Characterization of seven novel mutations causing factor XI deficiency

Michal Zucker, Ariella Zivelin, Meytal Landau, Ophira Salomon, Gili Kenet, Frederic Bauduer, Michel Samama, Jacqueline Conard, Marie-Hélène Denninger, Abu-Samra Hani, Micheline Berruyer, Donald Feinstein, Uri Seligsohn

From The Amalia Biron Research Institute of Thrombosis and Hemostasis, Chaim Sheba Medical Center, Tel-Hashomer and Sackler Faculty of Medicine, Tel Aviv University, Israel (MZ, AZ, OS, GK,US); Department of Biochemistry, George S. Wise Faculty of Life Sciences, Tel-Aviv University, Israel (ML); Department of Clinical Hematology, Centre Hospitalier de la Cote Basque, Bayonne, France (FB); Department of Biological Haematology, Hotel-Dieu Hospital, Paris, France (MS, JC); Department of Biological Haematology, Hopital Beaujon, Clichy, France (MD); Maccabi Health Service, Tel-Aviv, Israel (AH); Laboratoire D' Hématologie, Centre de Biologie Est, Groupe Hospitalier Est, Lyon, France (MB); Department of Hematology, The Keck School of Medicine, University of Southern California, Los Angeles, USA (DF).

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Correspondence:
Uri Seligsohn, Amalia Biron Research Institute of Thrombosis and Hemostasis, Chaim Sheba Medical Center, Tel-Hashomer, Israel 52621
E-mail: seligson@sheba.health.gov.il

ABSTRACT

Background and Objectives

Factor XI (FXI) deficiency is a rare autosomal recessive disorder, the main manifestation of which is injury-related bleeding. The disorder is rare in most populations, but common among Jews in whom two mutations, E117X and F283L, account for 98% of cases. Other mutations, C38R and C128X, are prevalent in French Basques and Britons, respectively. Additional sporadic mutations have been described in most parts of the world. The objective of this study was to identify the mutations in 15 unrelated FXI-deficient patients and characterize missense mutations by expression in baby hamster kidney (BHK) cells.

Design and Methods

Clinical and laboratory information and DNA samples were obtained from the patients and mutations were identified by sequencing. Missense mutations were expressed in BHK cells and their effect on FXI secretion and dimerization was assessed using enzyme-linked immunosorbent assay and immunoblotting.

Results

Of 16 mutations detected, seven are novel including two deletions, one splice site and four missense mutations. Expression of the four novel missense mutations (C58Y, Y427C, C527Y and V20A) in cells revealed no secretion of FXI-C58Y, Y427C and C527Y and secretion of only 22% of normal in the medium for FXI-V20A. Secretion of FXI from BHK cells harboring a previously reported E297K substitution cells was also impaired (4.5% of wild-type). Homodimerization was normal for all five mutants.

Interpretation and Conclusions

Defective homodimerization of FXI was previously recognized as a major mechanism for defective secretion of FXI from producing cells. In this study, five FXI missense mutations (four novel) were associated with impaired secretion albeit normal dimerization, underscoring the existence of other mechanisms for defective secretion.

Key words: factor XI, factor XI deficiency, factor *11* gene.

