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A Dysfunctional Factor X (Factor X San Giovanni Rotondo) Present at Homozygous and Double Heterozygous Level: Identification of a Novel Microdeletion (delC556) and Missense Mutation ($Lys^{408} \rightarrow Asn$) in the Factor X Gene A Study of An Italian Family

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Abstract

Low levels of factor X (F.X) were detected in a 4-year-old boy who experienced acute lymphoblastic leukemia and bleeding manifestations. Laboratory data suggested the presence of a dysfunctional F.X molecule. Two novel F.X gene mutations were identified in the proband that was double heterozygous for both: a microdeletion (delC556) in exon VI resulting in a frameshift leading to a termination codon at position 226. This deletion was found in six family members with reduced F.X antigen and activity levels. A second mutation characterised by a $G^{1344} \rightarrow C$ transversion in exon VIII was detected in the proband resulting in a $Lys^{408} \rightarrow Asn$ substitution. This latter mutation was present in several asymptomatic family members from the paternal and the maternal side. The proband's sister was homozygous for the $Lys^{408} \rightarrow Asn$ substitution and exhibited low F.X activity with a normal antigen level. The naturally occurring F.X $Lys^{408} \rightarrow Asn$ ($F.X^{K408N}$) variant was isolated from plasma of either homozygous or double heterozygous individuals. NH_2-terminal sequencing of the heavy chain of $F.X^{K408N}$ failed to show any sequence abnormality in patients who were also carriers of the delC556, suggesting that this latter lesion accounted for the lack of F.X synthesis. Purified F.X $Lys^{408} \rightarrow Asn$ had an identical behaviour to normal F.X as judged by SDS–PAGE and immunoblotting. Clotting assay using purified $F.X^{K408N}$ and F.X-deficient plasma resulted in a laboratory phenotype similar to that observed in a homozygous subject for F.X $Lys^{408} \rightarrow Asn$ substitution. This is the first characterisation of a naturally occurring F.X variant with a mutation at the COOH-terminal end of the molecule.

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Factor X (F.X) is a vitamin K-dependent protein that plays a pivotal role in the coagulation cascade [1]. It can be activated through either the intrinsic or the tissue factor (extrinsic) pathway. Factor Xa is composed of a 17-kDa light chain and a 45-kDa heavy chain associated through a disulfide bond. The light chain contains 11 γ-carboxyglutamic acid residues (Gla-domain region), whereas the heavy chain contains the activation peptide with 52 amino acids and the active site of factor Xa. Factor Xa associates with its cofactor factor Va, on a membrane surface in the presence of Ca^+, to form the prothrombinase complex that activates prothrombin to thrombin (for review see Refs. [1,2]). The human F.X gene is composed of eight exons spanning a region of 25 kb and is located on chromosome 13q34 [3,4].

Congenital F.X deficiency is a rare autosomal recessive disorder that is heterogeneous for both laboratory and clinical expression [3–5]. The classification of F.X deficiency is based on the results of functional and immunologic assays [3,4]. Three main groups of deficiency can be identified: the CRM^- group in which both antigen and activity are similarly decreased (''hypo''), while prothrombin time, activated partial thromboplastin time as well as Russell's viper venom (RVV) time-based assays are prolonged; the CRM^+ group, which has normal or near-normal antigen levels and factor X activity is decreased (''dys''); finally the CRM^R group in which both activity and antigen levels are reduced but antigen is markedly higher than activity (''hypo–dys''). Since F.X can be activated via both the extrinsic and the intrinsic pathways as well as by RVV, F.X variants (CRM+) may appear with different activity levels depending on the assay system used for the determination. Accordingly, a classification of F.X variants has been proposed by Fair and Edgington [6]. A similar simpler classification has been proposed by Girolami [7]. The lowest F.X levels are usually related to homozygous or double heterozygous genetic defects. Chromosomal abnormalities or gene deletions have been reported only in a few patients with F.X deficiency, the majority of genetic mutations being represented by missense mutations [3,4]. Clinical manifestations of F.X deficiency are even more variable and usually only homozygotes or double heterozygotes present haemorrhagic manifestations. However, while F.X levels between 10% and 40% of the normal are considered sufficient to prevent bleeding manifestations, homozygotes might exhibit levels higher than 10% activity. Thus, many individuals carrying an F.X mutation may remain undetected or develop haemorrhagic complications only when challenged or because of an additional problem. The estimated prevalence of homozygous F.X deficiency is about 1:500,000 but the heterozygotes are likely to be as frequent as 1:500 even though clinically asymptomatic [4]. We report in this study a family from the South of Italy (San Giovanni Rotondo, Foggia area, Apulia) in which two new genetic mutations, i.e., a missense mutation and a single nucleotide deletion in F.X gene have been identified resulting in CRM+ (''dys'') and CRM− (''hypo'') phenotypes, respectively.

