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Identification of two Novel Mutations in the Factor X Gene; A 5' Donor Splice-Site Mutation (IVS1+1G>T) and a Missense Mutation (Asp413Asn G>T) in Unrelated Palestinian Factor X Deficient Patients

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Abstract

Factor X deficiency is a rare autosomal disease with an estimated prevalence 1: 1,000,000. It is characterized by a reduction in factor X, an essential component of the prothrombinase complex responsible for converting prothrombin to thrombin. The aim of the study was to identify the molecular defects in the factor X gene in Palestinian factor X deficient patients. Nine unrelated Palestinian patients were identified by thrombin time [PT], activated partial thromboplastin time [APTT] and plasma factor X levels. All exons including exon/ intron border and promoter regions were PCR amplified, purified, sequenced and compared to the normal factor X gene. A novel splicing junction mutation IVS1+1G>T was identified in two patients resulting in a major distortion of the protein structure and function. A second novel missense mutation Asp413Asn G>T that apparently distorts the protein structure and affects its catalytic activity was identified in the family of one patient. Six patients proved to be homozygous of the previously identified c358 delG deletion mutation leading to a severely truncated protein that seems specific to our population. A putatively mild heterozygous mutation Ser105thr G>C was detected in two patients who suffer from the severe c358 delG deletion mutation.

Keywords: Blood coagulation; Factor X gene variants; Inherited coagulation disorder; Nucleotide deletion; Gla protein distortion and activity

Introduction

Factor X is a vitamin K dependent serine protease synthesized in the liver [1]. After activation by factor IXa [intrinsic pathway] or VIIa [extrinsic pathway], factor X becomes a major component of the prothrombinase complex which converts prothrombin into thrombin [2]. Deficiency in factor X is rare in the world affecting approximately one in million people [3]. The complete absence of factor X was shown to be fatal since $FX^{-/-}$ knockout mice failed to survive [3]. It is inherited as an autosomal recessive disorder resulting in low or absence of serum protein concentration and severe bleeding disorder [4]. Factor X deficiency is expressed in two major types where type 1 is characterized by a significant decrease in the level and function of the protein while type II results primarily from dysfunction of the protein. The factor X gene is located on chromosome 13 and consist of 8 exons [1,5]. Over 100 mutations have been reported thus far in the gene where missense mutations represent the majority of cases in addition to deletion/insertion, splice site and non-sense mutations that were also detected in patients from various populations. These include fifteen mutations identified in 13 unrelated Iranian families [6], four in two Chinese families [7,8] and several other mutations were reported in102 subjects from Europe and Latin America [3]. Furthermore, additional mutations in the factor X gene were detected in patients from the USA [9], Saudi Arabia [10], Hungary [11], Germany [12], Japan [13], France [14], Australia [15] and China [16,17]. Interestingly, a patient with severe factor X deficiency was found to suffer from deletion of exon 8 in the factor X gene in addition to deletion of exons 1 and 2 in protein Z gene located downstream from the factor X gene [18]. Six additional novel missense mutations were recently identified in six patients [19] and one additional nonsense novel mutation was identified in 15 factor X deficient patients [20]. Two additional missense mutations were identified in the factor X that result in moderate FX deficiency due to either decreased secretion [21] or reduced activity in the intrinsic pathway of the clotting cascade [22].

Materials and Methods

Seven male and two female patients from nine unrelated families were identified with severe factor X deficiency and history of bruising, painful swelling of joints and high PT and partial thromboplastin time PTT values. Genomic DNA was purified from patients and/or their parents from whole EDTA venous blood samples using MasterPureTM Genomic DNA Purification Kit (Epicenter Technologies, USA). All exons, exon–intron borders and the promoter regions were PCR amplified as described before [6]. Amplified DNA fragments were purified using the Wizard® SVGel and PCR Clean-Up System (Promega) and subjected to direct sequencing using an automated sequencer (ABI PRISM™ Model 310 Version 3.7). DNA sequences were read by Finch TV and compared to the factor X gene sequence stored in the UCSC Genome browser. BLAST search at NCBI and alignment was applied in order to identify homologies between the DNA sequences of the purified PCR products with Homo Sapiens factor X gene stored in the Gene Bank (http://www.ncbi.nlm.nih.gov/BLAST). The effect of the identified mutations on the protein sequence of factor X was predicted by the amino acid

translator program [EX PASY] bioinformatics resource portal translation tool.

