Identification of eight novel coagulation factor XIII subunit A mutations: implied consequences for structure and function

Vytautas Ivaskevicius,*[1] Arijit Biswas,*[1] Carville Bevans,[1] Verena Schroeder,[2] Hans Peter Kohler,[2] Hannelore Rott,[3] Susan Halimeh,[3] Petro E. Petrides,[4] Harald Lenk,[5] Manuele Krause,[6] Bruno Miterski,[7] Ursula Harbrecht,[1] and Johannes Oldenburg[1]

[1]Institute of Experimental Hematology and Transfusion Medicine, University Clinic Bonn, Bonn, Germany; [2]Hemostasis Research Laboratory, Department of Hematology, University of Bern, Switzerland; [3]Laboratory and Ambulance for Coagulation Disorders, Duisburg, Germany; [4]Hematology Oncology Center, Munich, Germany; [5]Pediatric Hospital, University of Leipzig, Leipzig, Germany; [6]German Clinic for Diagnostics, Wiesbaden, Germany; [7]Institute of Clinical Genetics, Oberhausen, Germany

ABSTRACT

Background
Severe hereditary coagulation factor XIII deficiency is a rare homozygous bleeding disorder affecting one person in every two million individuals. In contrast, heterozygous factor XIII deficiency is more common, but usually not associated with severe hemorrhage such as intracranial bleeding or hemarthrosis. In most cases, the disease is caused by *F13A* gene mutations. Causative mutations associated with the *F13B* gene are rarer.

Design and Methods
We analyzed ten index patients and three relatives for factor XIII activity using a photometric assay and sequenced their *F13A* and *F13B* genes. Additionally, structural analysis of the wild-type protein structure from a previously reported X-ray crystallographic model identified potential structural and functional effects of the missense mutations.

Results
All individuals except one were heterozygous for factor XIIIA mutations (average factor XIII activity 51%), while the remaining homozygous individual was found to have severe factor XIII deficiency (<5% of normal factor XIII activity). Eight of the 12 heterozygous patients exhibited a bleeding tendency upon provocation.

Conclusions
The identified missense (Pro289Arg, Arg611His, Asp668Gly) and nonsense (Gly390X, Trp664X) mutations are causative for factor XIII deficiency. A Gly592Ser variant identified in three unrelated index patients, as well as in 200 healthy controls (minor allele frequency 0.005), and two further Tyr167Cys and Arg540Gln variants, represent possible candidates for rare *F13A* gene polymorphisms since they apparently do not have a significant influence on the structure of the factor XIIIA protein. Future *in vitro* expression studies of the factor XIII mutations are required to confirm their pathological mechanisms.

Key words: factor XIII deficiency, FXIII-A, FXIII-B, structural analysis.

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**VI and AB contributed equally to this article*

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Correspondence:
Vytautas Ivaskevicius, Institute of Experimental Haematology and Transfusion Medicine, University Clinic Bonn, 53127 Bonn, Germany. Phone: international +49.0228.287.15175. Fax: international +49.0228.287.16087. E-mail: vytautas.ivaskevicius@ukb.uni-bonn.de

Introduction

Congenital factor XIII (FXIII) deficiency is chiefly caused by mutations in the *F13A* gene (95% of cases) and, more rarely, by *F13B* gene defects (5% of cases). The *F13A* gene, coding for the FXIII A protein subunit, occupies chromosomal position 6p24-25 and comprises 15 exons encoding a 731 amino acid protein.[1] Homozygous mutations in this gene usually result in severe FXIII deficiency (OMIM: +134570, +134580), which is a rare autosomal recessive bleeding disorder affecting approximately one person in every in two million individuals. Patients suffering from this condition are characterized by a life-long bleeding diathesis and impaired wound healing and affected females have a high incidence of spontaneous abortions.[2] In contrast, patients with heterozygous FXIII deficiency tend to be asymptomatic. However, under special conditions such as surgery, dental extraction or trauma, even heterozygous carriers of a mutation may bleed significantly. Heterozygous females may experience menorrhagia and post-partum hemorrhage requiring blood transfusion.[3,4]

In plasma, mature inactive FXIII circulates as a tetramer (A2B2) composed of two A subunits and two B subunits, while intracellular FXIII is a homodimer comprising two A subunits (A2). The FXIII A subunit belongs to a family of transglutaminases and has catalytic activity, while FXIII B subunits act as carrier molecules, stabilizing circulating A subunits against proteolysis and systemic clearance. The high-resolution structure of the FXIII A subunit was determined by X-ray crystallography.[5] FXIII A is composed of five distinct domains: an activation peptide (residues 1-37), a β-sandwich (38-183), a central core (184-513), and β-barrel 1 (514-628) and β-barrel 2 (629-731) regions.[6,7] The central core domain contains a catalytic triad comprising Cys314, His373 and Asp396 that interact with each other through a hydrogen bonding network.[8]