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Factor XI (FXI) is an important component of blood coagulation. Following its activation by thrombin on platelet surfaces, activated FXI cleaves its substrate, factor IX, which then leads through subsequent reactions to thrombin generation at the site of vessel wall injury. Thrombin generation gives rise to fibrin formation and to its stabilization by inhibiting fibrinolysis through activation of the thrombin activatable fibrinolysis inhibitor. FXI circulates in plasma as a homodimer consisting of two 80kD subunits. Each subunit comprises a light chain harboring a serine protease catalytic domain and a heavy chain consisting of four 90-91 residue tandem domains designated *apple domains*. Apple 4 domain is essential for dimerization of the two FXI subunits and was shown to regulate FXI secretion from producing cells.[1] Inherited FXI deficiency is an injury-related bleeding disorder manifested predominantly in tissues exhibiting fibrinolytic activity such as oral mucosa and the urinary tract.[2] FXI deficiency is rare worldwide but in Jews of Ashkenazi or Iraqi origin it is common.[3, 4] Two mutations, E117X (type II) and F283L (type III), account for approximately 98% of mutant alleles that cause FXI deficiency among Jews in Israel.[4] Two less common mutations were described in Jews: type I, a splice-site mutation and type IV, a 14bp deletion between exon 14 and intron N.[5, 6] Other relatively frequent mutations, C38R and C128X, were reported in French Basques and Britons, respectively.[7-9] More than 120 other sporadic mutations have been identified in different populations [*http://www.med.unc.edu/isth, http://www.factorXI.org*].[10]

Characterization of the growing number of FXI mutations has shed light on the relationship between structure and function of this protein, and has contributed to defining the importance of its various domains. In this study we detected the mutations in 15 unrelated patients with FXI deficiency. Altogether, 16 mutations were identified: eight missense mutations, three splice site mutations, four deletions and one nonsense mutation. Seven mutations are herein described for the first time, of which four (missense mutations) were found to impair FXI secretion despite normal dimerization.

Design and Methods

Patients

Fifteen unrelated patients with known FXI deficiency were examined in this study. Seven patients were from Israel (five Jews and two Arabs) and eight originated from the following countries: Portugal (n=2), France (n=2), Belgium (n=1), Mexico (n=1), Morocco (n=1) and Tunisia (n=1). The gender, age, FXI level and bleeding manifestations of these patients are shown in Table 1. Of these 15 FXI-deficient patients, 11 had severe deficiency (FXI activity < 15 U/dL) and four had partial deficiency (FXI activity 25-38 U/dL).

Mutation identification

DNA was extracted from whole blood of the patients' leukocytes by a standard procedure.[11] All patients were initially tested for the presence of types I-IV mutations by previously described methods.[3,5,6] Polymerase chain reaction amplifications of all exons and flanking exon-intron boundaries of the *F11* gene were performed and direct sequencing of amplified fragments was carried out by an automatic sequencer (ABI, Foster city, CA, USA). Sequences of specific primers and PCR conditions are available upon request (*seligson@sheba.health.gov.il*).

FXI activity and antigen measurements

FXI activity was determined by an activated partial thromboplastin time (aPTT)-based assay using severe FXI-deficient plasma as a substrate (FXI activity < 1 U/dL) and 1:10 to 1:320 dilutions of reference normal plasma for construction of a standard curve. A 1:5 dilution of patients' plasma yielded an assay sensitivity of < 1 U/dL. FXI activity in the medium of transfected cells was measured in undiluted samples and the standard curve for this assay was constructed by diluting reference plasma in Dulbecco's modified Eagle's medium (DMEM) yielding a sensitivity of < 0.1 U/dL. FXI antigen was measured in patients' plasma samples and in cell media and lysates by an enzyme-linked immunosorbent assay (ELISA). This assay was carried out using 1 μg/mL monoclonal anti FXI antibody as a capture antibody (Haematologic Technologies Inc., VT, USA), incubation with samples containing FXI antigen and, after washing with TBS-Tween buffer, binding of a 1:1000 dilution of polyclonal goat anti-human FXI IgG (Affinity Biologicals, Ontario, Canada). Detection was achieved using peroxidase-conjugated anti-goat IgG and *o*-phenylenediamine as a substrate of the peroxidase. Optical density at a wavelength of 490 nm was expressed in ng/mL. A standard curve was constructed by 1:25 to 1:800 TBS-0.2% BSA dilutions of reference normal plasma, yielding a sensitivity of < 6 ng/mL for the medium and < 30 ng/mL for the cell lysate.

