of the mutations, as well as for the polymorphisms. This confirms our hypothesis that there are multiple genetic factors determining the FVII levels. It is worth recalling that the heritability of FVII levels is high.[10] It is also interesting to point out that propositus 2 had FVII:C below 3% because of mutations carried by each of her alleles. However, individuals I:2 and II:1 had normal FVII levels and not 50% as might be expected in carriers of only one of the mutations. These data clearly show that FVII deficiency is complex, and the variability in levels could be the result of environmental factors working in concert with the mutations (Q100R, G331S and M298I).

It is noteworthy that these polymorphisms not only affect the function of FVII, but that they may also modify the hemostatic balance between bleeding and clotting. They may amplify the differences in FVIIa that have clinical implications, as suggested by the recent finding of an association between certain *F7* genotypes and myocardial infarction.[31-32] It is, therefore, likely that some of these polymorphisms are in linkage disequilibrium with the *F7* gene polymorphisms that we have identified, which might be physiologically relevant to the metabolism of FVII. In view of the proposed role of FVII as a risk factor for coronary heart disease[7,33] it is important to define the mechanisms which make these *F7* polymorphisms functional. Since the risk factor burden of individual polymorphisms may vary among populations, further studies in different populations may cast light on the question of whether the new *F7* polymorphisms identified here are important genetic risk factors for cardiovascular disease.

In conclusion, these results show that multiple genetic factors determine FVII levels. They also highlight that FVII deficiency is a complex disorder involving genetic and environmental interactions. If FVII levels are indeed risk factors for coronary heart disease, further analyses of these novel DNA variants should yield useful information for elucidating the quantitative role of genetic variation in FVII levels and relative genetic risk of cardiovascular disease in the general population.

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Contributions

MS-L performed laboratory analyses, wrote the paper and was involved in the design of the study, selection of patients analysis and interpretation of data. JMS, JM wrote part of the paper and were involved in analysis and interpretation of data. IC were involved in interpretation of data. JCS played a part in the selection of patients. EM-M and EM-S performed genetic analyses. CV was in charge of the plasma analysis. EM-M and EM-S performed laboratory analyses and analysed their results. All the authors revised the manuscript and contributed to its intellectual content. JF and JMS reviewed the paper and supervised the whole study.

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In the following paragraphs, Professor Cazzola summarizes the peer-review process and its outcomes.

What is already known on this topic

Patients with FVII deficiency may have no clinical symptoms or a range of manifestations up to severe hemorrhage. The molecular basis of this heterogeneity is poorly understood.

What this study adds

This study suggests that factor VII gene (F7) polymorphisms may play an important role in modulating FVII levels and clinical phenotype in patients with F7 mutations, thus accounting for variable genotype:phenotype relationships.