Original Contribution

Kurume Medical Journal, 63, 23-28, 2016

Factor X Deficiency with Heterozygous Mutations of Novel p.G435S and Known p.G244R in a Patient Presenting with Severe Umbilical Hemorrhage

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Received on 25 February 2016, accepted on 25 July 2016
J-STAGE advance publication 15 March 2017

Edited by YOSHIRO KODA

Summary: A 10-day-old male patient was referred to our hospital with severe umbilical bleeding. Prothrombin time (PT) and activated partial thromboplastin time (APTT) were prominently prolonged. Plasma coagulation factor X (FX) activity and antigen levels were 1% and 0.6%, respectively. A DNA sequence analysis of his leukocytes revealed a compound heterozygous state; known Gly244 to Arg (p.G244R) in exon 6 and a novel mutation of Gly 435 to Ser (p.G435S) in exon 8. A pedigree analysis showed that p.G244R originated from the paternal side, while p.G435S was from the maternal side. A p.G244R mutation was reported previously as $FX_{Debrecen}$ and this mutated protein was synthesized as a non-secretable protein.

The glycine at amino acid position 435 in the C-terminal region is completely conserved in the trypsin-like serine protease family, including thrombin, FVII, protein C, plasmin, trypsin, and chymotrypsin. In a three-dimensional structural model of FX, Gly 435 was located within the 11^{th} β-strand and buried in the back of the catalytic pocket. Therefore, the substitution to serine was expected to disrupt this structure. p.G435S FX was also predicted to be synthesized and exist in the cytoplasm, but not to be secreted into culture media by a cDNA expression assay. These two mutations may be responsible for the type 1 (null levels of both activity and antigen in plasma) FX deficiency with severe bleeding phenotype.

Key words Umbilical hemorrhage, missense mutation, compound heterozygote, amino acid alignment, three-dimensional structure model

INTRODUCTION

Coagulation factor X (FX) is a vitamin K-dependent protein that is produced by hepatocytes, circulates as a precursor of the serine protease, factor Xa, and is activated by F IXa and cofactor VIIIa, and/or by F VIIa + tissue factor on cell surface membrane phospholipids in the presence of Ca^{2+}. FXa converts prothrombin to thrombin with the assistance of F Va and Ca^{2+} on a suitable negatively charged phospholipid membrane, and exerts an important physiological role on hemostasis [1]. Mature FX is physiologically separated into a γ-carboxyglutamic acid (Gla) domain, epidermal growth factor domain, activation peptide, and catalytic domain [2].

The gene coding FX (*F10*) is located at chromo-

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Abbreviations: APTT, activated partial thromboplastin time; ELISA, enzyme-linked immunosorbent assay; FX, factor X; PCR, polymerase chain reaction; PT, prothrombin time.

some 13q34 and spans 27 kb. *F10* has been shown to contain 8 coding exons that correspond to 488 amino acids [3-4].

Congenital FX deficiency shows autosomal recessive inheritance with an incidence of one in 500,000 individuals. More than 100 mutations have been identified in the *F10* gene, the majority of which are missense mutations [5-7].

Patients with FX deficiency exhibit various bleeding tendencies. Previous studies conducted in Japan reported that most patients were aged 10 years or older and presented with subcutaneous hemorrhage, epistaxis, menorrhagia, gastrointestinal bleeding, and bleeding after surgery or trauma. However, the severity of FX deficiency varies from mild to serious, and depends on the degree of residual plasma FX activity [8-10].

We herein present a case of congenital FX deficiency that presented with massive hemorrhage from the umbilical stump, and a genetic analysis revealed compound heterozygosity with the missense mutations of novel p.G435S and known p.G244R.

CASE REPORT

History of present illness

No significant events were reported during the perinatal period or delivery. The patient was born by vaginal delivery at 40 weeks and 4 days gestation with a birth weight of 2876 g. The umbilical cord fell off naturally five days later. The patient was taken to hospital with umbilical hemorrhage nine days after being born. Although cautery treatment with silver nitrate temporarily stopped the bleeding, it recurred the following day. Since difficulties were associated with stopping the umbilical hemorrhage, the patient was referred to our hospital 10 days after being born.

Family history

There was no history of consanguinity and no family history of bleeding tendencies.