Expression of wild type or mutant FXI in baby hamster kidney cells

Human FXI complementary DNA (cDNA) in pBR322 vector was kindly provided by Dr. Dominic Chung from the University of Washington (Seattle, USA) and was subcloned into pcDNA3 (Invitrogen, Carlsbad, CA, USA) as previously described.[7] Mutations were introduced into the pcDNA3 vector using the QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions, using specific primers whose sequences are available upon request (*seligson@sheba.health.gov.il*). Baby hamster kidney (BHK) cells were grown in DMEM supplemented with 2 mg/mL L-glutamine and 5% fetal calf serum (Biological Industries, Beit-Haemek, Israel). Cells were transfected with 1 μg of either wild type (WT) or mutated pcDNA3-FXI using Lipofectamine reagent (Gibco, Paisley, UK) and selected by

Table 1. Mutations, origin and clinical and laboratory findings in 15 patients with FXI deficiency.

Patient	Origin	Gender/ Age	Plasma FXI activity (U/dL)	Plasma FXI antigen (U/dL)	Bleeding manifestations	Nucleotide (nt) change†	Mutation	Location or domain	Genotype
1	Non-Ashkenazi Jewish	F/2	< 1	< 1	Gingival	c.73-86del*/ c.403G>T	Frame shift/ E117X	Apple 1/Apple 2	Compound heterozygote
2	French	F/34	32	ND	None	c.113T>C	V20A	Apple 1	Heterozygote
3	Yemenite Jewish	M/19	25	ND	Hemoptysis	c.227G>A	C58Y	Apple 1	Heterozygote
4	Mexican	F/91	< 1	ND	None	c.595+3A>G	Splicing		Homozygote
5	Israeli Arab	F/15	29	23	Epistaxis, menorrhagia	c.943G>A	E297K	Apple 4	Heterozygote
6	Belgic	F/24	< 2	< 5	Bleeding after tooth extraction	c.943G>A/ c.1634G>A*	E297K/ C527Y	Appl 4/ Catalytic	Compound heterozygote
7	French	M/50	< 1	ND	Bleeding after appendicectomy	c.961-962delTG	Frame shift	Apple 4	Homozygote
8	AshenaiJewish	F/33	9	9	Epistaxis	c.1021G>A/ c.901T>C	E323K/ F283L	Apple 4/ Apple 4	Compound heterozygote
9	Poruguese	F/50	2	ND	None	c.1026G>T / c.1247G>A	Splicing/ C398Y	Apple 4/ Catalytic	Compound heterozygote
10	Moroccan	M/67	2	< 5	Epistaxis, bleeding after tooth extraction	c.1072delA	Frame shift	Catalytic	Homozygote
11	Portuguese	F/58	2	ND	None	c.1072delA / c.1247G>A	Frame shift/ C398Y	Catalytic/ Catalytic	Compound heterozygote
12	Tunisian	M	<1	ND		Exons 11-15 del*‡		Catalytic	Homozygote
13	Ashkenazi Jewish	F/44	<1	<1	Menorrhagia	c.1334A>G*/ c.403G>T	Y427C/ E117X	Catalytic/ Apple 2	Compound heterozygote
14	Ashkenazi Jewish	F/29	38	20	None	c.1480+2T>G*	Splicing	Catalytic	Heterozygote
15	Israel Arab	M/6	14	ND	Easy bruising	Undefined			