1. Materials and Methods

1.1. Case Report

The proband is a 4-year-old child of healthy nonconsanguineous Italian parents who was admitted to the hospital because of superficial hematomas of the legs and epistaxis. Myelocentesis and peripheral blood smear were consistent with a diagnosis of acute lymphoblastic leukemia CD10+. He was treated according to standard procedures with a good response (complete remission). Asparaginase was excluded from the therapeutic protocol in order to avoid additional coagulation problems (hypofibrinogenemia, etc.). Routine coagulation tests also showed on admission a prolongation of partial thromboplastin (PTT) and prothrombin time (PT), normal fibrinogen level and low platelet count (14,000/μl). Clotting factor assays were consistent with a remarkable reduction of F.X activity levels in the proband whereas the mother had 50% antigen and markedly reduced F.X activity while the father had apparent normal F.X values. Fresh frozen plasma and blood cells were collected form the proband and all available

family members for investigation. There was no family history of bleeding manifestations and the proband had not experienced haemorrhagic episodes in the past. The proband is now still in complete remission and platelet count is within the normal range. He has remained asymptomatic for bleeding over the past 2 years.

1.2. Materials

3,3′-Diaminobenzidine (DAB), 3,3′,5,5′-tetramethylbenzidine (TMB) was purchased from Fluka (Buchs, Switzerland). Benzamidine was from Sigma-Aldrich Europe (Milan, Italy). SDS, acrylamide, *N*,*N*′-methylene bisacrylamide, DTT, and prestained molecular weight standards were purchased from BioRad (Richmond, CA). Polyvinylidene difluoride (PVDF) membranes (Immobilon P) were obtained from Millipore (Bedford, MA). Antifactor X polyclonal antibody and HRP conjugate of polyclonal rabbit antifactor X were from Dako (Glostrup, Denmark). Factor X-deficient plasma was supplied by Dade-Behring, Milan, Italy. All other chemicals were analytical grade products from Merck (Darmstadt, Germany), unless otherwise stated.

1.3. Routine Tests

Plasma from the proband and the family members was collected in 1:10 Na citrate 3.8%, centrifuged at 3000 rpm for 10 min and stored in aliquots at −40°C until use.

Laboratory investigations including PT and aPTT, Thrombotest, Normotest and PP-test have been performed as previously reported [8–11]. Pooled normal plasma was obtained from 50 healthy subjects of both sexes aged 20 to 70 years.

1.4. Elisa for Factor X Antigen Determination

For solid phase immunoassays, polyclonal antibodies directed against human factor X were used as capture antibodies. Plastic 96-well microtiter plates were coated with antifactor X polyclonal antibodies from Dako diluted 1:400 (100 μl/well) in 0.1 M $NaHCO_3$, pH 9.0 and 0.5 M NaCl overnight at room temperature. After removal of the coating solution, the plates were washed five times with 50 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Tween-20 (ELISA buffer). A standard curve was obtained by serial dilutions of pooled normal plasma in the ELISA buffer. Samples were incubated for 2 h at room temperature. Following incubation, the plates were washed five times and incubated with 1:600 dilution of antifactor X polyclonal antibody horseradish peroxidase (HRP) conjugated (Dako) for 1.5 h at 25°C. The plates were washed five times with the Elisa buffer and HRP activity was detected by incubation with 0.003% H_2O_2, 0.1 mg/ml 3,3′,4,4′-tetramethylbenzidine (TMB), and 0.1 M sodium acetate (pH 5.5). The staining reaction was stopped by adding 100 μl 2 M H_2SO_4, and the optical density at 450 nm was determined.

1.5. Factor X Functional Assays

Chromogenic activity was measured in plasma using Stachrom X kit (Stago, Asnieres, France). Factor X was activated by RVV and amidolytic activity detected with the chromogenic substrate CBS 31.39 [12]. Clotting activity was assessed using both the extrinsic and the intrinsic pathways as well as activation by RVV [8–11]. Tests were repeated four times and in different samples from the same subject and mean values were calculated. Normal ranges for each functional test were obtained from 40 healthy subjects. Factor X functional assays were also performed after reconstitution of F.X-deficient plasma (Dade-Behring) with either purified normal F.X or F.X $Lys^{408} \rightarrow Asn$ (see below for the purification procedures).

1.6. Polymerase Chain Reaction (PCR)

Genomic DNA was prepared from leukocytes by standard procedures. Amplification of exons I through VIII and respective splice junctions of the factor X gene were performed using oligonucleotide primers kindly supplied by Dr. H. James (Tyler, TX) according to previously described methodologies [13].

1.7. DNA Sequencing

Sequencing was performed on an ABI PRISM 310 DNA sequencer (Perkin-Elmer, Foster City, CA) using ABI PRISM BigDye Terminator Cycle

Sequencing Reaction Kit with AmpliTaq DNA Polymerase FS (Perkin-Elmer). Forward and reverse primers were the same as those used for PCR amplifications. Sequencing data were analysed by the Sequencing Analysis 3.0 computer program (Perkin-Elmer). Results were compared with factor X sequence as reported in the GenBank database (GenBank accession number M14327).