In the present study we identified eight novel *F13A* gene mutations (six missense and two nonsense) by direct sequencing among 13 patients of Caucasian origin with clinically diagnosed FXIII deficiency. The possible impact of the missense mutations on the structure of the FXIII A subunit was studied using protein structure visualization tools to inspect the local wild-type protein environment around each mutant residue position in order to infer the structural impact and potential underlying molecular mechanisms of the mutational phenotype.

Design and Methods

Patients and controls

Our investigation was a genetic study (screening for mutations in *F13A* and *F13B* genes) in which we collected samples from 150 unrelated patients with suspected heterozygous FXIII deficiency [defined here by a reduced FXIII activity i.e <65% (normal range, 65-120%) and/or a history of bleeding] over a period of 5 years between 2004 and 2009. Mutations were not found in 127 of these 150 screened patients, suggesting that non-genetic causes or defects in other genes were responsible for their clinical phenotype. Ten patients were heterozygous for single mutations within the *F13A* gene and 13 individuals showed single mutations within the *F13B* gene. Only the mutations in the *F13A* gene are presented here; the mutations in the *F13B* gene will be published separately. The *F13* gene was also analyzed in a single patient with severe FXIII deficiency (<5% of normal FXIII activity). The clinical data and measured FXIII activity values for the ten index patients and three of their relatives in whom mutations were found in the *F13A* gene are presented in Table 1. We also genetically screened 200 healthy controls (blood donors from the greater Bonn region in Germany) for the presence of the point mutations identified in the index patients. All subjects gave informed consent to participation in this study, as required by the Declaration of Helsinki. The study was approved be the local ethics committee of the University of Bonn.

Determination of factor XIII activity, F13A polymerase chain reaction analysis and sequencing

FXIII activity for each subject was measured using a photometric assay.[9] Genomic DNA was extracted from peripheral blood using standard procedures. *F13A* and *F13B* gene exons and their flanking regions were amplified by polymerase chain reaction and sequenced using an ABI3130 sequencer as described previously by Ivaskevicius *et al.*[2,10] The amino acid positions are reported according to Ichinose *et al.*[1]

Multiple sequence alignment

The multiple sequence alignment of mammalian FXIII A orthologs was done using the MultiAlign program, (http://mendel.ethz.ch:8080/Server/MultAlign.html). The amino acid sequences of 11 mammalian species were downloaded from PubMed and aligned with Multialign.

Structural analysis

The crystallographic model of the recombinant human cellular coagulation FXIIIA zymogen (EC: 2.3.2.13, resolution solved to 2.1Å) was downloaded from the Protein Data Bank (data file 1F13)[11] for viewing, analysis and graphical rendering using UCSF Chimera 1.2306.[12] Ribbon models were created with the FXIIIA subdomains in different colors. Side chains of the catalytic residues, as well as the native residues at the positions of the reported human mutations, were depicted as van der Waals' spheres. Hydrogen bonds were inferred using the H-bond distance calculation algorithm of Mills and Dean[13] relaxing constraints by 0.4 Å and 20 degrees.

Results

Direct sequencing of the *F13A* gene from 13 patients among ten unrelated families revealed eight novel *F13A* mutations. Six of these were missense and two were nonsense mutations. With the exception of one, all individuals were heterozygous (average FXIII activity: 52% of normal). The homozygous individual suffered from severe FXIII deficiency (operationally defined as <5% of normal FXIII activity[2]). None of these patients had mutations in the *F13B* gene.

Missense mutations

Patient A had a point mutation (c.503A>G) in exon 4 resulting in substitution of a Cys residue by an aromatic Tyr at position 167 (Cys167Tyr) in the β-sandwich domain. Patient B was heterozygous for a point mutation (c.869C>G) in exon 7 resulting in substitution of a neutral Pro residue by a positively charged Arg at position 289 (Pro289Arg) in the central core domain. Patient D was heterozygous for a missense mutation in exon 12 resulting in substitution of a positively charged Arg by a neutral Gln residue at position 540 (Arg540Gln, c.1622G>A) in the

thermostable β-barrel 1 domain. A Gly to Ser substitution at position 592 (Gly592Ser) in exon 13 (β-barrel 1 domain) was found in three unrelated females (patients E, F and G). Three related patients (patients I and J are the son and daughter, respectively, of patient H) carried a missense mutation in the β-barrel 1 domain resulting in the substitution of a His residue by Arg (c.1835G>A, Arg611His). The youngest patient in this series, patient L, and his mother (patient M) were heterozygous for a point mutation in exon 13 resulting in substitution of a negatively charged and hydrophilic Asp by the neutral and hydrophobic Gly at position 668 (c.2006A>G Asp668Gly) in the terminal β-barrel 2 domain.

