Phenotype and genotype report on homozygous and heterozygous patients with congenital factor X deficiency

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ABSTRACT

Factor X deficiency is a severe rare hemorrhagic condition inherited as an autosomal recessive trait. It is one of the most severe recessive inherited coagulation disorders. We analyzed the clinical manifestations, laboratory phenotype and genotype in 10 patients with severe Factor X deficiency and in their heterozygous relatives. The most frequent bleeding episodes were hematomas (70%) and gum bleeding (60%). Fifty percent of the homozygous patients required blood transfusion and one-third of heterozygotes required treatment after surgery or delivery. The genetic characterization revealed six different missense mutations, two of which were novel: p.Glu69Lys and p.Asp103His. Haplotype analysis, performed with intra- and extra- *FX* gene polymorphic markers in Indian, Iranian and Italian patients with the same mutations failed to establish identity by descent, despite the same Caucasian origin. In conclusion, factor X deficiency was confirmed to be one of the most serious among rare bleeding disorders and genetically heterogeneous in different populations.

Key words: bleeding disorders, bleeding symptoms, factor X deficiency, *F10*, phenotype-genotype.

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Introduction

Factor X (FX), a vitamin K dependent plasma glycoprotein, plays a pivotal role in the coagulation cascade being the first enzyme in the common pathway of thrombin formation. FX is synthesized by the liver and circulates in plasma at a concentration of 8-10 μg as a two-chain protein with a 17-kDa light chain linked to a 45-kDa heavy chain.[1] The light chain contains a γ-carboxyglutamic acid (Gla)-domain necessary for a Ca2+-dependent conformational change associated with phospholipid binding, and two epidermal growth factor (EGF) domains. The heavy chain contains the catalytic serine protease domain, structurally homologous to that of other coagulation serine proteases.[2] FX is activated (FXa) by both factor VIIa/tissue factor and factor VIIIa/factor IXa. In turn, FXa, which forms the prothrombinase complex together with factor Va, catalyses thrombin formation.[3] The F10 gene (*F10*), located on chromosome 13q34, spans 27kb and contains eight exons, each of which encodes a specific protein domain.[3,4] To date, approximately 95 variants, comprising deletions, missense, frame shift and splice site mutations have been reported in *F10*.[5,6] FX deficiency is a rare hemorrhagic disorder, inherited as an autosomal recessive trait with a reported incidence of approximately 1:10[6] in the general population. It is more common in populations with a high rate of consanguineous marriages, with an 8 to 10 fold increase in frequency.[7] The disorder is diagnosed by a concomitant prolongation of the prothrombin time (PT) and activated partial thromboplastin time (APTT), and by the low level of FX activity. Its clinical presentation makes it among the most severe of rare coagulation defects, and typically includes hemarthroses, muscle hematomas, umbilical cord bleeding, gastrointestinal and central nervous system (CNS) bleeding.[6,8] In this study we evaluated the severity of bleeding symptoms and the relationship between genotype and laboratory phenotype in 10 patients from 7 unrelated Iranian families not included in previous studies. Mutation analysis identified six missense mutations: four previously described and two novel, one located in the (Gla)-domain and one in the EGF1 domain. A haplotype analysis was also

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performed in 14 patients with severe FX deficiency (7 from this study and 7 from previous studies, *www.rbdd.org*), carrying the same genetic alterations in order to verify whether or not a founder effect was present.

Design and Methods

Patients

Ten patients with severe FX deficiency from 7 unrelated families (Table 1) including 4 males (age range: 3-20 years, mean: 14) and 6 females (age range: 14-26 years, mean: 21) were investigated. Nine patients were the off-spring of first cousins, while one was from a marriage between second cousins (family G). To establish the type and severity of bleeding symptoms from each patient a specially tailored questionnaire was used. All patients and their family members were diagnosed at the Hematology and Thrombosis Unit, Hematology Research Center in Shiraz, Iran, by confirmation of prolonged PT, APTT and low FX coagulant activity. Frozen blood and plasma samples from these patients were then dispatched with dry ice to the Hemophilia and Thrombosis Center of Milan, Italy, where the FX antigen level and coagulation activity were measured using an in-house enzyme immunoassay (ELISA) and (APTT), respectively, as previously reported.[9] This study was approved by the Ethical Review Board of the IRCCS Maggiore Hospital, Mangiagalli and Regina Elena Foundation, Milan, Italy. Informed consent for the study was obtained from all patients.

