Molecular genetic analysis of severe coagulation factor XI deficiency in six Italian patients

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GIORGIA ZADRA
ROSANNA ASSELTA
MASSIMO MALCOVATI
ELENA SANTAGOSTINO
FLORA PEYVANDI
PIER MANNUCCIO MANNUCCI
MARIA LUISA TENCHINI
STEFANO DUGA

A B S T R A C T

Background and Objectives. Factor XI (FXI) deficiency is a rare autosomal recessive coagulopathy which is, however, frequent among Ashkenazi Jews. Two mutations, type II (Glu117stop) and type III (Phe283Leu), account for the majority of abnormal alleles in this population. The aim of this study was to analyze the molecular basis of FXI deficiency in six unrelated Italian probands with severe deficiency, a population hitherto largely unexplored.

Design and Methods. All patients showed unmeasurable functional FXI levels in plasma. Mutational screening was performed by sequencing. Haplotype analysis was performed using intragenic polymorphisms. Expression studies were performed by transient transfection in COS-1 cells.

Results. Sequencing identified two novel mutations: a nonsense mutation (Cys118stop) in exon 5 in two probands, and a 6-bp deletion (643-648delATCGAC) in exon 7 in one proband. The Cys118stop is predicted to cause FXI deficiency by a secretion defect and/or by increased mRNA degradation. The 6-bp deletion causes the loss of residues Ile197 and Asp198. There was a remarkable secretion impairment of the deleted FXI protein. In four of the six probands, the type II mutation was found. Haplotype analysis in patients carrying the type II mutation revealed that they share a common haplotype, perhaps derived from a Jewish ancestor.

Interpretation and Conclusions. The identification and characterization of two novel mutations widen the mutational spectrum of FXI deficiency. Haplotype analysis is compatible with a Jewish origin of the type II mutation. The high occurrence of the type II mutation among Italian patients will be helpful to direct future genetic screenings.

Key words: FXI deficiency, Italian patients, deletion, nonsense mutation, type II mutation.

From the Department of Biology and Genetics for Medical Sciences, University of Milan (GZ, RA, MM, MLT, SD), Angelo Bianchi Bonomi Hemophilia and Thrombosis Center and Fondazione Luigi Villa, Department of Internal Medicine, University of Milan and IRCCS Maggiore Hospital, Milan, Italy (ES, FP, PMM).

*Correspondence:
Dr. Stefano Duga,
Department of Biology and Genetics for Medical Sciences, via Viotti, 3/5 -20133 Milano, Italy.
E-mail: stefano.duga@unimi.it*

Factor XI (FXI) is a zymogen of a serine protease that participates in the early phase of blood coagulation as a catalyst in the conversion of factor IX (FIX) to activated FIX (FIXa) in the presence of calcium ions.[1,2] FXI exists in two different forms: plasma FXI, a glycoprotein primarily produced by hepatocytes[3] (molecular weight between 125 and 160 kDa) and platelet FXI (a 220-kDa product), whose site of synthesis and nature are still matters of debate.[4]

Among coagulation factors, human FXI is unique in that it is a dimer composed of two identical 80-kDa polypeptide chains linked by a disulfide bond, and circulates in plasma as a complex with high molecular weight kininogen (HMWK).[5] Upon activation, each monomer is cleaved into an amino-terminal heavy chain and a carboxy-terminal light chain, held together by three disulfide bonds.[6] Each light chain contains the catalytic domain of the enzyme and is homologous to the trypsin family of serine proteases. Each heavy chain, which mediates binding to HMWK and FIX, is composed of four tandem repeats of 90 or 91 amino acids (apple domains) with a characteristic disulfide-bonded organized structure.[7] The FXI gene (*F11*), located on the tip of the long arm of chromosome 4 (4q35.2),[8] consists of 15 exons and 14 introns (named A to N) spread over a genomic region of approximately 23 kb.[9]

FXI deficiency was first reported as a hemophilia-like syndrome by Rosenthal and co-workers in 1953.[10] However, this coagulopathy differs from classical hemophilia in that spontaneous bleedings, such as hemarthrosis and purpura, are rare and hemorrhages most often occur only after

trauma or minor surgery. Moreover, since it is inherited as an autosomal recessive trait (MIM *264900), both sexes can be affected. Homozygotes have severe FXI deficiency (less than 20%), whereas heterozygotes have partial FXI deficiency (20 to 50%).[11] Curiously, bleeding manifestations, which may range from a complete lack of symptoms to hemorrhages requiring blood transfusions, seem to correlate poorly with the level of plasma FXI activity. It has been hypothesized that platelet FXI (probably originating from an alternative splicing), may help to compensate for the absence of plasma FXI in asymptomatic individuals.[12] Another reasonable explanation for this paradox is that the one-stage FXI activity assay used does not reflect some effects of FXI that are crucial for hemostasis, such as those on the function of platelets and of the fibrinolytic system;[13] however this explanation does not account for the vast majority of FXI-deficient patients, who are *antigen negative*.

