Molecular analysis of multiple genetic variants in Spanish FXII-deficient families

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ABSTRACT

We studied 3 Spanish patients with <1% FXII levels. DNA sequencing of the whole F12 gene identified 15 genetic variants. Molecular analyses of F12 mRNA demonstrated that the deficiency was caused by 5281delG in exon 9 of Patient 1 (in the homozygous state) and the 6306delG in exon 12 and another deletion of 23 bp in intron 8 of Patient 2 (both in the heterozygous state). Finally, a G-8C transversion was found in the homozygous state in Patient 3. Based on previous data, including a mouse model, the G-8C might be responsible for the FXII deficiency. None of these variants were present in 40 controls.

Key words: F12 gene, SNPs, FXII deficiency, C46T.

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Factor XII (FXII) participates in the blood coagulation cascade by initiating the intrinsic pathway, fibrinolysis, and generating bradykinin and angiotensin.[1,2] FXII deficiency is a recessive Mendelian trait due to mutations in the *F12* gene that comprises 14 exons on chromosome 5.[3,4] The normal variation of FXII plasma levels exhibits a high heritability (67%) and is correlated significantly with thromboembolic disease.[5] Despite the genetic data, the function of coagulation FXII is poorly understood and controversial results regarding the clinical consequences of variation in FXII levels have been described. While lower FXII levels have been associated with a high risk of arterial and venous thrombosis[6] it has also been reported that patients with FXII deficiency may show no clinical symptoms. The present study examined the whole *F12* gene in 3 patients with a severe FXII deficiency. Therefore, the genetic alterations in the *F12*, together with the co-segregation of the mutation *C46T*, may yield further understanding of the role of FXII and how it affects the risk of thrombosis.

Design and Methods

Subjects and blood samples

This study included 3 unrelated patients with severe FXII deficiency (<1%). Patient 1 was a 34 year old woman who had suffered 4 miscarriages within the first quarter of pregnancy. Patient 2 (I-1; Figure 1) was a 49 year old woman who had suffered one miscarriage in the second quarter of pregnancy. She had a family history of thromboembolic disease. We recruited her son (II-1; Figure 1) and daughter (II-2; Figure 1), 24 and 21 years old respectively. Patient 3 (II-2; Figure 2) was a 58 year old woman who had suffered an embolism at the age of 57. We also recruited her 2 sisters (II-1 and II-6; Figure 2), who were 60 and 51 years old, a 32 year old daughter (III-1; Figure 2) and 2 nieces (III-5 and III-7; Figure 2) 25 and 15 years of age respectively. No consanguinity among any of the patients' parents was reported. All 3 patients exhibited normal values for the thrombophilic parameters (including functional antithrombin, amidolytic PC, total free and functional PS, functional assay for FVIII, APCR, the FVL, the G20210A F2, homocysteine levels, lupus anticoagulant, APTT test, anticardiolipin and antiphosphatidylserine antibodies) with the exception of prolonged APTT. All procedures were reviewed and approved by the Institutional Review Board of the Hospital de la Santa Creu i Sant Pau.

DNA and RNA analyses

DNA from the 3 patients and 40 healthy controls was isolated from peripheral blood leukocytes by a standard technique.[7] We analyzed the *F12* gene (including the promoter, exons, introns and the 3'-UTR) by PCR and direct sequencing of 4 overlapped fragments. The *F12* gene DNA variants were deter-

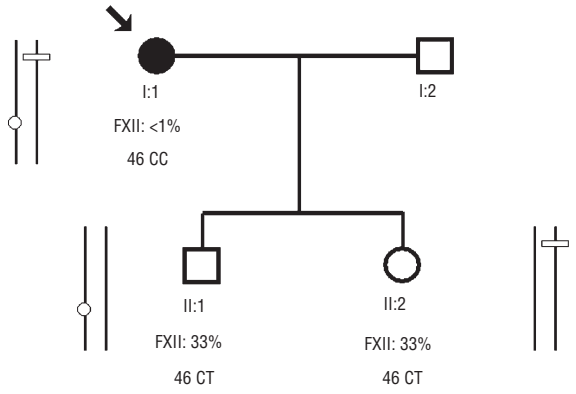

Figure 1. Family FXII-2 pedigree. The proband is identified by an arrow. FXII antigen levels are shown under each sex symbol. Vertical lines indicate the chromosomes. The 23bp deletion and the G deletion respectively are shown on the chromosomes. Therefore, Patient 2 (I:1) carries 2 deletions (one in each chromosome) and her offspring inherited 1 deletion each.