1.8. SDS–PAGE and Immunoblotting

SDS–PAGE was performed on 5–15% linear gradient gels, according to Laemmli [14]. After electrophoresis the gels were stained with silver, according to Morrissey [15], or blotted onto PVDF-membranes according to the method of Towbin et al. [16]. Samples were prepared from patients' plasma after Ba citrate adsorption and trichloroacetic acid precipitation. Pellets were dissolved in 20 μl 4 × sample buffer and applied to the gel under nonreducing or reducing conditions. In the latter case, 2 μl 1 M dithiothreitol was added to each sample. Immunoblot of isolated normal and variant F.X was also performed. Factor X was detected in the immunoblot using mouse antihuman F.X monoclonal antibody AHX-5050 (Haematologic Technologies, Essex Jct, VT). Bands were visualised using goat anti-mouse HRP (Biorad) as a second antibody. HRP activity was detected by DAB.

1.9. Isolation of Factor X Variant

The isolation of F.X variant and normal F.X was performed using immunoaffinity chromatography. Normal plasma (50 ml) and patients' plasma (either from homozygous F.X variant or double heterozygous F.X-deficient patients, separately, 50 ml) was incubated with 5 mM benzamidine and 0.01% soybean trypsin inhibitor. The vitamin K-dependent proteins were precipitated with $BaCl_2$ (8 ml of 1 M $BaCl_2$/100 ml plasma). Following centrifugation (20 min at 5200 × *g*) the barium citrate precipitate was resuspended in one-third of the original volume of 50 mM Tris (pH 8.0), 150 mM NaCl, 10 mM benzamidine, 100 mM EDTA, and dialysed twice against the same buffer containing only 10 mM EDTA and twice against 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM benzamidine. The preparation was loaded on column containing 2 mg of mouse antihuman factor X monoclonal antibody immobilised onto sepharose 4B (AHX-5050). The column was equilibrated with 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM benzamidine. The column was washed with the same buffer. Additional washing of the column with 1 M NaCl removed the non-specific bound proteins. Then, the protein was eluted with 3 M KCNS. Fractions containing F.X were pooled and dialysed three times against 20 mM Tris pH 7.4, 150 mM NaCl, 1 mM benzamidine. The preparation was then applied to a 0.3-ml column of Fast Flow Q sepharose to concentrate F.X. The column was eluted with 1.5 column volumes of 20 mM Tris pH 7.4, 1 M NaCl. Fractions containing F. X were pooled and dialysed against 20 mM Tris, 150 mM NaCl, pH 7.4. The purity of isolated F.X was assessed by silver staining of SDS–PAGE gel.

1.10. NH_2-Terminal Sequence Analysis

Isolated normal and variant factor X from either homozygous or double heterozygous patients were reduced with β-mercaptoethanol and blotted onto PVDF membrane as described above. The PVDF membrane was stained with Coomassie blue R-250 0.2% w/v in methanol 45% v/v and acetic acid 10% v/v. After destaining with methanol 90% v/v and acetic acid 7% v/v, the membrane was washed with water. The NH_2-terminal sequence of the variant F.X heavy chain molecule was determined by automatic Edman degradation on an Applied Biosystem 475A protein sequencing system (Applied Biosystem, Foster City, CA) equipped with a Blott Cartridge (Applied Biosystem) in the laboratory of Dr. Alex Kurosky (University of Texas, Medical Branch at Galveston, Galveston, TX). The phenyl-thiohydantoins amino acid was identified using high-performance liquid chromatography (HPLC) and compared to a chromatogram containing all the amino acids, except cysteine.

2. Results

The proband and 14 family members (Fig. 1) were screened for F.X levels and DNA analysis was

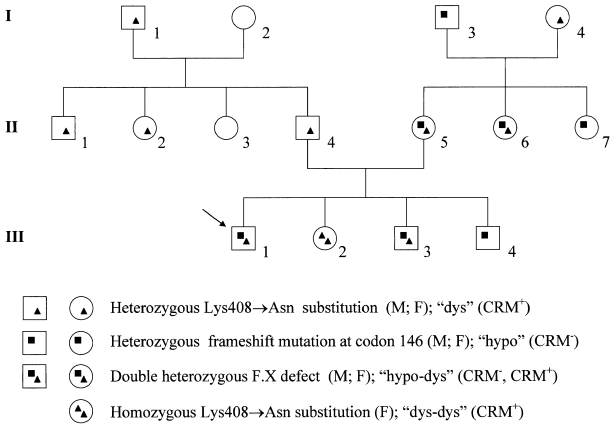

Fig. 1. Family pedigree in three generations. There was no consanguinity between the parents of the proband who is indicated by the arrow. This proband is double or compound heterozygous (''hypo–dys'') for heterozygous F.X deficiency (CRM−) and F.X abnormality (CRM+). The mother has a similar defect. This observation is due to the fact that the proband's father (II-4) is a carrier (heterozygous) for the same F.X abnormality ($Lys^{408} \rightarrow Asn$) present in the mother. The proband's sister (III-2) is homozygous for the F.X abnormality (''dys–dys'').