**Novel mutation; ND: not determined; †Nucleotide numbers are based on the Geebank file M13142 using the A (nucleotide 44) of the ATG initiator methionine as +1. ‡The boundaries of this deletion are not determined.*

a medium containing 0.7 mg/mL neomycin (Gibco). Stably transfected cells (5×10^6) were grown for 24 hours in 7 mL of media and thereafter media were collected for assays of FXI. For analyses of intracellular FXI, the cells were first washed with phosphate-buffered saline (PBS), and then lysed with 1 mL lysis buffer containing 10 mM Hepes (pH 7.5), 50 mM NaCl, 5 mM EDTA, 0.3 M sucrose, 1% Triton X-100, 0.5% deoxycholic acid sodium salt, 0.1% SDS and 1 tablet of protease inhibitor cocktail (Roche, Penzberg, Germany) per 10 mL. Cell lysates were incubated in ice for 5 min, centrifuged for 5 min at 20,800 g at 4°C to remove debris and then kept frozen at -20°C until assayed. To precipitate FXI from cell lysates or media, 250 µL kaolin solution (20 mg/mL saline) were added to 1 mL cell medium or lysate and incubated for 15 min at 37°C and then centrifuged at 14,000 g for 12 sec. FXI was consequently eluted from kaolin particles in 30 µL sample buffer (LifeGels, NSW, Australia) and heated to 95°C for 5 min. Thereafter, samples were centrifuged at 14,000 g for 5 min and the supernatant was kept at room temperature for western blot analysis performed instantaneously.

Western blot analysis

Because the recovery of FXI after kaolin precipitation varied substantially in experiments using the same samples, western blot analysis was only used as a qualitative method aimed to distinguish between disulfide bonded FXI dimers and non-disulfide bonded FXI. Samples were not reduced prior to western blot analysis. Samples were subjected to electrophoresis on 8% LongLife Gels (LifeGels), and then transferred to a polyvinylidene difluoride membrane (Immobilon P, Millipore, MA, USA) in transfer buffer (LifeGels) according to the manufacturer's instructions. FXI was detected by using 1:500 anti-FXI monoclonal antibody (Haematologic Technologies), and a 1:5000 dilution of peroxidase-conjugated anti-mouse IgG (Jackson, PA, USA). Immunoreactive bands on the membrane were detected using an enhanced chemiluminescence (ECL) kit (Pierce, IL) and X-ray film exposure.

Structural analysis of mutants

Structural analysis was performed using the crystal structures of the zymogen FXI (PDB code – 2F83)[12] and activated FXI (PDB code – 1XX9).[13] The solvent-accessible area for each residue in both FXI and FXIa structures was calculated using the SURFV program with a probe sphere with a radius of 1.4Å and default parameters.[14] Evolutionary conservation analysis was performed using the ConSurf web-server[15] (*http://consurf.tau.ac.il*). The calculations for the serine protease domain were carried out using the

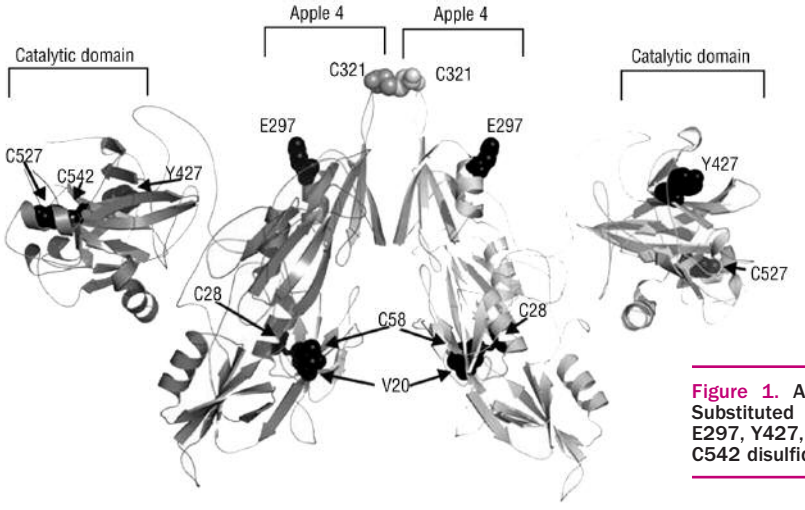

Figure 1. A ribbon representation of the FXI dimer.[12] Substituted residues of missense mutations (V20, C58, E297, Y427, C527) are depicted. The C58-C28 and C527-C542 disulfide bridges are shown.