Nonsense mutations

Nonsense mutations were observed in two patients. In patient C a nonsense mutation results in premature termination of the protein at amino acid position 390 (c.1171G>T, Gly390X) in the core domain. The second nonsense mutation (in patient K) was identified in exon 14 and results in premature termination of the protein at residue 664 (Trp664stop) within the terminal β-barrel 2 domain.

Screening of healthy controls

Using direct sequencing, we screened a control group of 200 healthy blood donors of German ethnicity for the presence of any of the identified mutations. Only Gly592Ser was found among the members of the control group. The heterozygous Gly592Ser variant was identified in two control individuals, corresponding to a minor allele frequency of 0.005 among the sampled population. Unfortunately, FXIII levels were not available for these two patients. To date, the Gly592Ser variant has not been reported in either the NCBI (National Library of Medicine at the National Institutes of Health) human mutation database or in the Seatttle single nucleotide polymorphism database (http://www.ncbi.nlm.nih.gov/sites/entrez, http://pga.gs.washington.edu).

Structural analysis

Figure 1 displays a backbone ribbon model of the crystallographic FXIIIA dimer from the 2.10 Å resolution X-ray data of Weiss *et al.*[11] indicating the locations of the six FXIIIA residues that were found to be mutated in our cohort of patients. Detailed views of the local molecular environments of each of the individual wild-type residues at each of these positions are shown in Figure 2A-F. Tyr167 is situated in the β-sandwich domain. It is the first amino acid of the short β-strand ranging from Tyr167 to Thr172. The tyrosine side chain extends into the loop formed by the amino acids Gly33-Leu45. In the figure, there is a gap in the loop since Arg37 and Gly38 were not resolved in the crystal model. The side chain of cysteine is smaller than that of tyrosine. Pro289 is part of a loop situated between α-helix and β-strand structures within the core domain, close to the catalytic center Cys314. The Pro289Arg mutation leads to the replacement of the rigid proline pyrolidine ring by the larger and charged arginine side chain. Arg540 is situated at the edge of barrel 1. It is part of the β-strand ranging from Phe533 to Asn542 which forms the middle strand of a three-stranded antiparallel β-sheet. Arg540 forms two hydrogen bonds with Asp519 on the neighboring strand. Arginine is the most polar amino acid with its positively charged side chain. This side chain, which is missed by the Gln540 mutant, points towards the surface of the molecule. Gly592 is part of the loop Gln590-Gln601 which connects two β-strands in barrel 1. Serine has a larger and more polar side chain compared to glycine. Arg611 is part of the β-strand running from Ala602 to Asn613

Table 1. A summary of patients' clinical data, FXIII values and gene defects. Patients I and J are the son and daughter of patient H, and patient M is the mother of patient L.

N.	Patient ID, age, (sex)	Symptoms	FXIII[1], % of normal	Nucleotide exchange	Predicted amino-acid substitution	Domain
1	A, 29, (f),	-	57-70	c.503A>G	Tyr167Cys (het)	β-sandwich
2	B, 10, (m)	subcutaneous shank hematoma due to trauma	46	c.869C>G	Pro289Arg (het)	core
3	C, 68, (f)	bleeding after dental extraction	49	c.1171G>T	Gly390X (het)	core
4	D, 32, (f)	-	50-61	c.1622G>A	Arg540Gln (het)	barrel 1
5	E, 28, (f)[2]	postpartum bleeding	61	c.1777G>A	Gly592Ser (het)	barrel 1
6	F, 42, (f)	menorrhagia, postpartum bleeding, bleeding after dental extraction	71	c.1777G>A	Gly592Ser (het)	barrel 1
7	G, 35, (f)	bleeding after breast fibrioadenoma surgery,	60	c.1777G>A	Gly592Ser (het)	barrel 1
8	H, 59, (m)	postoperative bleeding due to facial trauma	49	c.1835G>A	Arg611His (het)	barrel 1
9	I, 27, (m)	-	43	c.1835G>A	Arg611His (het)	barrel 1
10	J, 24, (f)	-	43	c.1835G>A	Arg611His (het)	barrel 1
11	K, 9, (f)[3,4]	umbilical stump bleeding, hemarthrosis,	<5	c.1994G>A	Trp664X (hom)	barrel 2
12	L, 3, (m)	bleeding due to craniostenosis surgery, mucous and subcutaneous bleeds, impaired wound healing	23-40	c.2006A>G	Asp668Gly (het)	barrel 2
13	M, 22, (f)	-	n.d.	c.2006A>G	Asp668Gly (het)	barrel 2