performed to detect the genetic defects. Table 1 summarises the main laboratory findings of the family members investigated. Four subjects were double heterozygotes for both F.X defects (CRM−/CRM+ or ''hypo–dys''), eight were heterozygous for either one (3 CRM− or ''hypo''; 5 CRM+ or ''dys''), one was homozygous CRM+ (or ''dys–dys'') and two had no F.X defects.

2.1. Factor X Deficiency Phenotype

The proband (III-1) had subnormal F.X antigen levels and exhibited low F.X activity regardless of the assay system used (Table 1). Reduction in F.X activity was more pronounced when activity was measured via the extrinsic pathway (9.2%) as compared to the reduction in activity via the intrinsic pathway (24%) or after RVV activation (43%). The chromogenic activity of the proband's molecule was also very low (22% of the normal value). These data suggest the presence of an abnormal F.X in the molecule in proband's plasma.

The proband's father exhibited high F.X antigen levels (134%) and near normal activity (Table 1). Chromogenic activity was within the normal range. A clear discrepancy was observed in this individual between the aPTT, the F.X antigen and the activities levels suggesting the possible presence of an abnormal F.X molecule. The proband's mother had approximately half the normal F.X antigen level and considerably reduced F.X activities regardless of the assay used (Table 1). These findings suggest the presence of a defective F.X allele in addition to a defective molecule in the proband's mother.

According to these preliminary data, we concluded that the F.X molecule found in the proband is most likely the result of a double heterozygous F.X defect: the first one a CRM^+ defect inherited from the paternal side and the second one a CRM^- defect inherited from the maternal side.

2.2. F.X Deficiency Genotype

Genotypic analysis resulted in a clear definition of the F.X deficiency in the proband's family. The underlying pathologic defects in the F.X gene were determined by direct sequencing of the eight exons encoding the F.X molecule and splice junctions of the F.X gene of the proband. Only two genetic abnormalities were observed when compared with the wild-type sequence and with the known polymorphisms for the F.X gene. The first genetic abnormality was found in exon VI (Fig. 2) consisting of a single nucleotide deletion at the first position of codon 146 (delC556). This deletion disrupts the reading frame after codon 146 and leads to a premature termination of translation at codon 226 (stop codon), 80 amino acids downstream. Therefore, the proband would have a defective truncated F.X molecule because of this frameshift mutation. The second mutation found in the proband's F.X exon VIII was a transversion ($G^{1344} \rightarrow C$) (Fig. 3). This transversion results in a $Lys^{408} \rightarrow Asn$ substitution (Fig. 3) in the F.X molecule. These two mutations are most likely responsible for the proband's phenotype.

DNA analysis of the F.X gene of the proband's father showed the presence of the transversion in exon VIII responsible for the $Lys^{408} \rightarrow Asn$ substitution while the proband's mother exhibited

Table 1. Main characteristics and laboratory findings of the family members investigated

Family member	Age (years)	PT (s)	aPTT (s)	F.X antigen (%)	F.X activity extrinsic (%)	F.X activity intrinsic (%)	F.X activity RVV (%)	F.X activity chromogenic (%)	Phenotype	Genotype
Normal values		14.4	30–40	80–120	80–120	80–120	80–120	80–120		
III-1, proband	4	30.4	43.5	72	9	24	43	22	CRM –; CRM+ ''hypo–dys''	Double heterozygous
I-1, grandfather, paternal side	68	22.8	54.1	125	73	72	120	76	CRM+ ''dys''	Heterozygous
I-2, grandmother, paternal side	68	14.7	42.9	135	120	160	170	143	N	N
I-3, grandfather, maternal side	71	19.3	43.3	54	60	66	57	61	CRM – ''hypo''	Heterozygous
I-4, grandmother, maternal side	69	16.2	36.0	116	76	110	94	78	CRM+ ''dys''	Heterozygous
II-1, uncle, paternal side	43	19.3	46.0	150	82	–	125	92	CRM+ ''dys''	Heterozygous
II-2, aunt, paternal side	41	19.2	50.0	102	68	62	90	72	CRM+ ''dys''	Heterozygous
II-3, aunt, paternal side	23	16.8	46.0	107	105	100	98	110	N	N
II-4, proband's father	45	16.6	46.9	134	78	73	110	89	CRM+ ''dys''	Heterozygous
II-5, proband's mother	43	28.5	48.4	55	8	17	31	20	CRM –; CRM+ ''hypo–dys''	Double heterozygous
II-6, aunt, maternal side	46	30.7	55.9	75	11	16	54	32	CRM –; CRM+ ''hypo–dys''	Double heterozygous
II-7, aunt, maternal side	34	17.5	49.2	67	74	50	60	73	CRM – ''hypo''	Heterozygous
III-2, proband's sister	12	36.1	48.0	98	16	18	56	20	CRM+ ''dys–dys''	Homozygous
III-3, proband's brother	21	30.0	50.5	62	11	30	33	20	CRM –; CRM+ ''hypo–dys''	Double heterozygous
III-4, proband's brother	16	16.3	33.6	56	70	43	55	62	CRM – ''hypo''	Heterozygous