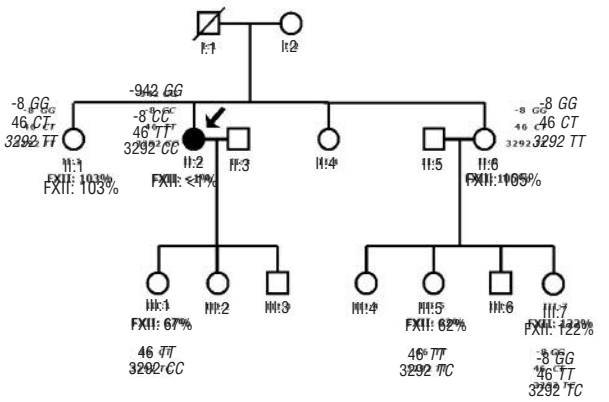

Figure 2. Family FXII-3 pedigree. The proband is identified by an arrow. FXII antigen levels of individuals are shown under each sex symbol. Genotypes are also shown under each sex symbol.

mined in family members by independent sequencing analyses. RNA analyses were performed in 7 healthy individuals, in the 3 patients, in the offspring of Patient 2 and in 7 normal healthy controls. Total RNA was isolated from blood using the PAXgene Blood RNA kit following the supplier's recommendations. Then, a RT-PCR was performed to obtain *F12* cDNA. The *F12* cDNA was sequenced as the genomic DNA. We used NetGene2 Server[8] for the predictions of splice sites in humans. Genetic variants were numbered according to the transcription initiation.

Results and Discussion

We analyzed the whole *F12* gene in 3 patients with severe FXII deficiency and 40 healthy controls. From these analyses we found that the *F12* gene was 7,476 bp long rather than the 12 kb as previously described.[3] This difference in length is due to intron 1 that contains 367 bp and not the 4677 bp that has been reported. This result was consistent in all of the individuals. To our knowledge, our study is the most extensive sequencing analysis that identified genetic variability in FXII-deficient families and it has allowed us to determine the correct sequence of the *F12* locus. We identified 15 genetic variants in the *F12* gene in the 3 patients (Table 1). It is worth noting that only 2 of the variants were located in exons and both were deletions. In addition, 11 out of the 15 DNA variants that we identified have not been published. In Patient 1, we found a G deletion at position 5281 in exon 9 (*5281delG*) in the homozygous state, affecting the codon 288 (confirmed as homozygous in the cDNA). Therefore, the pathologic effect could be attributable to the generation of a truncated protein by a premature stop codon (136 amino

Table 1. Known and novel allelic variants found in FXII deficiency patients.

DNA position	*Type*	*Change*	*Zone*	*Known/ Novel*	*Patient 1*	*Patient 2*	*Patient 3*	*dbSNP*
-941	SNP	A/G	Promoter	Novel	GG	AG	GG	ss65624554
-8	SNP	G/C	Promoter	Known	GG	GG	CC	ss65624557
46	SNP	C/T	4 bp before ATG	Known	TT	CC	TT	ss65624558
2697	SNP	C/T	Intron 2	Novel	TT	CC	TT	ss65624562
3163	SNP	C/T	Intron 2	Novel	CC	TT	CC	ss65624567
3261	SNP	G/T	Intron 2	Novel	TT	GG	TT	ss65624572
3272	SNP	T/C	Intron 2	Novel	CC	TT	CC	ss65624573
3275	SNP	A/G	Intron 2	Novel	GG	AA	GG	ss65624574
3292	SNP	T/C	Intron 2	Known	TT	CT	CC	ss65624575
5081	DEL	23BP	Exon 8-intron 8 junction	Novel	NN	N-DEL	NN	ss65624581
5281	DEL	G	Exon 9	Novel	DEL-DEL	NN	NN	ss65624582
5400	VNTR	8-9 G	Intron 9	Known	9-9	8-8	9-9	ss65624583
5459	SNP	T/C	Intron 9	Novel	TT	CC	TT	ss65624584
5951	SNP	T/C	Intron 10	Novel	CC	TT	CC	ss65624586
6305	DEL	G	Exon 12	Novel	NN	N-DEL	NN	ss65624589