Very recently, dominant transmission of FXI deficiency was described in two families. This unusual pattern of inheritance was due to two different missense mutations which were demonstrated to exert a dominant negative effect on wild type FXI secretion through intracellular heterodimer formation.[14]

FXI deficiency is a rare disorder in the general population (estimated incidence of 1 in 10^6),[15,16] however it is one of the most common inherited disorders among Ashkenazi Jews. Two prevalent mutations (the so-called type II and type III mutations) have been identified in the FXI gene of Ashkenazi Jewish patients.[17-19] The type II mutation is a nonsense mutation in exon 5, creating a premature termination codon (Glu117stop) while the type III mutation is a missense mutation in exon 9 resulting in a Phe283Leu amino acid substitution. Type II and type III mutations together account for most cases (95%) of FXI deficiency in Ashkenazi Jews, but are responsible for only 12% of cases of FXI deficiency in patients with no known Jewish ancestry.[20,21] Among the latter, a significantly higher level of allelic heterogeneity has been reported in different ethnic groups, remarkable exceptions being French Basques, French patients from Nantes and English patients, in whom different prevalent ancestral mutations were found.[22-24] So far, besides the type II and type III mutations, 56 additional causative mutations (31 missense mutations, 10 nonsense mutations, 7 splice-site mutations, 7 small insertion/deletion, and a single large deletion) have been found throughout the gene in both Ashkenazi Jews and in other populations.[14,22-43]

The aim of the present work was to identify the molecular basis of FXI deficiency in six unrelated Italian probands.

Design and Methods

Patients

Six probands with severe FXI deficiency, belonging to apparently unrelated families (as far as ascertainable from their personal history) were studied.

Proband 1 is a 36-year-old man from Milan. At the age of 23 years he was treated with fresh-frozen plasma infusions after cervical disc surgery; subsequently, he was treated with an antifibrinolytic drug after a dental extraction. No bleeding problems occurred after either operative procedure. Other family members had no bleeding tendency, except for the proband's sister who experienced excessive bleeding after tonsillectomy, at the age of 2 years, and had a FXI activity level in plasma < 1%.

Proband 2 is a 56-year old woman from Milan. She had normal menses and no bleeding tendency until the age of 24 years when a bleeding episode occurred after a dental extraction. This did not, however, require any transfusions. Subsequently, at the age of 52 years, she underwent a right knee arthroscopy under prophylactic treatment with fresh-frozen virus-inactivated plasma. More recently, prophylactic treatment was not administered to cover a further minor orthopedic procedure, and no bleeding complications occurred.

Proband 3 is a 62-year old man from Reggio Emilia. He frequently suffered from severe epistaxis, resolved by fresh-frozen plasma infusion. Excessive bleeding occurred after dental extractions and as a consequence of surgery.

Proband 4 is a 33-year old man from Reggio Emilia. No specific clinical data, except for ecchymoses, hematomas, and post-traumatic bleeding tendency, suggestive of a coagulation disorder, could be obtained. His 37-year-old brother also had severely reduced FXI levels in plasma (FXI antigen, 2%).

Proband 5 is a 30-year old man from Milan. He has a thalassemia trait. He has not shown any bleeding manifestations: no severe bleeding problems occurred after a car accident or dental extractions. Recently, he successfully underwent varicocele repair under prophylactic treatment with fresh-frozen virus-inactivated plasma. No bleeding tendency was reported for the remaining family members, except for a prolonged partial thromboplastin time (PTT) in the proband's sister, who also had FXI deficiency (FXI activity level, <1%; FXI antigen, unmeasurable).

Proband 6 is a 16-year old girl from Milan. She first came to medical attention at the age of 10 years because of a prolonged PTT found prior to a tonsillectomy. The diagnosis of severe FXI deficiency (FXI coag-

ulant activity <1%) was made on this occasion. During childhood and puberty she had recurrent episodes of epistaxis and suffered from diffuse ecchymoses.

Materials

pCDNA3/FXI expression plasmid, containing the full-length FXI complementary DNA (cDNA), was kindly provided by Dr A. Zivelin (Institute of Thrombosis and Hemostasis, the Chaim Sheba Medical Center, Tel-Hashomer, Israel). Oligonucleotides were purchased from Invitrogen (Carlsbad, CA, USA). REDTaq and BIO-Taq DNA polymerases were from Sigma (St Luis, MO, USA) and Bioline (London, UK), respectively.