Mutation analysis

Following DNA extraction,[10] the coding region, intron/exon boundaries and 5' and 3' untranslated regions of the *F10* were amplified by polymerase chain reaction (PCR).[9] *F10* mutations were analyzed by direct sequencing using an ABI Prism310 automated sequencer (PE Applied Biosystems, Milan, Italy). The identified mutations were confirmed by repeating the sequence and by restriction analysis. To confirm that novel genetic variants were not frequent polymorphisms, they were investigated in 120 alleles from an Iranian control population. Mutations were named in accordance with the standard international nomenclature guidelines recommended by the Human Genome Variation Society (HGVS at *http://www.hgvs.org/mutnomen/recs.html*), with nucleotide +1 as the A of the ATG translation initiation codon. The genomic (GenBank accession n. 12738260) and cDNA (GenBank accession n.M57285) sequences of *F10* were used as the reference sequences.

Haplotype analysis

Haplotype analysis was carried out on 14 patients with severe FX deficiency, with the same *F10* mutations. Seven were from this study and 7 had been previously genotyped by our group (*www.rbdd.org*). The extra and the intra-*F10* polymorphisms used in this study, and their associated symbols are shown in Table 2. The three extra-genic polymorphisms were on the *FVII* gene (*F7*), located 2.8 Kb upstream of the *F10*.[14]

Table 1. Bleeding symptoms and phenotype/genotype in the seven unrelated families with severe factor X deficiency.

Families and mutations	*Symptoms*	*FX:Ag (%)*	*FX:C (%)*	*Genotype*
Family A: c.61G>A, p.Gly21Arg (previously reported as Gly-20Arg)				
R1 proband	Gum bleeding, hemarthroses, hematomas	4	<1	homozygous
R2 sister	Gum bleeding, hemarthroses, hematomas	5	<1	homozygous
R3 brother	Gum bleeding, epistaxis, post-trauma hemarthroses and hematomas, post dental extraction and post circumcision bleeding	43	23	heterozygous
R4 brother	Epistaxis, hematuria, hemarthroses, post-dental extraction and post-circumcision bleeding	2	<1	homozygous
R27 mother	Post-dental extraction bleeding	44	46	heterozygous
R28 father	Asymptomatic	48	64	heterozygous
Family B: c.61G>A, p.Gly21Arg (previously reported as Gly-20Arg)				
R149 proband	Gum bleeding, epistaxis, ecchymoses, menorrhagia	<1	<1	homozygous
R150 mother	Asymptomatic	36	42	heterozygous
Family C: c.205G>A, p.Glu69Lys				
R5 proband	Gum bleeding, hemarthroses, hematomas, epistaxis, CNS bleeding, umbilical cord bleeding	19	<1	homozygous
R29 father	Asymptomatic	52	65	heterozygous
R30 mother	Asymptomatic	52	54	heterozygous
Family D: c.307G>C, p.Asp103His				
R432 proband	Gum bleeding, hematomas, not available menorrhagia, post-delivery bleeding		<1	homozygous
R433 father	Asymptomatic	not available	44	heterozygous
R434 mother	Prolonged post-delivery bleeding	not available	46	heterozygous
Family E: c.400G>A, p.Gly134Arg (previously reported as Gly94Arg)				
R651 proband	Post-circumcision bleeding, hematomas, post-dental extraction bleeding	3	<1	homozygous
R652 father	Asymptomatic	38	40	heterozygous
R653 mother	Asymptomatic	40	45	heterozygous
Family F: c.730G>A, p.Gly244Arg (previously reported as Gly204Arg)				
R348 proband	Post-circumcision bleeding, hemarthroses	<1	<1	homozygous
R349 mother	Prolonged post-delivery bleeding	47	50	heterozygous
R350 father	Asymptomatic	42	45	heterozygous
Family G: c1262G>A, p.Gly 421Asp (previously reported as Gly381Asp)				
R62 proband	Hematomas, hematuria	18	<1	homozygous
R64 sister	Gum bleeding, epistaxis, menorrhagia	29	<1	homozygous
R65 sister	Asymptomatic	37	39	heterozygous
R63 mother	Asymptomatic	44	50	heterozygous