acids downstream than from wild type stop codon) in both alleles. In Patient 2 we found a heterozygous deletion of 23 bp in intron 8. This deletion (5081-5103del23bp) started 2 bp downstream from the end of exon 8 covering 27% of the intron 8. Some information on the function which of this mutation came from a bioinformatic analysis indicated that the presence of this deletion is consistent with the loss of both wild type donor and acceptor splice sites of intron 8 (Table 2). In addition, while there is no alternative donor splice site in the mutated sequence, a cryptic acceptor splice site sequence appeared 48 bp upstream than the wild type (Table 2) indicating that the surrounding sequences are important in selecting the new or the wild type acceptor sites. A second deletion (6306delG in exon 12) was found in Patient 2, introducing a frameshift that removed the wild type stop codon of the protein. Therefore, the resulting protein had 43 aminoacids more than the wild type protein. Since this deletion was found in the homozygous state in the cDNA but in the heterozygous state in the genomic DNA, it seemed that only the allele carrying this mutation was being expressed. The patient's son and daughter (Figure 1) were heterozygous for each one of these deletions, which is in agreement with a heterozygous FXII deficiency in both individuals. In addition, although Patient 2 was homozygous for the normal allele (C/C) of the *C46T* mutation, both children were heterozygous having inherited the abnormal T allele from their father. The *C46T* mutation has been associated consistently with low FXII levels, therefore the co-existence of both mutations in these individuals (the deletion and the 46T), each mutation affecting a different allele, could explain the low (33%) FXII levels. Among the allelic variants found in Patient 3, only the mutations G-8C and *T3292C* were not detected in 40 unrelated controls. A relationship has been reported between severe FXII deficiency and these 2 mutations.[9] As in this previous report, since our patient was also homozygous for both mutations, we cannot define their possible function. Nevertheless, Hofferbert[9] suggested that the T3292C is responsible for the deficiency due to a cryptic splice site in intron 2, although expression analysis was not performed to confirm this hypothesis.[9] In our study, when we analyzed the cDNA of Patient 3, no splicing change was observed. Therefore, it is unlikely that the intronic T3292C caused a FXII deficiency as a result of aberrant splicing. More importantly, it has been reported that HNF4-α transcription factor plays a critical role in the regulation of the expression of the *F12* gene in the mouse.[10] The binding site for HNF4-alpha in the promoter of *F12* in the mouse (-64 AGACCTTTGCCCG -52) has a homologous binding site in the human *F12* (-16 AGACCTTTGGCCA-4) that involved the *G-8C* mutation. This observation suggests that FXII deficiency in this patient was due to the reduction of expression by the modification of a transcription binding site. Nevertheless, further experiments and functional assays for this G-8C mutation should be performed to clarify its affect on FXII levels.

The sister and nieces of Patient 3 (Figure 2) were normal for the G-8C mutation, but her daughter (III-1; Figure 2) was heterozygous for this mutation, which is consistent with a heterozygous FXII deficiency (FXII levels of 67%). Of special interest is the difference observed in FXII levels in both nieces, since they share the same genotypes with the exception of *C46T* mutation. The III-7 is heterozygous for the *C46T* genotype (C/T) and showed FXII levels of 122%, whereas her sister (III-5) is homozygous for the mutated T allele. This shows a notable reduction of her FXII levels (62%). No other polymorphisms or mutations were identified in this branch of Patient 3's family.

Based on our previous experience[11] and although the *F12* gene is expressed in liver, we took advantage of the ectopic transcription of *F12* gene in lymphocyte to analyze the effect of these mutations at the mRNA levels. Our hypothesis was that the deletions and splice-site mutations identified in these patients might be responsible for the FXII deficiency by alterating the mRNA levels (alternative splicing or exon skipping). However, neither alteration in the mRNA processing nor other

Table 2. Neural network predictions of splice sites in humans from NetGene2 World Wide Web Server (***Brunak et al., 1991; Hebsgaard et al., 1996***).

Donor Splice Sites, Direct Strand

pos 5'->3' phase strand	*confidence*	*5' exon intron 3'*
Normal allele	0.71	CCTTCTGCCG^GTGCGCCGCG
Del23bp allele	No donor site predictions above threshold	

Acceptor Splice Sites, Direct Strand

pos 5'->3' phase strand	confidence	5' exon intron 3'
Normal allele	0.65	GTGGCTACAG^GAACCCGGAC
Del23bp allele	0.69	TCCGCCCCAG^GGCTCCGGGC

changes in the coding region were found in the cDNA from these patients.

There are only a few reports describing the molecular basis of congenital FXII deficiency. The majority of them deal with amino acid substitutions in the coding region.[12-14] In our study, we did not detect any point mutation in the coding region, supporting the hypothesis that another kind of genetic variant (i.e., deletions) underlies the genetic basis of FXII deficiency. In addition, as reported in studies of other genes,[11] the phenomenon of allelic exclusion could be a mechanism by which the *F12* allele is not expressed resulting in the FXII deficiency. This mechanism by which a newly created premature termination codon results in a decrease in concentration of steady-state cytoplasmic mRNA is not yet understood.

In addition to these mutations, we detected the *C46T* mutation co-segregating in the patients and their families. Although the effect of this mutation on the FXII levels should be irrelevant in our patients due to the weighty effect of the rare mutations, the *C46T* mutation is clearly important in determining FXII levels in the patients' relatives. In fact, although the members of these 2 families showed wide variability in FXII levels, the concentration of FXII in plasma were perfectly correlated with their respective genotypes, especially when the *C46T* genotype was taken into account.