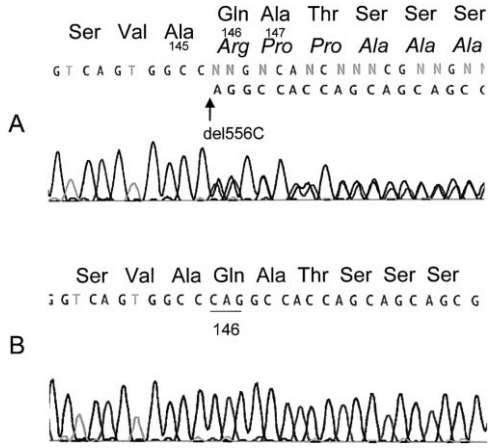

Fig. 2. Sense strand DNA sequences of exon VI of proband's (Panel A) and normal (Panel B) factor X genes. Panel A: DNA sequences of the two alleles appear to be superimposed starting from codon 146 as the result of a single nucleotide deletion at position 556 C (arrow) in one allele. The patient is heterozygous for the defect. This leads to a shift in the reading frame predicting the synthesis of an abnormal molecule (see amino acid sequence in italics) with a termination of translation downstream at codon 226. Panel B: DNA sequence of exon VI of homozygous wild-type allele around codon 146 (underlined) is shown for comparison. Nucleotide and codon numbering as used by Messier et al. [17].

both, the nucleotide deletion at codon 146 of the F.X gene that could explain F.X antigen levels of about 50% (CRM−) but also the mutation in exon VIII found in the proband and in his father responsible for $Lys^{408} \rightarrow Asn$ substitution. We thus concluded that the latter mutation may be responsible for the reduced F.X activity levels (CRM+). No other mutations could be detected in the proband's parents.

One proband's sister (III-2) was homozygous for $Lys^{408} \rightarrow Asn$ substitution and as a consequence had normal antigen level but reduced activity (CRM+) (Figs. 1 and 3, and Table 1). One of the proband's brothers (III-3) was double heterozygous with the same phenotype (i.e., CRM+ and CRM−) (Table 1) and the other (III-4) had only the single nucleotide deletion in exon VI (CRM−). Collectively these data demonstrate that deletion at nucleotide 146 results in the production of an unstable F.X molecule that is not present in plasma (CRM−), whereas the $Lys^{408} \rightarrow Asn$ substitution is responsible for the impaired catalytic activity of the F.X molecule (CRM+).

These conclusions were verified following the study of the proband's grandparents and aunts. From the maternal side, the grandfather (I-3) has the deletion in exon VI on one allele while the other one is normal (phenotype CMR−). The grandmother (I-4) has the $Lys^{408} \rightarrow Asn$ substitution in exon VIII and as a consequence a phenotype CRM+. One aunt (II-6) has the double defect with the corresponding phenotype (CMR−, CMR+) while the other (II-7) only the deletion in exon VI corresponding to decreased antigen level (see Table 1 and Fig. 1). From the paternal side, the grandfather (I-1) has the defect in exon VIII corresponding to the $Lys^{408} \rightarrow Asn$ substitution (CRM+) resulting in decreased F.X activity whereas the grandmother has a normal F.X genotype without any mutations. Two additional family members (II-1 and II-2) had the same genotype and phenotype as the proband's father (heterozygous CRM+) and therefore decreased F.X activity levels (Table 1 and Fig. 1).

2.3. Characterisation of F.X $Lys^{408} \rightarrow Asn$

To verify the properties of the mutant molecule the variant F.X molecule was purified using procedures described in the Methods section. For the isolation of F.X $Lys^{408} \rightarrow Asn$, plasma from either the proband's mother (II-5; double heterozygous) or the proband's sister (III-2; homozygous) was used. In addition, normal F.X was isolated from pooled normal plasma. The F.X preparations obtained following isolation using an anti-factor X immunoaffinity column were pure and homogeneous as judged by silver staining (Fig. 4, panel A). No heterogeneity in the factor X light or heavy chain was observed. No F.X molecule corresponding to a truncated F.X (representing the CRM− phenotype) could be detected in our preparations. The heavy chain of the proband's F.X molecule had an NH_2-terminal sequence identical to the wild type (Fig. 4, panel B). These data demonstrate that individuals who are double heterozygous CRM−, and CRM+ possess one F.X molecule in their plasma that is the result of the CRM+ allele. The CRM− allele does not produce a circulating F.X molecule.