Results and Discussion

Clinical manifestations

The clinical symptoms and the results of the laboratory phenotype and genotype characterization are shown in Table 1. Five patients out of 10 were diagnosed because of bleeding episodes (R1, R149, R5, R348 and R64), and the other 5 were diagnosed on routine examination. Each of the homozygous patients has had history of at least one hospital admission due to a severe bleeding and half of them required blood transfusion (R5, R149, R348, R432 and R651). The most frequent symptoms among homozygotes were hematomas (present in 70%), gum bleeding (60%) and hemarthroses (50%). Only one homozygote has had CNS and umbilical cord bleedings, confirming previously reported data.[16,17] As regards heterozygotes, 4 out of 14 (29%) (R3, R27, R434 and R349) who have had dental extraction, surgery or delivery without prophylactic replacement therapy showed post-operative bleeding which required treatment with fresh-frozen plasma (FFP). R3, with mild FX deficiency (FX:C 23%), has had post-traumatic hemarthroses and hematomas, post-dental extraction and post-circumcision bleeding, spontaneous epistaxis and gum bleeding. In order to rule out the presence of other coagulation defects, further coagulation assays were performed, but no additional defects were identified (*data not shown*). However, this subject also developed a mental retardation following encephalitis which probably led to clumsiness and post-traumatic bleedings. His mother (R27) also had post-dental extraction bleeding with 46% of FX:C. The other two heterozygotes subjects (R434 and R349) had prolonged post-delivery bleedings and have been treated with FFP.

Phenotype and genotype analysis

Six different homozygous candidate mutations were identified, two of which were novel. The first mutation was p.Glu69Lys, consisting of a G to A transition (c.205G>A) in exon 2 encoding the Gla-domain, identified in patient R5, an 11-year old boy. The vitamin K-dependent γ-glutamyl carboxylase catalyses the post-translational modification of specific glutamic acid residues to γ-carboxyglutamic acid (Gla) residues in the Gla-domain.[18] The mutation at codon 69 affects one of the glutamic acid residues. Two previously reported mutations on *F7* and *FIX* gene (*F9*), the p.Glu69Lys in FVII and the p.Glu70Lys in FIX caused severe FVII deficiency and severe hemophilia B, respectively.[19,20] These two mutations were originally reported as Glu29Lys in FVII and Glu30Lys in F9. The second novel mutation was p.Asp103His, identified in patient R432, a 19 year old woman, consisting of a G to C transversion (c.307G>C) in exon 4 encoding the EGF1 domain. Correct post-translational modifications, such as γ-carboxylation, glycosylation and β-hydroxylation, are essential for the FX protein to be fully functional, because they may alter physical and chemical properties, folding and thus protein stability. The β-hydroxylation site of FX is located in the first EGF domain at Asp103 residue, resulting in a β-hydroxyaspartic acid. The replacement of a histidine at this residue will probably result in the loss of FX function by affecting β-hydroxylation and thereby causing a severe FX deficiency. The remaining four mutations had been previously reported and were all identified in the homozygous state. The c.61G>A, p.Gly21Arg mutation, previously reported as Gly-20Arg in a patient from Santo Domingo,[21] was found in patients R1, two of his siblings (R2 and R4) and in patient R149 (families A and B respectively) with undetectable FX:C. This severe FX deficiency is probably due to the inability of signal peptidase to cleave the mutated FX, therefore being directly responsible for the severe clinical phenotype.[22] The c.400G>A, p.Gly134Arg mutation, identified in patient R651 (family E), was previously reported as Gly94Arg in association with Asp95Glu, in two homozygous siblings with FX:C <1 and FX:Ag level of 3-4%,[9] similar to patient R651 (this study). The c.730G>A, p.Gly244Arg mutation was found in patient R348 (family F) who had post-circumcision bleeding at the age of 40 days. The same mutation was first identified by Bereczky *et al.*[23] (reported as Gly204Arg) in a 1-year old boy affected by severe FX deficiency, whose ethnic group was not given, and also by Peyvandi *et al.* in a 17-year old Iranian woman with severe bleeding and FX:C <1%.[9] The last mutation, c1262G>A, p.Gly421Asp, identified in patient R62 (family G), was previously reported as Gly381Asp[24] in 3 members of an Omani family with severe FX deficiency, similar to our patient and his sibling. The comparison of genotype and laboratory phenotype among our patients and other previously characterized patients carrying the same genetic alterations, confirmed that each different mutation was associated with similar laboratory phenotypes. Some differences were found only for mutation p.Gly21Arg, identified in two families from this study and in a patient from Santo Domingo previously reported by