Table 2. Expression of novel missense mutations in BHK cells.

*Mutation **	*FXI antigen in lysate ng/mL (mean±SEM)*	*FXI antigen in medium ng/mL (mean±SEM)*
WT (n=4)	225±17	213±25
C58Y (n=3) **	258±19	0
Y427C (n=4)	80±8	0
C527Y (n=3)	133±22	0
V20A (n=3)	165±35	46±8

**n denotes the number of transfections; **this mutant antigen was detected by capture antibodies composed of three different monoclonal antibodies kindly provided by Dr. Joost Meijers.*

structure of FXIa (PDB code – 1XX9), and were based on an alignment of 200 serine protease sequences collected from the SWISSPROT database[16] and default parameters. The conservation analyses of the FXI apple domains were based on 40 non-redundant apple domain sequences collected from the UNIPROT database.[17]

Results

Of 16 mutations identified in 15 patients, seven are novel (Table 1). Four patients were homozygotes, four were heterozygotes and six were compound heterozygotes. In one patient (#15) no mutation was identified within exons 1 to 15 or in the promoter region. Of the compound heterozygotes, three were of Jewish origin and each carried one of the common Jewish mutations (type II in patients #1 and 13, and type III in patient #8) as well as another mutant allele (Table 1); two were Portuguese (patients #9 and 11) both harboring a C398Y mutation and another mutant allele, and one was of Belgian origin (patient #6).

Of the seven novel mutations, four were missense mutations, two were deletions and one was a splice site mutation. Figure 1 shows the location of the missense mutations on a FXI dimer derived from the crystal structure.[12] Substitution of C58Y resulted in abrogation of FXI secretion (Table 2). This mutation disrupts the C58-C28 bond that is one of the three disulfide bonds responsible for correct folding of the apple 1 domain (Figure 1). Apple 1 comprises the recognition site for the monoclonal antibody used to detect FXI antigen by western blot analysis and in the ELISA. This probably accounted for our inability to show a FXI band in western blots (Figure 2B). Indeed, a mixture of three different monoclonal antibodies that bind to epitopes of other FXI apple domains did detect normal amounts of FXI-C58Y in cell lysates by ELISA (Table 2) although less conspicuous FXI dimer in western blots (Figure 2C). The second novel missense mutation, Y427C, was identified in a compound heterozygote (#13 in Table 1) who also carried the type II mutation. Expressing Y427C in BHK cells resulted in abrogation of FXI secretion and was associated with a reduced amount of FXI in lysed cells (Table 2 and Figure 2). Western blot analysis revealed that the mutated FXI in cells was a 160 kD dimer (Figure 2B). The third novel missense mutation was a C527Y substitution located in the catalytic domain of FXI (Figure 1). It was identified in a compound heterozygote (#6 in Table 1) who also bore an E297K substitution. Expression of C527Y in BHK cells revealed intact dimerization, a reduced amount of FXI in lysed cells and total lack of secretion from the cells (Table 2 and Figure 2). The fourth novel missense mutation, V20A, was identified in a heterozygote (#2 in Table 1). Substitution of FXI-V20 by A in BHK cells caused profoundly decreased FXI secretion (22% of WT secretion by cells) despite the presence of slightly reduced amounts of FXI dimer within the cells (Table 2 and Figure 2). The fifth novel mutation was a splice site mutation, c.1480+2T>G. This mutation was identified in a heterozygous Ashkenazi Jewish patient (#14 in Table 1) who had partial FXI deficiency. The sixth mutation was a 14 bp deletion in exon 3 identified together with type II mutation in a non-Ashkenazi Jewish patient (#1 in Table 1). This deletion includes codons 7 to 11 and leads to a frameshift creat-