Results

Clinical description of patients

All patients, except patient B, suffered from prolonged PT and APTT indicating a defect in the common coagulation pathway that was verified by undetectable levels of plasma factor X. These patients have a history of easy bruising on their limbs due to minor trauma and epistaxis, bleeding from umbilical stump, joint swelling and pain. Most patients result from consanguinity marriages. Patients treatment include fresh frozen plasma once or twice a month due the lack of factor X concentrate in the local hospitals. Patient B was a newborn male patient who was diagnosed with severe factor X deficiency 4 months after birth following a severe brain bleeding condition and died shortly before genomic DNA could be obtained for genetic analysis. Alternatively, blood samples were obtained from his parents to identify the defect in the factor X gene which resulted in their child clinical condition.

Genetic analysis

All 8 exons and exon/intron borders of the factor X gene were amplified and subjected to direct sequencing followed by comparison to the wild type factor X gene sequence. The results from two patients (20 and 22) revealed the presence of a novel homozygous donor splice-site mutation in exon 1/intron 1 border (IVS1+1G>T) as shown in Figure 1A and 1B. All other exons and exon/intron sequences in addition to the promoter region showed no variation from the normal gene sequence. A second novel homozygous missense mutation Asp 413Asn G>A was predicted in the gene of the diseased patient (B), who suffered from major brain bleeding due to severe factor X deficiency, from the DNA sequence of his parents as shown in Figure 1G and 1H. This mutation lies in the catalytic domain of the factor X protein responsible for its activity. Six of the nine patients examined in our study (patients 1,9,14,27,32,X) were found to be homozygous for the c302delG mutation previously described by Livnat et al. [23] in three Palestinian factor X deficient patients as shown in Figure 1E and 1F. This mutation results in an early stop codon leading to a severely truncated protein and seems predominant in the Palestinian population. Furthermore, an apparently mild missense mutation Ser105Thr G>C was detected in exon 6 in patients 14 and 27 as shown in Figure 1C and 1D. Both patients suffer from the c302delG deletion mutation and are heterozygous for this mutation and therefore has no relevance to their clinical symptoms. Table 1 summarizes the list of the identified mutations in the present study including their nature, location, nucleotide change and effect. In addition to the identified mutations, several polymorphisms were detected in the promoter and exons 3,7 and 8 of the factor X gene in seven of examined patients as described in Table 2. The polymorphism in exon 8 in patients 9 and 14 (G>A corresponding to T293T) represents a new polymorphism in the gene while the polymorphisms in the promoter region and exons 3 and 7 were reported before. The sequence of the indicated exons and intron/exon borders from thirty normal individuals with no signs of bleeding disorder did not reveal any of the identified mutations detected in the patients. This provides further confirmation that the identified mutations in the factor X gene form these patients are responsible for the expressed clinical abnormalities and do not represent normal variation in the gene.

Sample	Mutated exons	Mutation Type	Nucleotide change	Codon/aa change
1	4:Homozygous	Frame shift (deletion)	c308 delG	Stop codon
9	4:Homozygous	Frame shift (deletion)	c308 delG	Stop codon
14	4:Homozygous 6:Heterozygous	Frame shift (deletion) missense	c308 delG G→C	Stop codon Ser→Thr
20	1:Homozygous	Base substitution	G→T	5' donor splicing site
22	1:Homozygous	Base substitution	G→T	5' donor splicing site
27	6:Heterozygous 4:Homozygous	Missense Frame shift (deletion)	G→C c 308 delG	Ser→Thr Stop codon
32	4:Homozygous	Frame shift (deletion)	c 308 delG	Stop codon
X	4:Homozygous	Frame shift (deletion)	c 308 delG	Stop codon
B	8:Homozygous	Base substitution	G to A (p.D413N)	Asp to Asn

Table 1: Mutations identified in the Factor X gene.