Table 2. List of the extra- and intra-*F10* genetic markers and their associated symbols.

Gene region	*Polymorphism*	*Symbols*		*References*
F7 promoter	g.-323_-324insCCTATATCCT*	del	A1	11
		ins	A2	
F7 promoter	g.-122C>T	T	B1	12
		C	B2	
F7 exon 8	g.10976C>A, p.Arg353Gln*	G	M1	13
		A	M2	
F10 promoter	g.-343_-348del TTGTGA	ins	C1	14,15
		del	C2	
F10 promoter	g.-222C>T	C	D1	14,15
		T	D2	
F10 promoter	g.-220C>A	C	E1	14,15
		A	E2	
F10 exon 7	c.792C>T, p.=	T	F1	4
		C	F2	

**Agreed symbols as indicated by FVII Mutation Database site (http://193.60.222.13/polymorphisms.htm) maintained by Hemostasis Research Group based at the MRC Clinical Sciences Center.*

Table 3. Results of the haplotype analysis.

F10 mutation	*Subjects*	*Report*	*Origin*	*Haplotypes*						
				F7				*F10*		
				g.-323_-324ins CCTATATCCT	*g.-122C>T*	*g.10976C>A, p.Arg353Gln*	*g.-343_-348del TTGTGA*	*g.-222C>T*	*g.-220C>A*	*c.792C>T, p.=*
	R1			A1A1	B1B1	M1M1	C1C1	D1D1	E2E2	F1F1
c.61G>A,	R2	This study: family A	Iran	A1A1	B1B1	M1M1	C1C1	D1D1	E2E2	F1F1
p.Gly21Arg	R4			A1A1	B1B1	M1M1	C1C1	D1D1	E2E2	F1F1
	R17	www.rbdd.org	India	A1A1	B1B1	M1M1	C2C2	D1D1	E1E1	F2F2
	R149	This study: family B	Iran	A1A1	B1B1	M1M1	C1C1	D1D1	E2E2	F1F1
	R5	This study: family C	Iran	A1A1	B1B1	M1M1	C2C2	D2D2	E1E1	F1F1
c.205G>A,	R201			A1A2*	B1B2*	M2M2	C1C1	D1D1	E1E1	F1F1
p.Glu69Lys	R202	www.rbdd.org	Italy	A1A2*	B1B2*	M2M2	C1C1	D1D1	E1E1	F1F1
	R203			A1A2*	B1B2*	M2M2	C1C1	D1D1	E1E1	F1F1
	R651	This study: family E	Iran	A1A1	B1B1	M1M1	C2C2	D2D2	E1E1	F1F1
c.400G>A,	R209	www.rbdd.org	Iran	A1A1	B1B1	M1M1	C2C2	D2D2	E1E1	F1F1
p.Gly134Arg	R210			A1A1	B1B1	M1M1	C2C2	D2D2	E1E1	F1F1
c.730G>A,	R348	This study: family F	Iran	A2A2	B2B2	M2M2	C1C1	D1D1	E1E1	F1F1
p.Gly244Arg	R171	www.rbdd.org	Iran	A1A2	B1B2	M2M2	C1C1	D1D1	E1E1	F1F1