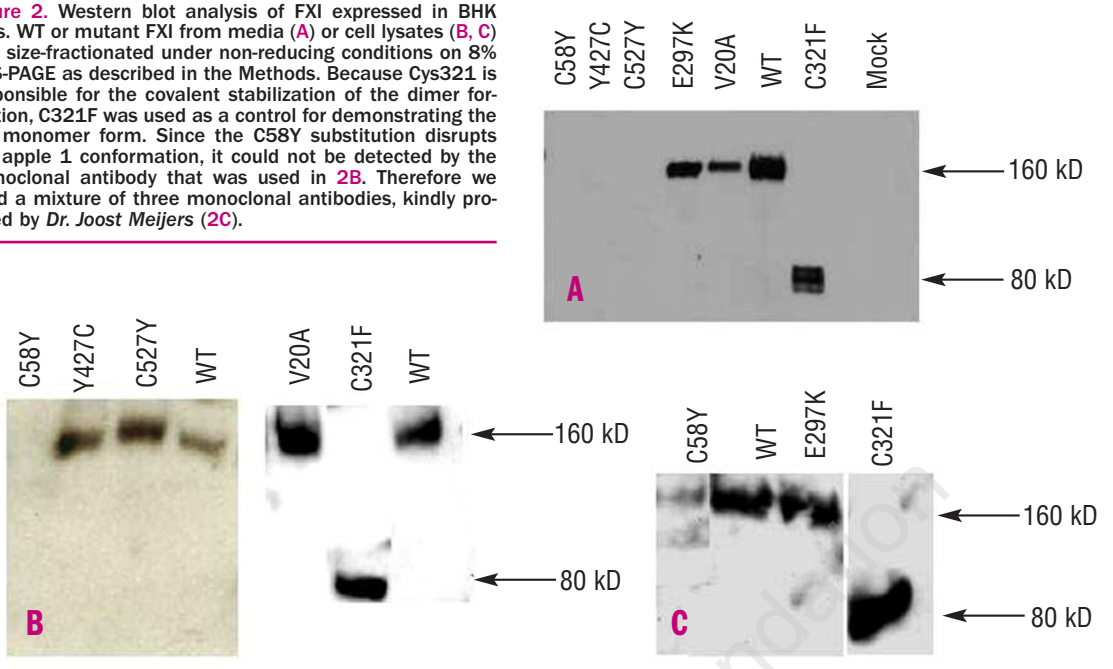

Figure 2. Western blot analysis of FXI expressed in BHK cells. WT or mutant FXI from media (A) or cell lysates (B, C) was size-fractionated under non-reducing conditions on 8% SDS-PAGE as described in the Methods. Because Cys321 is responsible for the covalent stabilization of the dimer formation, C321F was used as a control for demonstrating the FXI monomer form. Since the C58Y substitution disrupts the apple 1 conformation, it could not be detected by the monoclonal antibody that was used in 2B. Therefore we used a mixture of three monoclonal antibodies, kindly provided by *Dr. Joost Meijers* (2C).

ing a premature stop at codon 13. The seventh mutation was a large deletion of exons 11-15. It was identified in an homozygous patient (#12 in Table 1) whose FXI activity was <1 U/dL. This deletion includes the entire catalytic domain of FXI and probably involves an additional stretch of DNA beyond exon 15, the last exon of the *F11* gene. Of the remaining nine mutations detected in this study (Table 1) and previously reported,[5,18-22] four were missense mutations (F283L, E297K, E323K and C398Y). Three of these four missense mutations have been characterized by expression studies[20,21,23] whereas the fourth, E297K,[19] has not been expressed, and was, therefore, characterized in this study. Its expression in BHK cells revealed impaired secretion (4.5% of WT FXI) despite the presence of 70% of WT FXI antigen in cell lysate, which was shown to be a dimer by western blot analysis (Figure 2).