Symptoms at the initial diagnosis

The general condition of the patient was good, and his vital signs were normal; no abnormal findings were detected in a physical examination. Bleeding occurred from the umbilical stump, and it was difficult to stop this bleeding by compression alone (gauze with a large amount of blood and exudate weighed approximately 100 g).

Laboratory findings at the hospital visit revealed prominent prolongations in prothrombin time (PT) (131.6 seconds), activated partial thromboplastin time (APTT) (107.9 seconds), and the hepaplastin time (HPT) (<10%). The cross-mixing test using plasma from the patient and a normal subject showed a deficiency pattern, but not the inhibitor type.

A vitamin K deficiency was initially suspected due to the prolongations in PT and APTT; therefore, vitamin K was administered by an intramuscular injection, but was not effective. Although the activities of FII, FV, and fibrinogen were within normal limits, plasma activity and antigen levels of FX were <3% and 0.6%, respectively. Therefore, the patient was diagnosed with type 1 FX deficiency. The patient's family tree and their FX activities are shown in Figure 1. The administration of 20 ml/kg fresh frozen plasma (FFP) markedly shortened both APTT (107.9 to 38.6 sec) and PT (131.6 to 17.2 sec). Since umbilical bleeding recurred, FFP was administered until the cessation of hemorrhaging 17 days after birth.

The patient is now 2 years and 6 months old, and has had normal growth and developmental milestones. Prothrombin complex concentrate preparations were only administered when bleeding occurred. However, since multiple subcutaneous hematomas with induration developed and excessive traumatic hemorrhage often occurred, the patient was started on regular replacement therapy every week.

MATERIALS AND METHODS

FX activity was measured by a clotting assay using FX-deficient plasma. FX antigen levels were measured using a sandwich enzyme-linked immunosorbent assay (ELISA) with an A0373 anti-human FX rabbit polyclonal antibody (Dako, Tokyo, Japan), and was calculated from a prepared standard curve using a Dade Ci-Trol normal human plasma (Siemens, Munich, Germany).

Genomic DNA was extracted from the peripheral blood leukocytes of the patient and his family by standard techniques after obtaining informed consent from his family members. This study was approved by the Institutional Review Board at Kurume University (Research number 162). All exons and exon-intron boundaries of the *F10* gene were amplified by the polymerase chain reaction (PCR). Primer sets were designed as described by James et al. [11]. A forward and reverse direct sequencing analysis of all PCR products was performed with the Big Dye sequencing kit (PE Applied Biosystems, Warrington, UK) using an ABI Prism 310 DNA sequencer (PE Applied Biosystems). Mutant cDNAs were generated by site-directed mutagenesis of wild-type FX cDNA inserted in pME18S-

FL3 (Toyobo, Osaka, Japan) using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). COS-1 cells (Riken Cell Bank, Tsukuba, Japan) were grown in 35-mm diameter dishes and were transiently transfected with 5μg of wild-type or mutant vectors using the lipofectamine method (Lipofectamine2000 Transfection Reagent, Invitrogen, CA) [12].

After being incubated for 72 hours in 2 ml of serum-free media, media were harvested, and cells were washed two times with 1×PBS and suspended in Cell Lysis Buffer (10x)(Cell Signaling, Beverly, MA) (10 mM Tris, pH 7.6, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM sodium vanadate, and 1 mM N-phenylmethyl sulfonyl fluoride). One μg of protein was applied to 12% SDS-PAGE. A rabbit anti-human FX (H-120) polyclonal antibody (Santa Cruz, TX) was used in the Western blot analysis (ECL Prime Western Blotting Detection Reagent, RPN 2232) (Sigma Aldrich, St. Louis, Mo). The amino acid numbering of FX started at the initial translated methionine. The cDNA nucleotide number was also counted from the initial ATG [3]. The cited nucleotide numbers indicating the positions of the individual mutations were based on GenBank accession number NM_000504.3.

The alignment of amino acids around the mutated position and its functional effects on amino acid substitution were predicted by three types of predicting software: PolyPhen-2 v2.1.0r367 [13], SIFT [14] and MutationTaster [15].

The three-dimensional structure of the FX mutant protein was analyzed by the homology modeling software MOE [16] based on the X-ray structures of trypsinogen and FXa (PDB 1TGT and 1G2M).