Blood collection and genomic DNA extraction

This study was approved by the Institutional Review Board of the University of Milan. After acquiring informed consent, blood samples from all individuals were collected for biochemical and genetic analyses. Peripheral venous blood was collected in 1:10 volume of 0.11 M trisodium citrate, pH 7.3. Plasma was obtained by centrifugation at 2000 g for 10 min and aliquots were stored at -80°C until use. Genomic DNA was extracted from whole blood using the Nucleon BACC1 Kit (Amersham Pharmacia Biotech, Uppsala, Sweden).

Coagulation tests

FXI coagulant activity (FXI:C) was measured by a one-stage method based on a modified partial thromboplastin time using a lyophilized immunodepleted human plasma deficient of FXI (Hemoliance, Salt Lake City, UT, USA). FXI antigen (FXI:Ag) was assayed by an in-house enzyme-linked immunosorbent assay (ELISA) based on goat anti-human FXI polyclonal antibodies and peroxidase-conjugated IgG (Affinity Biological Inc., Hamilton, Ontario, Canada). FXI levels were expressed in both tests as percentages of pooled normal plasma from 30 normal male and female individuals. The detection limits of the FXI functional and immunologic assays were 1% and 0.1%, respectively.

Polymerase chain reaction (PCR) and DNA sequencing

PCR were performed on 200 ng of genomic DNA in a standard volume of 50 μL. The reaction mixture contained 1× reaction buffer (10 mM Tris [tris (hydroxymethyl) aminomethane]-HCl, pH 8.3, 50 mM KCl, 0.01% gelatin), 1.5 mM $MgCl_2$, 0.4 μM of each primer, 200 μM deoxynucleoside triphosphates and 1.25 U RED-Taq DNA polymerase (Sigma). The PCR were carried out on a PTC-100 thermal cycler (MJ-Research, Watertown, MA, USA) under standard conditions. Primers were designed on the basis of the known genomic sequence of *F11* (Ensembl accession number ENSG0000088926). Primer sequences, as well as the specific PCR conditions for each primer couple, are available on request. Sequencing reactions were performed directly on PCR products purified by ammonium acetate precipitation. Sequence analysis was carried out on both strands by means of the BigDye Terminator Cycle Sequencing kit and an automated ABI-3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Factura and Sequence Navigator software packages (Applied Biosystems) were used for mutation detection.

Haplotype analysis

Two single nucleotide polymorphisms (SNPs), one in intron A (-231C>T) and one in intron E (*HhaI* polymorphism), and two dinucleotide repeat polymorphisms, one in intron B $(CA)_{8-13}$ and one in intron M $(AT)_{7-11}$ of *F11* were analyzed.[44-46] Polymorphisms in introns A, E, and B were genotyped by sequencing the relevant PCR product. The microsatellite marker in intron M was PCR-amplified using a 6-Fam-labeled primer. Labeled products were separated on an ABI-3100 genetic analyzer (Applied Biosystems) and analyzed by the GeneScan software (Applied Biosystems). Oligonucleotide primers and PCR conditions are listed in Table 1.

Expression of the FXI-Δ197-198 mutant protein

The pCDNA3/FXI plasmid was used as a template to obtain a mutant vector expressing a FXI protein lack-

Table 1. Oligonucleotide primers used in *F11* haplotype analysis.

Primer	*Sequence*	*Location (intron)*	*Annealing temperature (°C)*
FXI-ex1-F	5' GCAAATACGCCTTGAAATGC 3'	5'UTR	55
FXI-IVS2-R	5' TTGCAGTGTGATTTCCCTCT 3'	B	
FXI-IVS2-F	5' FamAAGTAGTGAACACAGCCTCC 3'	B	56
FXI-IVS2-R2	5' TTTCCACCTGTAATCCCAGC 3'	B	
FXI-IVS5-F	5' GTAAGAAGGACTTAGCCA 3'	E	53
FXI-IVS5-R	5' CACATGAAGAAGGAGAGTG 3'	E	
FXI-IVS13-F	5' GATAATCGCTTGAACTTGGG 3'	M	55
FXI-IVS14-R	5' CCCCAACGCATTAAGCATTC 3'	M	

UTR: untranslated region; Fam: 6-carboxyfluorescein; F: forward; R: reverse.

Table 2. Characteristics of probands with FXI deficiency.