Samples	Exons	Nucleotide change	SNPs Reference Number(dbSNP build 137)
1	3	T→C (at position 24)	rs2251102
9	8	G→A (at position 55)	T293T -Silence Variant(Synonmous Variant)
14	3	T→C (at position 24)	rs2251102

	8	G→A (at position 55)	T293T -Silence Variant(Synonmous Variant)
20	Promoter 3 7	T→C (at position -139) C→T (at position 139) C→T (at position 139)	 rs563964 rs2251102 rs5960
22	_	_	
27	_	_	
32	7	A→G (at position 170)	rs2031184
X	3 7 7	T→C (at position 24) G→A (at position 55) A→G (at position 192)	 rs2251102 rs5960 rs2031184

Table 2: Identified SNPs in the Factor X Gene.

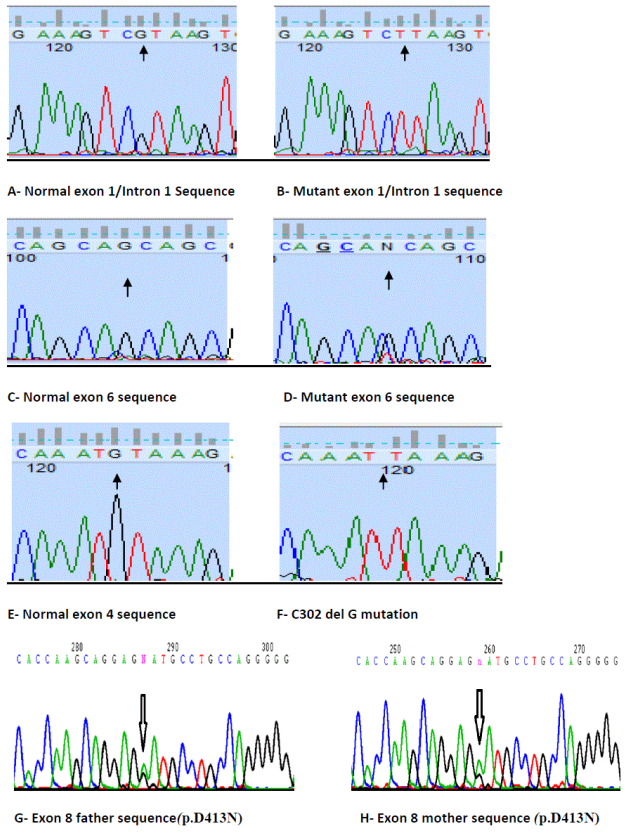

Figure 1: DNA sequences profile of the indicated exons in the factor X gene illustrating the specific identified nucleotide mutations.