RESULTS

The patient had the inherited type 1 deficiency of FX (activity; <3%, antigen; 0.6%) and developed clinically severe hemorrhage from the umbilical stump 9 days after being born. The FX activities of the father and mother were 47% and 60%, respectively (Fig. 1). A family study was conducted and both parents were suspected to be heterozygous for an FX deficiency.

Identification of defects in the F10 Gene

Two heterozygous mutations were identified by a direct sequencing analysis of all 8 exons and exon-intron boundaries in the F10 gene (Fig. 2). We found a heterozygous missense mutation (c.730G→A, p.G244R) in exon 6 which had been previously reported as $FX_{Debrecen}$ from Hungary [17]. The other heterozygous missense mutation was c.1303G→A, p.G435S in exon 8, which has not been reported previously.

p.G244R was detected in a heterozygous state in the father, but was absent in the mother. p.G435S was detected in a heterozygous state in the mother, but was

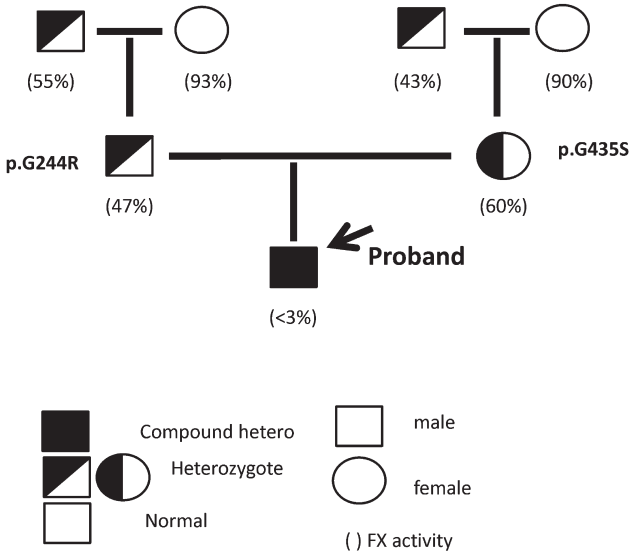

Fig. 1. The pedigree of the patient with FX deficiency
The propositus was a compound heterozygote of p.G244R originating from the father and p.G435S from the mother. Plasma FX activity levels were shown under each square or round box.

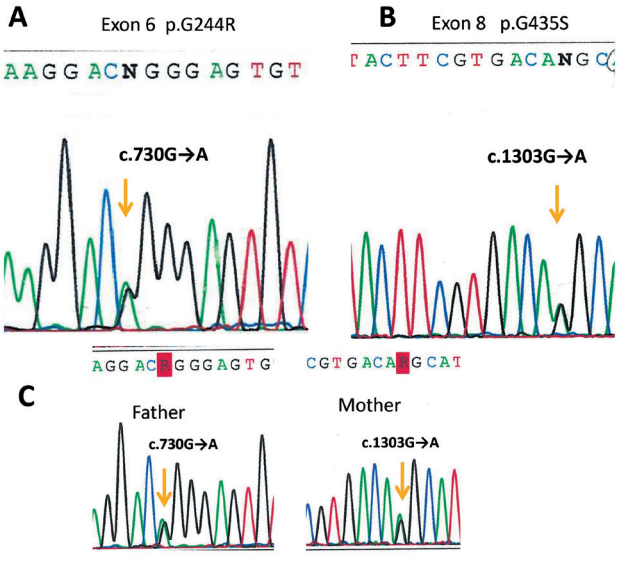

Fig. 2. Genomic sequencing analysis
Leukocyte DNAs from the propositus and the parent were directly sequenced after the amplification of exons from the F10 gene by PCR. The sequencing analysis revealed heterozygous missense mutations in exon 6 and exon 8.

absent in the father (Fig. 2). We investigated the allele frequency of these mutations by UCSC genome browser (http://genome.cse.ucsc.edu/), and found an allele frequency 0.01647% for c.730G→A, and no result for c.1303G→A.