	Probands					
Characteristics	*P1*	*P2*	*P3*	*P4*	*P5*	*P6*
City of origin	Milan	Milan	Reggio Emilia	Reggio Emilia	Milan	Milan
Gender	M	F	M	M	M	F
Present age, y	36	55	61	32	29	15
FXI activity levels, %	<1	<1	<1	<1	<1	<1
FXI antigen levels, %	0.4	0.4	0.2	5	0.5	0.3
Severity of symptoms	mild	mild	moderate	moderate	asymptomatic	mild

ing amino acids Ile[197] and Asp[198] (pCDNA3/FXI-Δ197-198). The deletion was created by a slight modification of the QuickChange Site-Directed Mutagenesis Kit protocol. The mutagenic oligonucleotides used to introduce the Δ197-198 deletion were FXIdel(ID)-F (5'-CGGTGTTTGCAGACAGCAACAGTGTCATGGCTCCCGATGC-3') and FXIdel(ID)-R (5'-GCATCGGGAGCCATGACACT-GTTGCTGTCTGCAAACACCG-3'). The modification of the original protocol consisted in the use of 40-mer primers, bridging the deletion, which ensured stable annealing on both sides of the deleted region.

The deleted plasmid was checked by sequencing. pCDNA3/FXI and pCDNA3/FXI-Δ197-198 were extracted by the EndoFree Plasmid Maxi Kit (Sigma). African green monkey kidney COS-1 cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin) and glutamine (1%), and grown at 37°C in a humidified atmosphere of 5% CO_2, and 95% air, according to standard procedures. In each transfection experiment equal numbers of cells were transiently transfected with LIPOFECTAMINE 2000 reagent (Invitrogen) in 6-well plates, with pCDNA3/FXI or with pCDNA3/FXI-Δ197-198 or with equimolar amounts of both plasmids, essentially as described by the manufacturer. Twenty-nine hours after transfection, cells were washed twice with phosphate-buffered saline (PBS) and cultured for additional 48 hours in serum-free medium supplemented with glutamine, antibiotics, 5 mM $CaCl_2$, and 5 mg/mL bovine serum albumin.

For each transfection (performed twice in duplicate) a mock experiment, with the unrelated pUC18 plasmid as a negative control, was set up.

FXI antigen measurement in conditioned media and in cell lysates

FXI antigen levels were evaluated by ELISA, as described above, both in conditioned media and in cell lysates. Standard curves were constructed with reference plasma diluted 1:100 to 1:6400 in Tris-buffered saline (50 mM Tris, 150 mM NaCl), pH 7.5. Conditioned media were collected in pre-chilled tubes containing a protease inhibitor mixture (Complete; Roche, Basel, Switzerland), centrifuged to remove cell debris, and stored at -80°C until use. To obtain cell lysates, cells were washed three times with pre-chilled PBS and lysed for 1 hour on ice with lysis buffer containing 1× PBS, 1.5% Triton X-100, and 1× Complete.[47] Cell lysates were centrifuged to remove cell debris. FXI activity was measured in about 10x concentrated medium as described above (see coagulation tests). Media were concentrated by Centricon Plus-20 centrifugal filters, containing a cellulose membrane with a molecular weight cut-off of 10 kDa (Millipore, Bedford, MA, USA).

The FXI specific activity (expressed as a percentage) was calculated as the ratio between FXI:C and FXI:Ag levels, both measured in concentrated media.

Results

Patients' data

Six Italian FXI-deficient probands were studied and their main demographic and clinical characteristics are summarized in Table 2. All probands had unmeasurable plasma levels of FXI functional activity and very low FXI antigen levels (ranging from 0.2 to 5%).

Mutational screening

In all six analyzed probands, mutational screening was performed by sequencing the whole *F11* coding region, as well as exon-intron boundaries and about 300 bp of the promoter region. Sequencing of *F11* identified two novel mutations. The first one was a C→A transversion in exon 5 corresponding to cDNA position 408 (numbering according to GenBank accession number NM000128, starting from the first nucleotide of the ATG start codon), which causes a nonsense mutation at amino acid 118 (Cys118stop, numbering omits the signal peptide). This mutation, found in three patients (proband 1 and his sister, and proband 6) in the homozygous state (Figure 1), involves the codon just downstream of that altered by the type II mutation (Glu117stop). It is predicted to cause the synthesis of a protein lacking a portion of the heavy chain and the