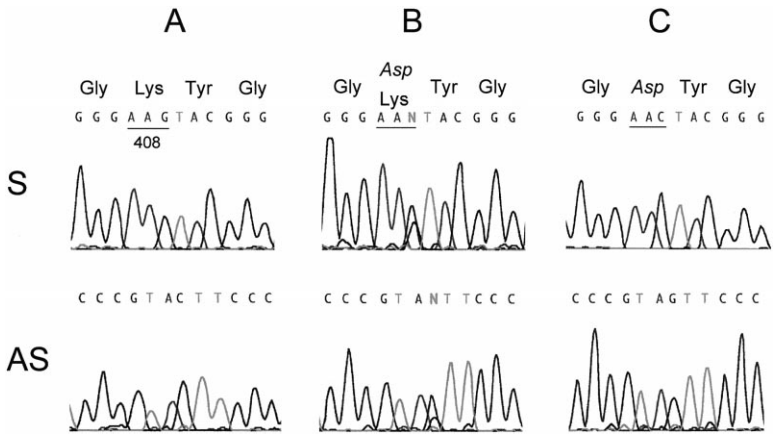

Fig. 3. Sense (S) and antisense (AS) strand DNA sequences of exon VIII of the F.X gene. Panel A: Sequence of homozygous wild type allele around codon 408. Panel B: The chromapherogram of sense strand reveals the presence of two superimposed peaks in the third position (at nucleotide 1344) of codon 408 (underlined), one being a G (wild-type allele), the other a C (mutated allele). Complementary nucleotide peaks are present in the antisense strand. This pattern is consistent with a heterozygous mutation that predicts the synthesis of an abnormal F.X molecule with a $Lys^{408} \rightarrow Asn$ substitution and may be responsible for the CRM+ phenotype. Nine family members have this mutation, four of them in association with the defect in exon VI (see Table 1 and Fig. 1). Panel C: The sense strand DNA sequence shows only one peak consistent with a G to C transversion at position 1344 in codon 408 (underlined) of both alleles of the F.X gene. This homozygous genotype predicts the synthesis of an abnormal F.X molecule in patient's plasma with an Asn at position 408 in the heavy chain. The antisense strand sequence confirms the presence of a homozygous defect. This pattern was observed in one of the proband's sisters (III-2) who has a typical CRM+ phenotype. Nucleotide and codon numbering are as used by Messier et al. [17].

Following reconstitution of F.X -deficient plasma with purified F.X $Lys^{408} \rightarrow Asn$, the data obtained demonstrated a similar activity profile to the one obtained using plasma from the homozygous carrier of F.X $Lys^{408} \rightarrow Asn$ (subject III-2). This data confirm that the F.X $Lys^{408} \rightarrow Asn$ substitution is responsible for the laboratory phenotype found in the family members who were carriers of the abnormal molecule.

2.4. SDS–PAGE and Immunoblotting

Fig. 5 shows a comparison of the F.X molecules obtained from normal plasma (lanes 4 and 8), the plasma of the proband (lanes 1 and 5), the plasma of the proband's father (lanes 2 and 6), and the proband's mother (lanes 3 and 7) under reducing and nonreducing conditions. All F.X molecules are constituted of similar heavy and light chains. Altogether these findings demonstrate that (1) deletion at codon 146 of the F.X gene results in an unstable F.X molecule that is not present in plasma resulting in a phenotype presenting lower antigen levels; (2) the amino acid substitution $Lys^{408} \rightarrow Asn$ is responsible for the decreased F.X activity present in individuals carrying the CRM+ phenotype.

3. Discussion

The data presented here describe a 4-year-old boy suffering from acute lymphoblastic leukemia and carrying double heterozygous F.X defect. The patient suffers from hematomas and epistaxis complications. It is noteworthy that the proband had also severe thrombocytopenia due to the presence of acute lymphoblastic leukemia (ALL) that may have contributed to or caused bleeding symptoms in itself. It is important to note that none of other family members, carriers of homozygous or double heterozygous F.X defects (Table 1), had ever experienced bleeding manifestations. This observation prompted us to investigate the genetic basis of the genetic defects in this family.