c., cDNA sequence – nucleotide one is the A of the ATG-translation initiation codon; [1]normal range for FXIII activity: 65-120%; [2]patient E was heterozygous for GCG/ACCTGCAGgtaa variant in untranslated exon 1; [3]prophylactic treatment with plasma derived FXIII; [4]consanguineous parents; hom, homozygous for the corresponding mutation, het, heterozygous for the corresponding mutations formed by the affected amino acid residues.

which is situated within the β-sheet structure of the barrel 1 domain. It is involved in key inter-domain stabilizing hydrogen bonds. Asp668 represents the C-terminal amino acid of the β-strand beginning with Leu660. Its large side chain extends into the space between the barrel 2 domain and the core domain. Its replacement by a Gly molecule would disrupt the inter-domain hydrogen bonded network that it forms with the residues on the transglutaminase core domain.

Multiple sequence alignment

The multiple sequence alignment of mammalian FXIII A orthologs is shown in Figure 3. Conserved residues are shown on a black background.

Discussion

While the causative nature of the Gly390Stop and Tyr664Stop nonsense mutations is obvious, that of the missense mutations remains less clear. Here we present some data from protein modeling to support the belief that these missense mutations are responsible for the patients' clinical phenotype. However, expression studies would be required for a definitive assignment of the causative nature of the mutation.

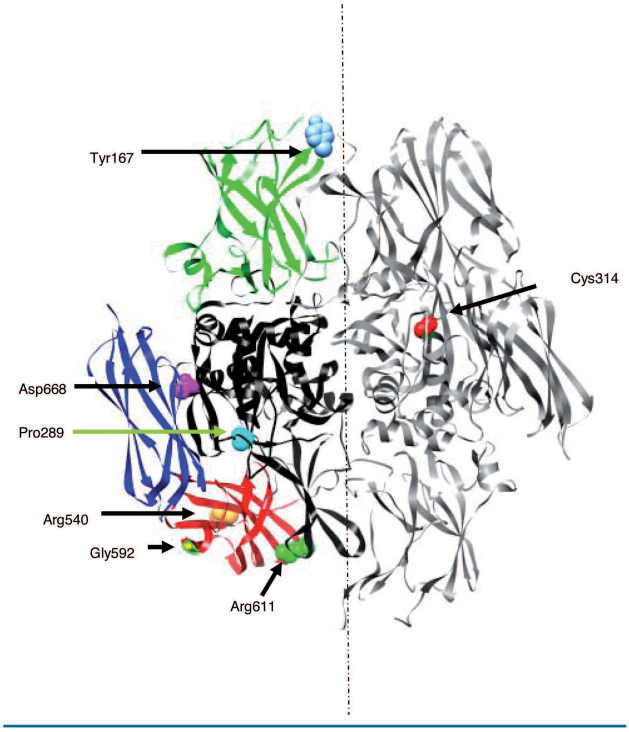

Figure 1. Backbone ribbon diagram of the FXIIIA dimer (from a model published by Weiss *et al.* (1998) built from X-ray crystallographic data collected to 2.1 Å resolution). The two monomers lie to the left and right of a central line drawn from top to bottom through the dimer (*i.e.*, the monomers are rotationally symmetric in the plane of the page about a point at the geometric center of the dimer). Four of the five domains of one subunit (monomer) are colored for identification (the N-terminal signal peptide domain is not visible): β-sandwich domain, green; central core domain, black (for the right FXIIIA monomer; depicted in gray for the left FXIIIA monomer for contrast as the dimer interface is chiefly comprised of the two central core domains); β-barrel 1 domain, red; β-barrel 2 domain, blue. Residues labeled on the left side are the wild-type amino acids at the positions of the mutations and are depicted as CPK space-filling spheres representing the van der Waals' surfaces of the side chains. The spheres are also differently colored for different residues. Due to PDB file constraints the Gly592 residue is shown as a yellow ribbon and not as a sphere. The catalytically active Cys314 is labeled on the right side. The other catalytic residues (His373 and Asp396) are not shown for visual simplification.