An important issue to be addressed in our study is the clinical status of these patients. Two of them had suffered miscarriages (an expression of a thrombophilic condition) and one had a familial history of thrombosis. The third patient suffered a deep venous thrombosis. It is interesting to note that most of the patients with FXII deficiency and venous thrombosis had some associated congenital or acquired risk factors.[15,16] In our study, with the exception of prolonged APTT, all 3 patients were normal for the basic thrombophilia parameters (including FVL and G20210A F2 mutations). Further studies, including follow-up of these families, should help clarify the role of FXII levels and the genetic determinants on the risk of cardiovascular disease. In conclusion, we believe that this molecular genetic study is a good illustration of the complexity of the relationship between phenotype and the risk of disease.

Authors' contributions

JMS, JF, and CM designed the study. CM and EM-M performed experimental work. JMS and CM wrote the manuscript. All authors contributed to the interpretation of data, approved the final version of the manuscript and declared that they have no potential conflict if interest.

Conflicts of Interest

The authors reported no potential conflicts of interest.

References

1. Kaplan AP, Castellino FJ, Collen D, Wiman B, Taylor FB Jr. Molecular mechanisms of fibrinolysis in man. Thromb Haemost 1978;39:263-83.
2. Griffin JH, Cochrane CG. Recent advances in the understanding of contact activation reactions. Semin Thromb Hemost 1979;5:254-73.
3. Cool DE, Edgell CJ, Louie GV, Zoller MJ, Brayer GD, MacGillivray RT. Characterization of human blood coagulation factor XII cDNA. Prediction of the primary structure of factor XII and the tertiary structure of b-factor XIIa. J Biol Chem 1985; 260:13666-76.
4. Royle NJ, Nigli M, Cool D, MacGillivray RT, Hamerton JL. Structural gene encoding human factor XII is located at 5q33-qter. Somat Cell Mol Genet 1988;14:217-21.
5. Souto JC, Almasy L, Borrell M, Gari M, Martinez E, Mateo J, et al. Genetic determinants of hemostasis phenotypes in Spanish families. Circulation 2000;101:1546-51.
6. Halbmayer WM, Mannhalter C, Feichtinger C, Rubi K, Fischer M. The prevalence of factor XII deficiency in 103 orally anticoagulated outpatients suffering from recurrent venous and/or arterial thromboembolism. Thromb Haemost 1992; 68: 285-90.
7. Miller SA, Dyckes AA, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acid Res 1988; 1215.
8. Brunak S, Engelbrecht J, Knudsen S. Prediction of human mRNA donor and acceptor sites from the DNA sequence. J Mol Biol 1991;220:49-65.
9. Hofferbert S, Muller J, Kostering H, von Ohlen WD, Schloesser M. A novel 5'-upstream mutation in the factor XII gene is associated with a TaqI restriction site in an Alu repeat in factor XII-deficient patients. Hum Genet 1996;97:838-41.
10. Inoue Y, Peters LL, Yim SH, Inoue J, Gonzalez FJ. Role of hepatocyte nuclear factor 4a in control of blood coagulation factor gene expression. J Mol Med 2006;84:334-44.
11. Soria JM, Berg LP, Fontcuberta J, Kakkar VV, Estivill X, Cooper DN, et al. Ectopic transcript analysis indicates that allelic exclusion is an important cause of type I protein C deficiency in patients with nonsense and frameshift mutations in the PROC gene. Thromb Haemost 1996; 75:870-6.
12. Kanaji T, Kanaji S, Osaki K, Kuroiwa M, Sakaguchi M, Mihara K, et al. Identification and characterization of two novel mutations (Q421 K and R123P) in congenital factor XII deficiency. Thromb Haemost 2001; 86:1409-15.
13. Ishii K, Oguchi S, Moriki T, Yatabe Y, Takeshita E, Murata M, et al. Genetic analyses and expression studies identified a novel mutation (W486C) as a molecular basis of congenital coagulation factor XII deficiency. Blood Coagul Fibrinolysis 2004;15:367-73.
14. Oguchi S, Ishii K, Moriki T, Takeshita E, Murata M, Ikeda Y, et al. Factor XII Shizuoka, a novel mutation (Ala392Thr) identified and characterized in a patient with congenital coagulation factor XII deficiency. Thromb Res 2005;115:191-7.
15. Girolami A, Randi ML, Gavasso S, Lombardi AM, Spiezia F. The occasional venous thromboses seen in patients with severe (homozygous) FXII deficiency are probably due to associated risk factors: a study of prevalence in 21 patients and review of the literature. J Thromb Thrombolysis 2004;17:139-43.
16. Roldan V, Gonzalez-Conejero R, Marin F, Pineda J, Vicente V, Corral J. Five prothrombotic polymorphisms and the prevalence of premature myocardial infarction. Haematologica 2005;90:421-3.