Our data demonstrate that the first defect found in exon VI of the F.X gene is a novel single-nucleotide deletion (delC556) affecting codon 146 in the region encoding for the activa-

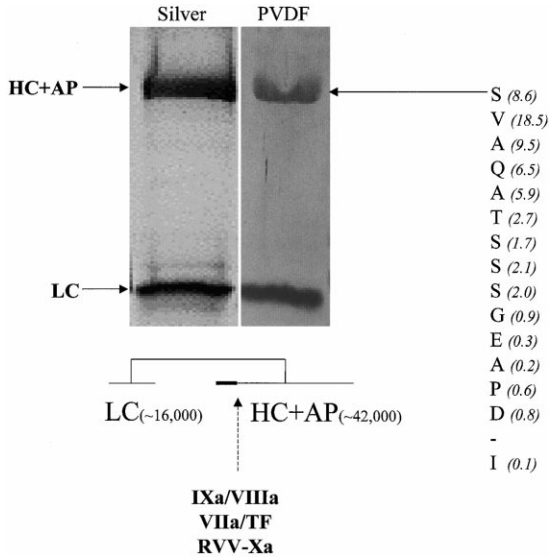

Fig. 4. Characterisation of purified F.X $Lys^{408} \rightarrow Asn$ ($F.X^{K408N}$). $F.X^{K408N}$ was purified as described in the Methods section. Panel A shows the purified molecule stained with silver and the PVDF membrane that was used for the sequencing of the heavy chain. Panel B depicts the first 15 amino acids of the heavy chain of F.X encompassing the activation peptide (numbers in parenthesis represent pmol of amino acid at the corresponding cycle), whereas Panel C shows the activation of F.X by the intrinsic and extrinsic pathways as well as by RVV-Xa. HC + AP identifies the heavy chain and activation peptide of F.X whereas LC identifies the light chain of F.X.

tion peptide. The resulting frameshift mutation is predicted to lead to the synthesis of an abnormal molecule truncated at codon 226. No truncated F.X molecules could be detected in the plasma from these patients. In addition, following purification the proband's molecule showed normal electrophoretic mobility. Additionally, purified F.X from patients had an identical NH_2-terminal sequence to the normal molecule. Thus, no abnormal primary structure of or around the activation peptide region that is the putative site of mutation in the CRM − individuals could be demonstrated. As a consequence, it must be concluded that the affected allele (delC556) accounted for the lack of synthesis and/or secretion of F.X whereas the other allele (wild type) encoded for the synthesis of patient's F.X, which is normal with respect to the activation peptide region. This interpretation was consistent with the fact that the deletion was present in all family members who possessed the heterozygous CRM − phenotype (partial defect of F.X synthesis).

The second defect identified in the proband's factor X gene is a novel missense mutation involving codon 408 in exon VIII corresponding to a $G^{1344} \rightarrow C$ transversion in the F.X gene that results in a $Lys^{408} \rightarrow Asn$ amino acid substitution in the F.X molecule. $Lys^{408} \rightarrow Asn$ is a semiconservative amino acid substitution close to the serine active site of the molecule. The data from the proband together with the data obtained from the proband's sister (III-2, Table 1), who is homozygous for the mutation, demonstrate that the $Lys^{408} \rightarrow Asn$ substitution in the F.X molecule is responsible for the dysfunctional F.X phenotype (low F.X activity).

Peptide inhibition studies using selected amino acid sequences from the COOH-terminal end of the F.X molecule demonstrated that this region is involved in both F.X activation as well as prothrombin activation [18]. This mutation was found to be present at the homozygous level in one proband's sister who exhibited a

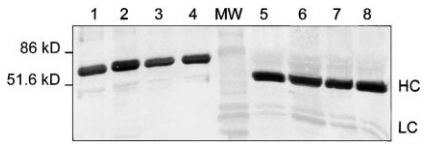

Fig. 5. SDS–PAGE and immunoblot of F.X under nonreducing and reducing conditions. Samples were prepared from patients' plasma (100 μl) as described in Materials and Methods. Barium citrate eluates (20 μl) from the proband III-1 (lane 1), the proband's father II-4 (lane 2), the probands' mother II-5 (lane 3) and pooled normal plasma (PNP) (lane 4) were separated on 5–15% gradient SDS–PAGE gel and blotted onto PVDF membranes. Lanes 5 to 8: same order as in lanes 1 to 4 but samples were reduced with 2 μl of 1 M dithioteithrol (DTT). Factor X was detected with an anti-F.X monoclonal antibody (HX-5050) 4 μg/ml in buffer (Tris 20 mM, NaCl 150 mM, pH 7.4, Tween-20 0.1%) and revealed by 1:500 dilution of goat antimouse antibody–HRP conjugated in the same buffer. HRP activity is visualised with DAB. No significant differences were observed in the apparent MW of F.X heavy and light chains among patients and PNP. Because of their double heterozygosity, the proband (lane 1) and his mother (lane 3) plasma contained only F.X $Lys^{408} \rightarrow Asn$. The proband's father (lane 2) had both normal and F.X $Lys^{408} \rightarrow Asn$; however, the two forms cannot be distinguished with regard to their respective MW. HC: heavy chain. LC: light chain of F.X. MW: molecular weight markers.