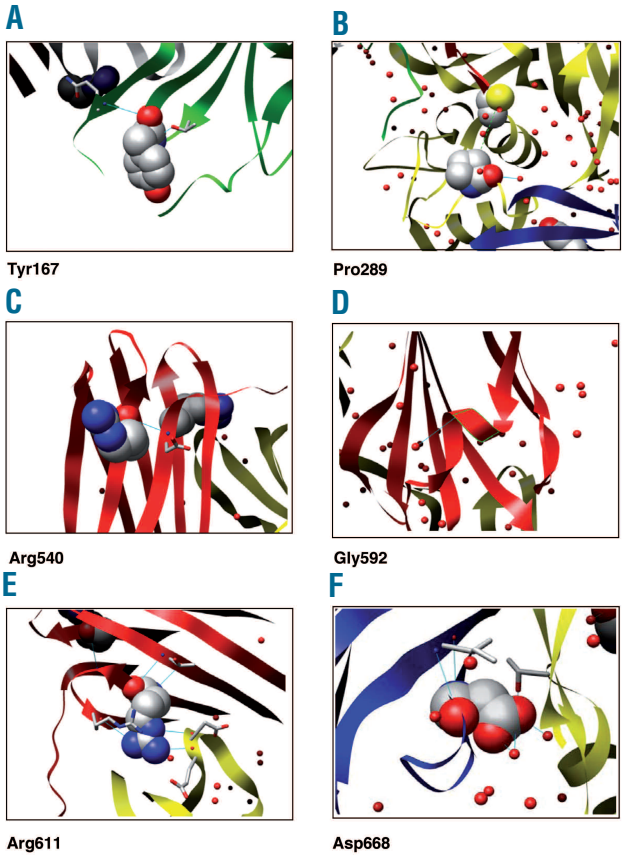

Figure 2. Close-up views of the local molecular environments of six wild-type residues at the positions of the human FXIIIA mutations. (Refer to Figure 1 for orientation of mutated residue positions with respect to the entire FXIIIA monomer.) (A) Tyr167 shown with hydrogen bonds (thin blue lines) to nearby β-staves (dark green ribbons, arrowheads point in the N-terminal to C-terminal direction of the primary protein sequence. The green loop at the bottom left relative to Tyr167 is broken due to unresolved residues in the X-ray crystallographic data. (B) Pro289 (lower CPK spheres with red (oxygen) and blue (nitrogen) peptide backbone atoms shown) lies in a large loop of the catalytic core domain (yellow backbone ribbons) and at a distance of ~9 Å (green dashed line) from the catalytic residue Cys314 (CPK spheres with sulfer atom colored yellow). (C) Arg540 (left cluster of CPK spheres in the foreground) shows hydrogen bonds and the adjacent β-staff in the red β-barrel 1 domain. The two blue-colored nitrogens of the guanidinium group are foremost visible and localize a unit positive charge on the solvent-accessible surface of FXIIIA. (Arg611 appears as another cluster of CPK spheres to the right of Arg540 and behind two nearby β-staves.) (D) Gly592 is depicted as a green-outlined section of the α-helical turn of red ribbon located at the center of the panel. No CPK spheres are shown because the side-chain of glycine is a single hydrogen atom (not resolved in the 2.1 Å X-ray crystallographic data). (E) Arg611 (CPK spheres) lies on the opposite side of the β-barrel 1 domain from Arg540 and is similarly solvent-exposed on the protein surface. However, it forms multiple hydrogen bonds with two adjacent β-staves of the β-barrel 1 domain (red) as well as with an exposed β-staff (yellow) of the adjacent core catalytic domain β-lever. (F) Asp668 (CPK spheres) shares hydrogen bonds with nearby residues in the β-barrel 2 domain (blue) as well as with an adjacent threonine side-chain oxygen of the core catalytic domain (yellow).

Missense mutations in the N-terminal β-sandwich domain (Tyr167Cys)

Tyr167 is 100% conserved among 11 mammalian species (Figure 3). However, it is not conserved among the other (TGZ, TGX, B4.2, TGE, TGC, TGK, TGP) transglutaminases.[14] Tyr167 is located in a short, two-amino acid loop connecting two anti-parallel β-strands at the distal end of the N-terminal immunoglobulin-like β-sandwich domain (Figure 2A). A somewhat larger loop connecting two additional β-strands of the same domain wraps around Tyr167, but the X-ray data do not resolve two residues, Arg37 and Gly38, which likely make van der Waals' contacts with it. The planar side chain ring of Tyr167 is positioned to possibly make hydrophobic contact with and stabilize the peptide backbones of Arg37 and Gly38. Thus, the Tyr167Cys mutation would most likely cause disruption of the local neighborhood structure including the small loop in which the mutation occurs as well as the larger loop that surrounds the mutated residue. It is important to note that Arg37-Gly38 is the cleavage site for thrombin in the FXIII A subunit and the Tyr167Cys mutation may, therefore, have an impact on the cleavage of thrombin. The Tyr167Cys mutation might also expose a reactive cysteine thiol at the distal end of the N-terminal β-sandwich domain which would provide the distinct possibility of forming covalent disulfide cross-links with other cysteine-containing proteins, possibly resulting in protein aggregation or other generally pathological effects.