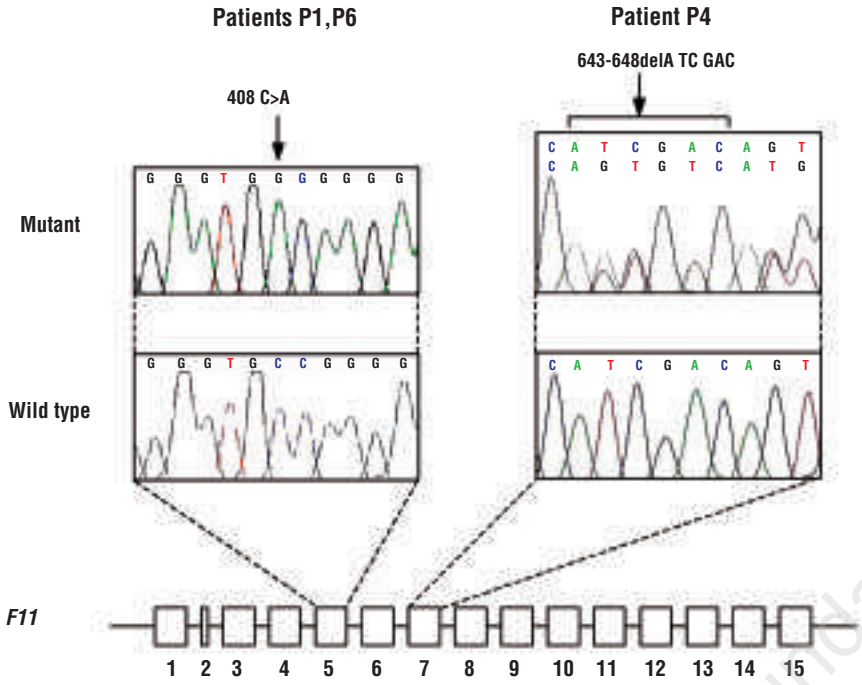

Figure 1. Novel mutations in *F11* identified in the analyzed probands. Electropherograms showing the mutations identified in probands P1, P6, and P4 (upper panels) and the corresponding wild-type sequences (lower panels) are reported over a schematic representation of *F11*. The 408C→A transversion (left), whose nucleotide position is indicated by an arrow, was present in the homozygous state in both P1 and P2, while the 6-bp deletion (right) was found in the heterozygous state in P4. The position of the newly identified mutations on a schematic representation of *F11* is also indicated. Exons (approximately to scale) are represented by boxes, while introns (not to scale) are represented by lines.

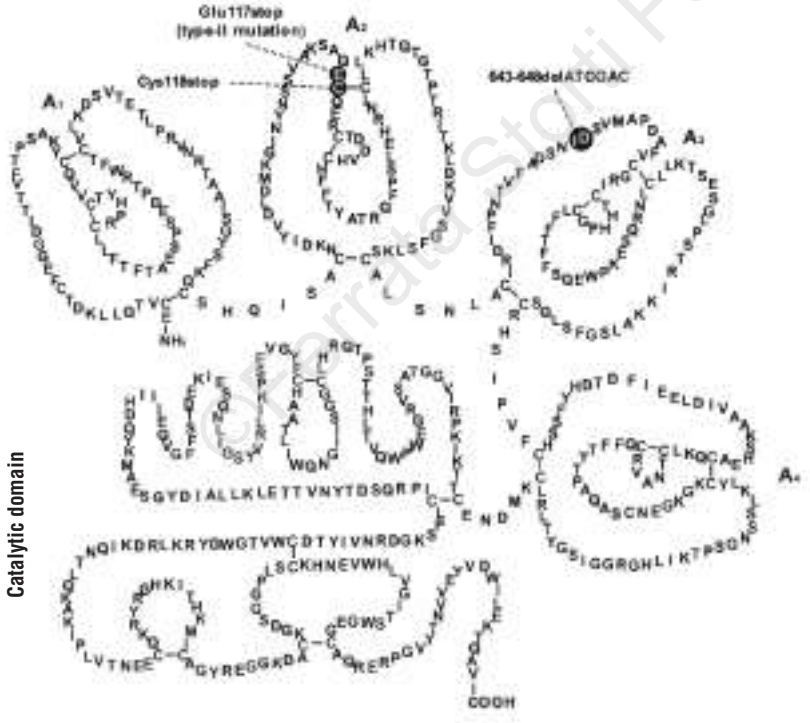

Figure 2. Amino acid sequence of FXI showing the position of the identified mutations (Glu117stop, Cys118stop, 643-648delATCGAC). Schematic representation of the primary structure of the FXI molecule showing the presence of the four apple domains (A1-A4) at the N-terminus and of the catalytic domain at the C-terminus. Disulfide bonds are also indicated. The mutations, identified in the six unrelated Italian probands, are located in the second (Glu117stop, Cys118stop) and in the third apple domain (643-648delATCGAC).

whole light chain (Figure 2). The second newly identified mutation was an in-frame 6-bp deletion (643-648delATCGAC) in exon 7 and was found in a compound heterozygous patient (proband 4) (Figure 1). The 643-648delATCGAC mutation causes the loss of amino acids Ile[197] and Asp[198] in the third apple domain of the heavy chain, which contains the binding site for FIX and platelets (Figure 2). In the remaining patients, the type II mutation (a G→A transition at cDNA position 403 leading to the Glu117stop mutation),[15] widely diffused among FXI-deficient Jews but fairly rare among non-Jewish patients, was found.