normal antigen level and an F.X activity of about 16%, which is twice as much as of the proband's plasma F.X level. These data suggest that while the $Lys^{408} \rightarrow Asn$ mutation did not alter the synthesis, transportation, secretion and stability of the F.X molecule it considerably affected its activity, resulting in marked reduction of F.X activity regardless of the type of assay used. Thus, even at the homozygous level, the F.X $Lys^{408} \rightarrow Asn$ still exhibits a residual activity ($\sim$16% of the normal) sufficient for an individual to be asymptomatic for bleeding manifestations. These data are in complete agreement with data obtained in vitro and in simulated situations, demonstrating that F.X activity as little as 0.1% of the total F.X zymogen concentration is enough to promote normal coagulation [19]. Thus, it is not surprising that the proband's sister is asymptomatic. The proband's bleeding problems are most likely unrelated to the F.X defect(s) alone and are the consequence of the three defects expressed together, i.e., the two F.X defects and the low platelet count (i.e., thrombocytopenia) due to acute lymphoblastic leukemia.

Although congenital F.X deficiency is known to be associated with haemorrhagic conditions since the 1950s [20,21], the molecular basis of this disorder is still under investigation. Since heterozygous and even homozygous F.X-deficient patients may remain asymptomatic, a reliable estimate of the prevalence of this deficiency among the general population is still not available. A limited number of F.X gene mutations have been described so far, the most comprehensive databases being published in 1997 [3,4] that included about 30 genetic mutations responsible for F.X defects. In addition Millar et al. [22] have recently reported on the F.X gene sequencing of 14 unrelated individuals with factor X deficiency (12 familial and 2 sporadic cases) yielding a total of 13 novel mutations.

Regardless of the underlined genetic defect, F.X activity levels above 10% are rarely associated with severe bleeding manifestations even though in the presence of other risks for bleeding. Individuals with these mutations may remain undetected unless challenged (trauma, surgery, low platelet count due to haematologic diseases, etc.).

Microdeletions have been reported as the cause of F.X deficiency in only two cases so far [23,24]. CRM+ F.X variants have been described, the first and best studied being F.X Friuli that, in its homozygous state, was associated with a moderate bleeding tendency [8,9,11,13,25–27]. This variant consists of a $Pro^{343} \rightarrow Ser$ substitution within the heavy chain of F.X. Homozygous F.X Friuli patients presented with a normal antigen level and a normal or near-normal activity by means of RVV assay but a severely reduced (4–9%) function in the intrinsic and extrinsic pathways. In addition, small chromogenic substrates were inefficiently cleaved by F.X Friuli [26]. Factor X $Lys^{408} \rightarrow Asn$ appears to be different from F.X Friuli because of the higher activity levels, the abnormal RVV activity assay pattern and the absence of bleeding symptoms.

Four of the subjects belonging to the family here described have a ''hypo–dys'' phenotype resembling a CRM^R defect. This phenotype derives from two different genetic mutations, a microdeletion resulting in the CRM − phenotype (''hypo'') and a missense mutation accounting for the CRM+ phenotype (''dys''). These four family members were all asymptomatic for bleeding events.

It can be observed that a certain heterogeneity in coagulation times and F.X levels has been found among carriers of same single or combined defects. Our data, however, represent the mean values of repeated assays carried out on different samples belonging to each individual. Discrepancies between global tests and specific factor assays are frequent and often due to the fact that other coagulation factors, besides factor X, may influence the PT and aPTT. In addition, F.X levels may reflect different expression of F.X gene depending on the presence of the mutation and the inheritance pattern.

In conclusion, genotype–phenotype relationship has been investigated in an Italian family with F.X deficiency. Two novel DNA mutations that contribute to the definition of the genetic basis of F.X deficiency have been identified. The clinical relevance of many mutations in the F.X gene, however, remain to be assessed since the majority of homozygous F.X defects reported are paucisymptomatic or even asymptomatic. Interestingly, F.X targeted mice with a

total deficiency in blood coagulation F.X presents partial embryonic lethality and fatal neonatal bleeding, suggesting the crucial role of F.X function [28]. On the other hand, complete deficiency of factor V in mice is also incompatible with survival [29]. However, factor V levels as low as 0.1% result in mice that survive the perinatal period and mature to adulthood [19,30,31]. Thus, prothrombinase is required for survival. It is likely that genetic mutations that cause total F.X deficiency in humans may be incompatible with life as well.

Very few symptomatic individuals with total or severe deficiency in the components of prothrombinase have been identified to date. In most if not all the cases these individuals were detected because of an additional deficiency/problem in addition to their deficiency (i.e., the present study) that oblige them to consult a physician. Previous and recent data has established that as little as 0.15 nM prothrombinase is enough to promote normal coagulation [19,32]. Thus, even in the case of a severe deficiency factor V- and factor X-deficient individuals may go unnoticed until challenged.

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