Missense mutations in the transglutaminase core domain (Pro289Arg)

Pro289 is situated in the largest extended loop of FXIIIA, joining two α-helices of a linear sequence of four consecutive α-helices in the FXIIIA transglutaminase core domain approximately 9 Å from the catalytic core Cys314 residue (Figure 2B). Mutation of Pro289 to Arg represents a non-conservative amino acid substitution that would result in steric implications due to the much larger side chain and introduction of a formal positive charge in the local protein environment. The heterozygous patient's reduction of more than 50% of FXIII activity suggests that the phenotype of the Pro289Arg mutant protein might be non-functional, and that the wild-type allele contributes entirely to the observed FXIII activity. Additionally, the 100% conservation of the Pro289 residue among 11 mammalians (Figure 3) and high (85%) conservation among other transglutaminases points to the importance of this residue to FXIIIA structure and function.[14]

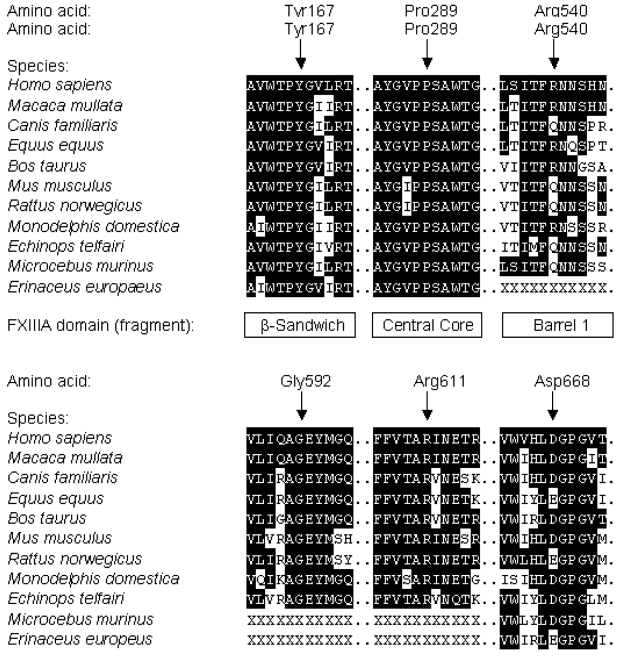

Figure 3. Multiple sequence alignment of *F13A* orthologues. Conserved residues are shown on the black background.

Missense mutations in the first β-barrel domain (Arg540Gln, Gly592Ser, and Arg611His)

Arg540 is situated on the solvent accessible distal surface of the FXIIIA second β-barrel domain and forms one