Table 3. *F11* haplotype of probands with FXI deficiency.

Marker	Location	Probands P1		P2		P3		P4		P5		P6	
-231C>T[#]	intron A	T	T	C	C	C	C	C	T	C	C	T	T
-138A>C[#]	intron A	C	C	A	A	A	A	A	A	A	A	C	C
(CA)n	intron B	9	9	11	11	11	11	11	11	11	11	9	9
Glu117stop[c] (type-II)[@]	exon 5	–	–	+	+	+	+	+	–	+	+	–	–
Cys118stop[c]	exon 5	+	+	–	–	–	–	–	–	–	–	+	+
430T>C*	exon 5	T	T	T	T	T	T	T	C	T	T	T	T
-431G>A (*Hha*I)	intron E	A	A	G	G	G	G	G	G	G	G	A	A
-361C>T[#]	intron E	T	T	C	C	C	C	C	C	C	C	T	T
643 648delATCGAC*	exon 7	–	–	–	–	–	–	–	+	–	–	–	–
801A>G+	exon 8	A	A	G	G	G	G	G	G	G	G	A	A
1191T>C*	exon 11	T	T	C	C	C	C	C	C	C	C	T	T
(AT)n	intron M	9	9	9	9	9	9	9	9	9	9	9	9
1812G>T*	exon 15	G	G	G	G	G	G	G	G	G	G	G	G
1839G>A*	exon 15	G	G	G	G	G	G	G	G	G	G	G	G

[#]Nucleotide positions of the intronic polymorphisms have been numbered starting from the nearest splicing junction. [c]Numbering refers to the amino acid sequence omitting the signal peptide. [@]Numbering refers to the cDNA sequence (GenBank, accession number NM000128), starting from the first nucleotide of the ATG start codon. The "Jewish" haplotype is represented in gray boxes, other polymorphisms are indicated in white boxes. The mutations (bold) found in the six analyzed probands are represented in the corresponding position within F11 haplotypes as black boxes.

Haplotype analysis

To establish whether or not the probands bearing the type II mutation share the same haplotype, four intragenic polymorphic sites (-231C>T in intron A, $(CA)_{8-13}$ in intron B, *Hha*I polymorphism in intron E, and $(AT)_{7-11}$ in intron M) were analyzed. These were previously reported to define a common haplotype shared by all Jewish FXI-deficient patients carrying the type II mutation.[44-46] Genotyping was performed both by sequencing and by fragment analysis. In particular, analysis of *Hha*I polymorphism, carried out by sequencing, allowed the identification of the polymorphic nucleotide within the HhaI restriction site: it is the third of four bp recognized by *Hha*I (-431G→A). The G→A substitution prevents the enzyme from cutting.

In haplotype construction, seven additional SNP, identified during mutational screening, were included: -138A→C in intron A, 430T→C in exon 5, -361C→T in intron E, 801A>G in exon 8, 1191T→C in exon 11, 1812G→T and 1839G→A in exon 15. For each mutation, haplotype analysis revealed a founder effect (Table 3). In particular, the type II mutation segregates with the same haplotype [-231C, $(CA)_{11}$, -431G (*Hha*I), $(AT)^9$] previously reported in Ashkenazi, Iraqi, Yemenite, Syrian, and Marrocan Jewish patients carrying the type II mutation, demonstrating that the mutation was of the same ancestry.[46]

The Cys118stop mutation was associated with the same haplotype in both probands 1 and 6, suggesting a common origin also for this mutation. This haplotype differs from the type II mutation haplotype at each analyzed marker, except for the AT repeat in intron M and for three SNP (one in exon 5 and two in exon 15).

Proband 4, who is a compound heterozygote for the type II and the novel 643-648 delATCGAC mutations, resulted homozygous for all markers except for the -231C→T and 430T→C polymorphisms in intron A and exon 5, respectively. The presence of a T and a C at markers -231C→T and 430T→C therefore defines a new haplotype associated with the 6-bp deletion.