Discussion

Factor X plays a central role in the blood coagulation cascade due to its involvement in the intrinsic and extrinsic pathways of this vital process. In the present study, we investigated putative mutations in the factor X gene in nine unrelated Palestinian patients who suffer from bleeding disorder due to severe reduction in plasma factor X levels. Six patients were found to suffer from a deletion mutation that was previously identified in three unrelated Palestinian patients [24] that seems predominant in the Palestinian population since it was not detected in patients from other ethnic groups. A novel mutation which disturbs the 5' donor splice site in intron 1(IVS1+1G>T) was detected. Evidently, the consensus sequence at the 5' splicing donor site is (C/A)AGgt(ag)agt (exonic nucleotides are represented in capital while intronic nucleotides are represented by lowercase), however, in case of the factor X gene, the sequences of all splice sites are predominantly GT-AT which is most dominant in the mammalian genome [25,26]. Mutations in the 5' donor splice site are predominant over mutations in the 3'acceptor site mostly affecting the G nucleotide at the +1 location [26]. Consequently, mutations in the conserved G nucleotide is expected to cause exon loss, inclusion of the intron sequence in the translation sequence or identification of an alternative splice site [24] which will significantly affect the normal expression and function of the protein. A related mutation (IVS1+1G>A) was previously identified in the factor X gene leading to the absence of any detected transcript which was confirmed by in vitro ectopic transcript analysis [11]. A similar effect is projected here form the G>A mutation reported here in the same location in the gene. Another related mutation (IVS1+3A>T) was previously identified in the factor X gene resulting in a significant decrease in the plasma protein level [6]. The donor splice-site sequence mutation primarily results in inclusion of intron 1 in the coding sequence leading to the identification of an early stop codon leading to the production of a severely truncated protein or unstable mRNA that is unable to code for a biologically active factor X protein. This is confirmed by the absence of detectable plasma factor X in the plasma of our study patients. Evidently, similar mutations were reported in the donor exon border splicing sequences of several genes which result in significant disturbances in the expression and function of the coded proteins. They include a G>A mutation in intron 7 of the human Rh50 glycoprotein gene [27], a G>A mutation in intron 2 in the CYP21 gene [28], a G>C mutation in intron 1 of the peripherin/rds gene [29], a cryptic donor splice site in intron 19 of the thyroglobulin gene [30], a C>T mutation and A>G mutation in intron 26 of the Von Willebrand factor gene [31] and a G>A mutation in intron 25 of the angiotensin 1-converting enzyme gene [32]. Moreover, another comparable mutation in the factor X gene (IVS5+1G>A) was reported in a patient who suffer from compound heterozygous mutations along with a deletion mutation (Asp409del) and characterized by the absence of detectable transcript that was further confirmed by ectopic

transcript analysis [17]. The authors projected a likely mechanism of the observed clinical expression where the mutation potentially resulted in the production of an unstable transcript that was quickly destroyed by the NMD pathway [33]. The absence of detectable factor X in the plasma of our patients with the G>T mutation at this location results primarily from a similar defective mechanism leading to the absence of stable and translatable transcript. Eventually, this mutation may provide the reason for the utilization of an alternative splicing site that significantly disrupts the splicing efficiency and producing unstable transcript leading to the observed phenotype. The majority of mutations affecting this nucleotide at this location lead to the loss of an exon as reported in the ATM gene which results in exon 8 skipping that was associated with frame shift causing premature termination of the translated message and the expression of ataxia telangectsis [34]. A similar mutation was also reported in exon 10 of the factor V gene which resulted in frame shift and premature translation termination [35].

The novel missense mutation detected in exon 8 which results in the replacement of acidic amino acid with a neutral polar residue (Asp413Asn) lies in the critical catalytic domain of the factor X protein. The nature of this shift in the amino acids type causes a significant change in the molecular structure of the 185-189 loop similar to the Asp409 deletion mutation that was reported previously [17]. This mutation will result in impairment of the catalytic activity of the FX protein due to the expected negative effect it has on Na^+ binding to the indicated loop. Similarly, the Arg347His mutation, causes a mutation in the same catalytic domain of the F X protein (8), that results in a dramatic decrease in FX protein activity.

Conclusion

Two mutations were identified in the factor X gene. A novel homozygous splicing mutation in the 5' donor site (IVS1+1G>T that results in a significantly distorted protein and a homozygous missense novel mutation in exon 8 (Asp413Asn G>T). A heterozygous mild mutation was also identified in exon 6 of the gene Ser105thr (G>C). In addition, a previously identified deletion mutation I exon 4 c358 delG which results in an early stop codon was detected in our investigation that appears specific to our population.

Conflict of Interest

The authors of this paper declare they have no conflict of interest. Sponsors of the work had no role in any part of the study including the work design, sample collection and processing data analysis and submission of the final manuscript for publication.

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