Discussion

In this study we identified 16 mutations in the *F11* gene in 15 patients with FXI deficiency. Seven mutations are novel and four of those are missense mutations (V20A, C58Y, Y427C and C527Y). The four missense mutations and a previously reported uncharacterized missense mutation[19] were expressed in BHK cells and analyzed in order to discern the associated functional defects. Two mutations (V20A and C58Y) are in residues located in FXI apple 1 domain, two are in the catalytic domain (Y427C and C527Y) and the one previously reported (E297K) is in the apple 4 domain. All five mutations were associated with intact FXI dimerization but impaired secretion from BHK cells. For three mutations (C58Y, Y427C and C527Y) there was no secretion at all, while for one mutation (V20A) there was partial secretion (Table 2). Residue C58 is involved in one of the three disulfide bonds that are essential for proper folding of the apple 1 domain. Consequently, the C58Y substitution disrupting the C58-C28 appeared to be responsible for the totally impaired secretion despite the presence of FXI in lysed cells. Two other substitutions of C58 were recently reported by Mitchell *et al.*[18] One, a C58F substitution, was detected in a homozygous patient whose FXI activity was <1% of normal, and the second, a C58R substitution, was identified in a heterozygous patient whose FXI activity was 42%. Other disulfide bond disruptions by mutations in cysteine residues were reported in apple 1 (C38R) and apple 3 (C237Y)[7] with both displaying, similarly to C58Y, intact FXI production but impaired secretion.

Residue 427 is not conserved and is located at a distance from the catalytic site (Figure 1). Conceivably, substituting tyrosine, a large aromatic amino acid by a small cysteine is responsible for disruption of hydrophobic interactions that are necessary for normal FXI secretion. Moreover substitution by cysteine could lead to the formation of abnormal disulfide bonds resulting in structural damage that could affect secretion. Indeed this Y427C mutation completely abolished FXI secretion in spite of intact dimerization of FXI within the cells (Table 2, Figure 2).

C527 forms a highly conserved disulfide bond with C542 (Figure 1). Substitution of such a highly conserved residue by tyrosine probably causes structural instability, leading to reduced antigen level within the cells as

well as to complete disruption of FXI secretion.

Substitution of V20 by alanine is a rather mild change. However, the positions corresponding to V20 in all other analyzed apple domains are occupied by a residue larger than alanine and the crystal structure of FXI[12] indicates that V20 has several important contacts (including one with C58; Figure 1). Consequently, it seems reasonable to assume that these contacts are disrupted by the V20A substitution. Comparison of homologous sequences indicates that E297 is not a conserved residue. It is an exposed residue located on the surface of the apple 4 domain but it is not included in the dimerization interface.[12] Conceivably, in the E297K mutation the substitution of a negatively charged glutamic acid by a positively charged lysine is the cause of the misfolding that impedes the secretion of FXI. Substitution of E297 by K resulted in secretion of only 4.5% of WT FXI as measured by ELISA. This very low secretion of FXI was not evident by western blot analysis which exhibited a rather intense band (Figure 2A). However, this apparent discrepancy, also observed with the V20A mutant stems from our finding that variable amounts of FXI are produced by kaolin precipitation and thus western blot analysis cannot be regarded as a quantitative measure (*see Methods*). Previous studies suggested that FXI dimerization is essential for FXI secretion.[1,23] This assertion emanated from the original study of the type III mutation (F283L) in which FXI dimerization was defective and so was the secretion from cells.[23] In the present study five missense mutations (C58Y, Y427C, C527Y, V20A and E297K) were shown to abrogate or reduce FXI secretion despite intact dimerization. These data indicate that secretion of FXI from producing cells can be impaired by mechanisms other than defective dimerization, as was also suggested by Pugh *et al*.[21]

Authors' contributions

All foreign participants and Abu-Samra Hani from Jaffa, Israel, contributed samples from one patient each. ML had made the structural analysis of this study; MZ and AZ arranged and organized the research amd wrote the manuscript.

Conflicts of Interest

The authors reported no potential conflicts of interest.

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