Expression of wild-type and Δ197-198 recombinant FXI in COS-1 cells

To evaluate whether the deletion of Ile^{197} and Asp^{198} affects FXI secretion, the mutant FXI-Δ197-198 protein was expressed in COS-1 cells. To this purpose, deletion mutagenesis was performed on the pCDNA/FXI plasmid to produce the pCDNA3/FXI-Δ197-198 vector, using a modification of the *QuickChange* protocol, as described in the *Design and Methods*. Following transient transfection with either pCDNA3/FXI or pCDNA3/FXI-Δ197-198 or with equimolar amounts of both expression plasmids (to mimic the heterozygous condition), serum-free conditioned media and cell extracts were analyzed for the presence of FXI antigen by ELISA.

In media conditioned by cells expressing FXI-Δ197-198, FXI antigen levels were reduced to 17% of the wild type, demonstrating a remarkable impairment of FXI secretion (Figure 3A). Co-transfection with equimolar amounts of wild-type and mutant FXI-expressing plasmids partially restored extracellular FXI antigen (to about 63% of the wild type) (Figure 3A). In cell lysates, no significant difference was observed between the wild-type and mutant FXI antigen levels (Figure 3A).

FXI coagulant specific activity was measured on concentrated conditioned media by an assay based on

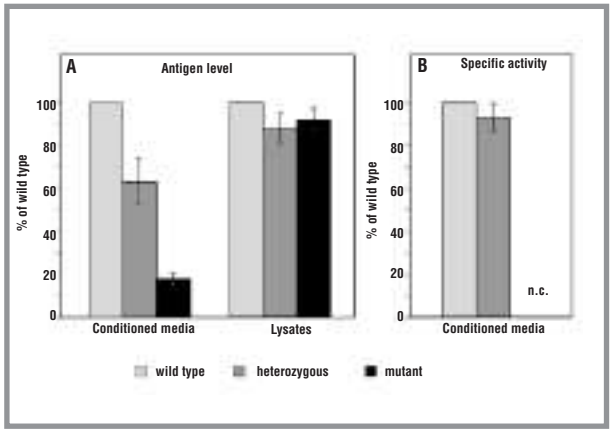

Figure 3. Transient expression of wild-type and mutant FXI protein in COS-1 cells. pCDNA3/FXI (wild type), pCDNA3/FXI-Δ197-198 (mutant) or equimolar amounts of both plasmids (heterozygous condition) were transiently transfected in COS-1 cells. Equal numbers of cells and equal amounts of plasmids were used in transfection experiments, as described in Design and Methods. (A). Antigen levels of recombinant FXI were measured both in conditioned media and in the corresponding cell lysates by an ELISA assay. Bars represent relative concentrations of protein in media and cell lysates compared to the mean for wild type FXI (set as 100%). Results are given as means ± standard deviations. (B). The specific activities of recombinant proteins were determined by calculating the ratio between FXI activity (measured by a one-stage method based on a modified partial thromboplastin time) and FXI antigen levels, both assayed in about 10x concentrated media. Bars represent means ± standard deviation of 4 independent experiments, each performed in duplicate. The mean value of wild-type FXI was set as 100%; n.c.: not calculable.

thromboplastin time, and the results are reported in Figure 3B. The activity of the FXI-Δ197-198 mutant protein was below the detection limit of the assay (<1%), even after 10-fold concentration of conditioned media. FXI molecules secreted by cells co-expressing wild-type and mutant cDNA had a specific activity similar to the wild-type recombinant FXI. To exclude possible artefacts caused by the concentration step, the coagulant activity of the wild-type recombinant FXI was also measured in conditioned medium as such. The calculated specific activity resulted in good agreement with data obtained from the concentrated medium (*data not shown*).

Discussion

To our knowledge, only two Italian FXI-deficient patients have so far been analyzed,[29,35] therefore this represents the first study exploring the molecular basis of severe FXI deficiency in a cohort of six unrelated Italian probands.

Both functional and antigen levels of FXI were markedly reduced in all analyzed patients, confirming the diagnosis of quantitative FXI deficiency. All patients had mild symptoms such as epistaxis and excessive oozing after dental extractions; only two of them required fresh-frozen plasma treatment after surgical procedures. Sequencing of *F11* identified two novel mutations: a nonsense mutation (Cys118stop) in exon 5 in two homozygous probands and an in-frame 6-bp deletion (643-648delATCGAC) in exon 7 in a compound heterozygous proband. The Cys118stop mutation involves the codon immediately downstream of that altered by the type II mutation. The truncation at residue 118 is predicted to cause the synthesis of a protein lacking the third and fourth apple domains of the heavy chain (involved in the binding of FXI to FIX and in the dimerization process, respectively)[48] and the whole light chain (containing the catalytic domain). The virtual absence of FXI protein truncated at residue Cys118 in blood may be due to a secretion defect of the truncated protein, which might be recognized as abnormal by the quality control system of secretory proteins, a stringent mechanism that prevents the secretion of misfolded and incompletely assembled proteins.[49]