**Indicates the A2 and B2 maternal alleles that cosegregates with p.Glu69Lys mutation. Graphs indicate members of the same family which need to be visualized.*

Watzke *et al.*[21] All patients from this study, as well as the patient reported by Watzke, had undetectable FX:C with minimum conserved FX:Ag levels (2-5%), with the exception of patient R149 with both FX:C and FX:Ag level <1%. In the latter case the discrepancy might be due to the modifying effect of F10 polymorphisms, even though the analysis of three extra- and four intra- genic polymorphic markers (Table 2) revealed a similar pattern in all the Iranian patients (R1, R2, R4 and R149) carrying this mutation. Phenotype analysis in families C and G showed that they were affected by a type II FX deficiency. This means that the mutations p.Glu69Lys and p.Gly421Asp partially affect the secretion pathway of FX, and mainly the FXa activity in the prothrombinase complex, resulting in a severe hemorrhagic phenotype.

Haplotype analysis

Data obtained from the analysis of three extra- and four intra-*F10* markers in 14 homozygous or compound heterozygous patients are shown in Table 3. Mutation p.Gly21Arg, present in three families (two from Iran and one from India) was associated with two different haplotypes: i) A1, B1, M1, *C1*, D1, *E2, F1* and ii) A1, B1, M1, *C2*, D1, *E1, F2* according to the different geographical origin. Furthermore, the patient from India was the only one carrying the F2 allele for the *F10* polymorphism located in exon 7. The mutation p.Glu69Lys, identified in homozygous state in patients R5 and in heterozygous state in previously characterized Italian patients (R201, R202 and R203), was associated with two different haplotypes: i) *A1, B1, M1, C2, D2,* E1, F1 in the Iranian family and ii) *A2, B2, M2, C1, D1,* E1, F1 in the Italian family (*unpublished*). The latter haplotype co-segregated with the p.Glu69Lys mutation identified in the Italian probands, and inherited from the maternal side. Mutation p.Gly134Arg, identified in two families coming from southern Iran, was associated with the same haplotype A1, B1, M1, C2, D2, E1, F1. Mutation p.Gly244Arg was identified in two different Iranian probands: R348 from southern Iran, investigated in this study, and R171, from northern Iran, who had been previously investigated (*www.rbdd.org*). In patient R348, the p.Gly244Arg mutation was associated with haplotype A2, B2, M2, C1, D1, E1, F1. By contrast, patient R171, as well as his parents, resulted heterozygotes for the polymorphisms located on the promoter region of *F7* (A and B). Therefore, in this patient the haplotype analysis was not informative. The haplotype analysis showed that patients coming from different geographical areas carrying the same gene mutations had different haplotypes. However, also two patients coming from the same geographical area (R171 and R348, one from northern and one from southern of Iran) carrying the same p.Gly244Arg mutation had different haplotypes. Since one of the consequences of a founder effect is the loss of an allele in a small sample of individuals taken from a larger population, and since this haplotype analysis was carried out in only two families, we could not completely exclude or confirm a founder effect. The haplotype analysis carried out on another two unrelated Iranian kindreds from southern Iran, both with the p.Gly134Arg mutation, is consistent with a founder effect. In conclusion, haplotype analysis in families with severe FX deficiency, carrying the same FX gene mutations, did not confirm identity by descent in Iranian, Indian and Italian patients, even though these populations share a common Caucasian origin.

Authorship and Disclosures

MK collected clinical data, analyzed data and wrote the paper, MM performed genotype, haplotype and new mutations analysis, analyzed data and wrote the paper, AA performed genotype analysis, SS performed the phenotypic characterization, FP designed the research and revised the manuscript critically. The authors reported no potential conflicts of interest

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