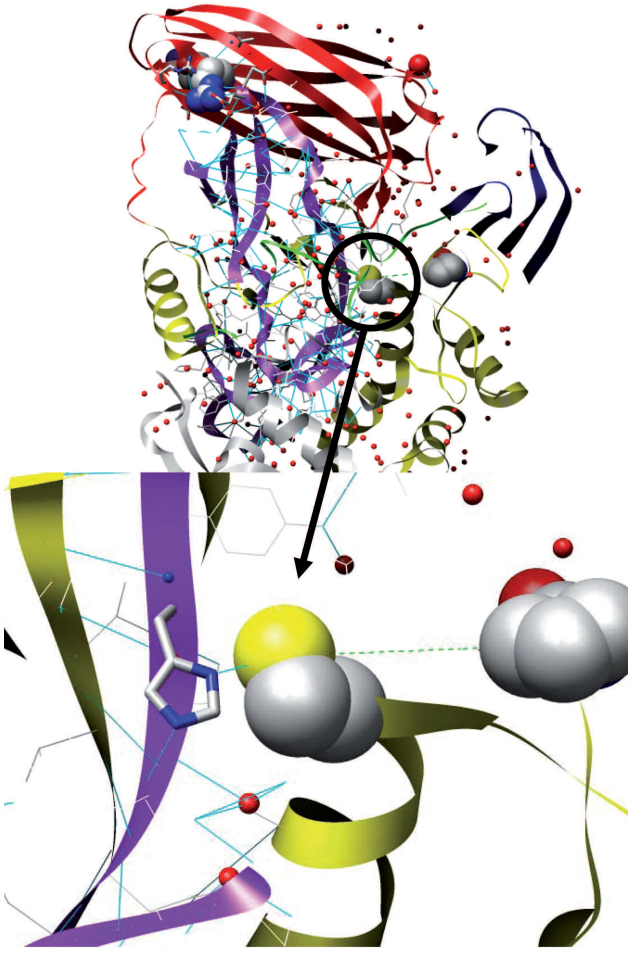

Figure 4. Views of the core catalytic domain β-lever, its extensive H-bond network and functional coupling to the active site catalytic residue Cys314. Upper panel: view of one FXIIIA monomer showing relative orientations of the core catalytic domain (yellow) surrounded by the β-barrel 1 (red) and β-barrel 2 (blue) domains; the β-sandwich domain is not shown for visual simplification. The "β-lever" is depicted in purple (109 residues in length) along with the extensive H-bond network stabilizing its structure and coupling it to the nearby active site Cys314 (shown as CPK spheres in the black circle). Lower panel: detailed view of the H-bond formed between His373 (pentagonal stick representation attached to the purple β-lever) and catalytically active Cys314 (central CPK spheres with sulfur colored yellow). Pro289 appears ~9 Å to the right of Cys213 (distance indicated by a green dashed line).

H-bond between its peptide backbone and the backbone of Asp519 on the adjacent β-sheet (Figure 2C). Although the mutation of Arg540 to Gln eliminates one positive formal charge from the protein surface, the heterozygous patient's measured FXIII activity of between 50% and 61% is consistent with only minimal residual activity contributed by the mutant allele. This suggests that the Arg540Gln mutation does not result in production of completely inactive protein, it is likely that overall function of the catalytic domain is not compromised, but that other factors are responsible for the reduced FXIII activity measured in this patient. Interestingly, Arg540 is not conserved among the other transglutaminases and five of the ten aligned FXIIIA mammalian orthologs have a Gln residue at this position indicating that this variant might be an infrequently occurring natural polymorphism. Previously, a missense mutation was described at the neighboring amino acid residue 541 (Asn541Lys) which was assumed to cause incorrect folding leading to FXIII deficiency.[15]

The Gly592Ser mutation was found in three unrelated females of German nationality. Two of these patients (patients E and G) showed reduced FXIII activity (60-61% of normal), while the third, patient F, had "borderline" reduced FXIII activity (71%). Gly592 lies in a small one-and-a-half turn α-helix situated on the solvent accessible surface of the FXIIIA first β-barrel domain. The Gly592 peptide backbone forms an H-bond with a nearby crystallographically defined water molecule, but does not form any helix-stabilizing H-bonds with neighboring residues (Figure 2D). The three heterozygous patients bearing the Gly592Ser mutation have residual FXIII activity of 60-71% suggesting some contribution from the mutant allele that might not directly affect the catalytic activity of the core transglutaminase domain. The peripheral location of this mutation on the surface of FXIIIA, similar to the surface location of the Tyr167Cys and Arg540Gln mutations, might contribute to reduced pathophysiological function. The Gly592 residue is 100% conserved among nine of the aligned mammalian species (Figure 3), although no conservation was recognized among seven more distantly related transglutaminases.[16] Interestingly, screening of 200 German blood donors revealed two individuals carrying the heterozygous (one affected allele) Ser592 variant, suggesting the possibility of a rare polymorphism at this position.

Arg611 is located in the FXIIIA first β-barrel domain at the interface shared by that domain and the transglutaminase core catalytic domain (Figure 2E). In addition to two H-bonds shared with Thr550 and an H-bond shared with Arg616 on adjacent β-strands of the first β-barrel domain, Arg611 shares two further H-bonds, one each with Glu356 and Asp357 located on an adjacent β-strand that belongs to the transglutaminase core domain and extends directly into the core catalytic reaction site. In fact, this β-strand extends uninterrupted along a continuous segment of 109 contiguous amino acid residues. We have named this long β-strand segment the "β-lever" as it forms a rigid-body extending from the surface interface between the transglutaminase core and first β-barrel domains near Arg661 to the catalytic core active site Cys314 (Figure 4). The β-lever is structurally stabilized along its 109-amino acid residue length by 83 intra-strand H-bonds and coupled to the surrounding protein by an additional 190 H-bonds. Astonishingly, His373, located on the β-lever strand deep in the core domain, shares an H-bond with the catalytic site Cys314. Of the five H-bonds involving Arg661, according to the FXIIIA X-ray structural data, the substitution of His for Arg661 would probably not affect the two peptide backbone-stabilized H-bonds shared between Arg661 and Thr550, but would certainly eliminate two of the three remaining H-bonds formed with Glu356 and Asp357, likely resulting in destabilization of the rigid structural coupling, mediated by Arg661, between the first β-barrel and glutaminase core domains. Accordingly, the Arg611His mutation might cause a structural perturbation of Cys314 in the catalytic site a great distance away, mediated by a direct physical coupling of both residues to the rigid, H-bond stabilized β-lever. Interestingly, the clinical data for the three patients found to have a heterozygous Arg611His mutation, resulting in only about half of normal FXIII activity (range, 43%-49%), suggest that the FXIIIA protein produced by the mutant allele may be enzymatically inactive. Astonishingly, the Arg611His mutation represents the only FXIIIA surface residue alteration among all of the mutations reported to date that apparently causes a complete loss of catalytic activity by acting at a great distance from the catalytic site through the intricately H-bond stabilized β-lever motif of the glutaminase core domain. To date, no homozygous (His/His611) individual has been reported.