Moreover, since premature termination codons are known to frequently affect the metabolism of the corresponding mRNAs, transcripts carrying the Cys118stop mutation might be committed to degradation by the nonsense mRNA-mediated decay. This surveillance mechanism, which efficiently degrades transcripts carrying premature termination codons, protects organisms from the deleterious dominant negative or gain of function effects of truncated proteins.[50] In any case, should the Cys118stop FXI be expressed, the resulting protein would be completely non-functional, since it would lack the domains responsible for binding to FIX, dimerization and catalytic activity of FXI.

The in-frame 6-bp deletion was identified in a compound heterozygous proband, who also carried the type II mutation. The finding of a three-nucleotide repeat (ACA) located at the 5' and 3' ends of the deleted fragment might explain the 6-bp deletion by slippage and mispairing at the replication fork during DNA synthesis.[51] This mutation causes the loss of amino acids Ile[197] and Asp[198] in the third apple domain of the heavy chain, leading to FXI deficiency presumably by altering the proper folding of the third apple domain and consequently impairing FXI secretion. Expression of a recombinant deleted FXI in COS-1 cells enabled the demonstration of the effect of Ile[197] and Asp[198] removal on protein secretion. In media conditioned by

cells expressing the mutant FXI, antigen levels were reduced by 83% relative to the wild-type. These results are suggestive of impaired secretion of the mutant protein and fit well with the FXI antigen level measured in the proband's plasma (5%), which was significantly higher than that in any of the other analyzed probands. Co-transfection experiments with equal amounts of mutant and wild-type expression plasmids partially corrected the secretion defect (antigen levels in conditioned media rose from 17% to 63% of the wild type). The intracellular levels of FXI-Δ197-198 (similar to those measured in the wild type) suggest that the secretion defect caused by this two-amino acid deletion does not cause intracellular accumulation of the mutant protein.

The coagulant activity of secreted wild-type and mutant FXI was evaluated after concentration of conditioned media. However, the coagulant activity of the mutant protein was not measurable, whereas the specific activity calculated from media conditioned by cells co-expressing the wild-type and the mutant proteins was indistinguishable from the specific activity of wild-type recombinant FXI. These results demonstrate that the coagulant activity of recombinant FXI-Δ197-198 is strongly impaired and might explain the small discrepancy between FXI activity (<1%) and antigen (5%) levels measured in the plasma of proband 4. In the remaining patients, the type II mutation, very common in FXI-deficient Jews, was found in the homozygous state. Previous haplotype studies revealed that the type II mutation, responsible for the majority of cases of FXI deficiency in Ashkenazi Jews but also highly common in Iraqi Jews and frequent in Sephardic Jews and Arabs, is invariantly associated with a single haplotype.[16,46] Given these findings, it was speculated that this mutation might have originated in an ancient Jewish founder approximately 2500 years ago, before the divergence of the major segment of Jews.[16] This mutation, besides being found in Jews, has also now been reported in non-Jewish individuals from England[52] and Portugal,[33] and in both cases it was associated with the same *Jewish* haplotype.

In order to identify the origin of the type II mutation in the Italian population, a haplotype comprising a series of 11 polymorphisms (nine biallelic and two microsatellite markers) spanning the entire F11 was determined. This analysis revealed that all individuals carrying the type II mutation shared the same haplotype found in Jewish patients, thus revealing the Jewish origin of the mutation also in the Italian patients. The high frequency of this mutation in the Italian population suggests that testing for the presence of type II mutation should be the first genetic screening in FXI-deficient patients.

The same polymorphic markers were genotyped in the two probands bearing the newly identified Cys118stop mutation, who were found to share an identical haplotype (different from that of patients bearing the type II mutation), thus suggesting a common origin for the Cys118stop mutation. Screening of a higher number of FXI-deficient patients will be necessary to reveal the existence of a founder effect also for this mutation in the Italian population.

All the authors participated in the conception and design of the present study, in the analysis and interpretation of data, and in revising the manuscript. GZ and RA were responsible for PCR amplifications, sequence analysis, site-directed mutagenesis, expression experiments, and drafting the manuscript. ES and PMM enrolled patients in the study, collected the clinical histories, and evaluated the clinical phenotypes. FP was responsible for measurement of FXI antigen and functional levels. MM, PMM, and MLT were involved in the discussion of the results and correction of the manuscript. SD was responsible for the conception of the study and the interpretation of results, and supervised the entire study. The authors reported no potential conflicts of interest.

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