Missense mutations in the second β-barrel domain (Asp668Gly)

Asp668 is situated in a three-staff β-pleated sheet belonging to the second β-barrel domain that forms an interface with the transglutaminase core domain (Figure 2F). This residue shares seven H-bonds with neighboring residues including two peptide backbone H-bonds with Ile706 and an H-bond with Gly669 within the second β-barrel domain. Three further H-bonds are shared with two nearby water molecules. One H-bond is shared between the Asp668 backbone and the Thr293 side chain oxygen situated in a loop region belonging to the transglutaminase core domain. Interestingly, Pro289 also lies in the same loop and, thus, the loop is coupled to the catalytic Cys314 through a local H-bond network. The Asp668Gly mutation would probably not affect the H-bonds shared with Ile706 in the second β-barrel domain. However, no side chain-mediated H-bonds can be formed by the mutant Gly side chain comprising a single hydrogen atom. Accordingly, there would be no H-bonds with the two nearby water molecules, as well as no H-bond shared with either Gly669 or Thr293 of the neighboring transglutaminase core domain. The FXIII activity data available from one of the two heterozygous patients with Asp668Gly mutation (patient L; FXIII activity was not determined for patient M), exhibiting only 23%-40% activity with respect to that of homozygous wild-type control patients, suggest that the mutant protein results in a dominant-negative effect on functional FXIIIA produced by the wild-type allele. The low 10%-27% residual FXIII activity reflects a greater decrease of FXIIIA function than would be expected from a single mutant allele that results in only loss of function for its protein product. One mechanism that would explain the lower than expected FXIII activity for the heterozygous Asp668Gly mutation is a direct influence of the mutant protein on the overall function of the FXIIIA dimer within the FXIII A2B2 protein complex. Two other missense mutations (Leu660Pro, Leu667Pro) located

in the same barrel 2 domain region have been previously predicted to cause aberrant folding of the protein and possibly rapid degradation following translation.[16]

Relevance of heterozygous mutations on phenotype

Examining the clinical data of the 12 heterozygous patients we recognized bleeding episodes in seven (58%) cases. Most of the hemorrhages (6/7) had occurred following surgery, dental extraction or trauma (Table 1). Our data indicate that patients with heterozygous deficiency might be at risk of bleeding upon provocation (e.g. surgery, trauma). Pre-operative screening of FXIII activity in patients with a bleeding history would show whether strategies are needed to prevent peri- and post-operative bleeding complications in those patients.

Conclusions

In conclusion, in the present study we identified eight novel *F13A* gene point mutations, of which six were unique missense mutations and two were nonsense mutations. On the evidence of *in silico* structural analysis, all of the missense substitutions seem to have a significant impact on the protein structure. However, three variants (Gly592Ser, Tyr167Cys, Arg540Gln) could, possibly, be non-synonymous, rare gene polymorphisms. Given the absence of homozygous individuals for these variants, *in vitro* expression analysis as well as screening of a larger population of healthy controls are required for further elucidation of the molecular mechanisms of these mutations. Our data also indicate that the frequency of subjects with heterozygous *F13A* gene mutations and only mildly reduced FXIII activity in the normal population may be higher than currently believed.

Authorship and Disclosures

VI, AB, and JO were the principal investigators and take primary responsibility for the paper. HR, SH, PEP, HL, MK, BM, and UH collected the clinical data and blood samples. AB and CB provided the structural analysis. VI, AB and JO analyzed the data and wrote the paper. VS, HPK provided coagulation assays. VI coordinated the research.

The authors reported no potential conflicts of interest.

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