Wild-type and mutated FX expression study

The protein expression study of wild-type or mutated FX was performed by the transfection of expression vectors in which either cDNA was inserted into Cos-1 cells. Cells and media were harvested after 72 hours in serum-free medium, and aliquots were applied to SDS-PAGE followed by Western blotting. As shown in Figure 3, both mutated proteins (p.G244R and p. G435S) were expressed in the cytoplasm of transfected cells as well as wild-type FX; however, these were not secreted into media. These results suggested that these mutated proteins were synthesized, but could not enter a secretory pathway.

Amino acid alignment around G435 and structure analysis of p.G435S FX

The amino acid glycine at 435 (homologous position 211 in chymotrypsin, most common representative in the comparative study of the amino acid alignment and protein structure among trypsin-like serine proteases) was completely conserved among the mammalian trypsin-like serine protease family (Fig. 4).

To predict the functional effects of the mutated FX with G435S, we used three types of mutation function software. This mutated FX was expected to have an adverse effect (damaging score, 1.000; sensitivity: 0.00; specificity: 1.00) by an analysis with Polymorphism Phenotyping version 2*. The same results were obtained from the other pathogenicity software; deleterious (score: 0, median: 3.33) by SIFT, and disease-causing (p-value: 1) by MutationTaster. Similar results from p.G244R were also obtained with a mutation functional analysis by these three software applications. Therefore, the glycine at 435 was considered to be indispensable for maintaining the structure and function of normal FX.

Three-dimensional structure of FX G435S

Gly 435 was located in the vicinity of the catalytic S1 site and within the 11th β-strand, and was buried in the back of the active pocket. Gly435 and Thr434 joined with His423 and constituted a part of the β-bulge in the antiparallel β-sheet. The three-dimensional protein model of the replacement of Gly to Ser at 435 (chymotrypsin number 211) was constructed using the homology modeling software MOE (Fig. 5). When analyzed using a space filling model (Fig. 5B), Ser435 appeared to interfere with the nearby Phe403 (chymotrypsin number 181). This model suggested that this missense mutation could cause crucial structural instability and secretion disturbances.

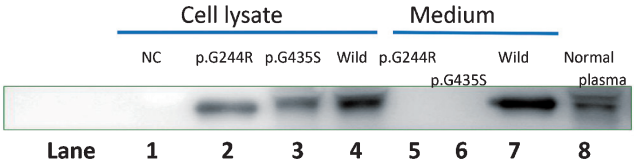

Fig. 3. Western blot analysis of cell lysates and media after cDNA transfection into COS-1 cells
One microgram each of proteins from cell lysates and media was applied to SDS-PAGE. The proteins were transblotted to the membrane and reacted with a polyclonal rabbit anti-human factor X antibody.
Lanes 1 to 4 contained the proteins from cell lysates and lanes 5-7 from media. Lane 1 contained the proteins from the non-treated COS-1 cell lysate. Lanes 2 and 5 contained those from COS-1 cells transfected with mutated cDNA (p.G244R). Lanes 3 and 6 contained those from mutated cDNA (p.G435S). Lane 7 contained proteins from COS-1 cells transfected with wild-type FX cDNA. Lane 8 contained those from normal human plasma. Mutated FX was synthesized and present in the cytoplasm, but was not secreted into media.

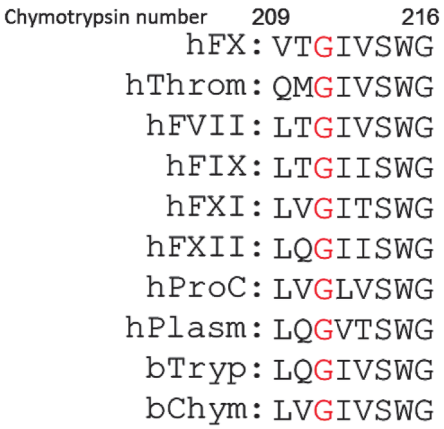

Fig. 4. Amino acid alignment around glycine 435 of human FX
Gly435 was strictly conserved among mammalian trypsin-like serine proteases.
Abbreviations: hFX: human factor X, hThrom: human thrombin, hFVII: human factor VII, hFIX: human factor IX, hFXI: human factor XI, hFXII: human factor XII, hProC: human protein C, hPlasm: human plasmin, bTryp: bovine trypsin, bChym: bovine chymotrypsin

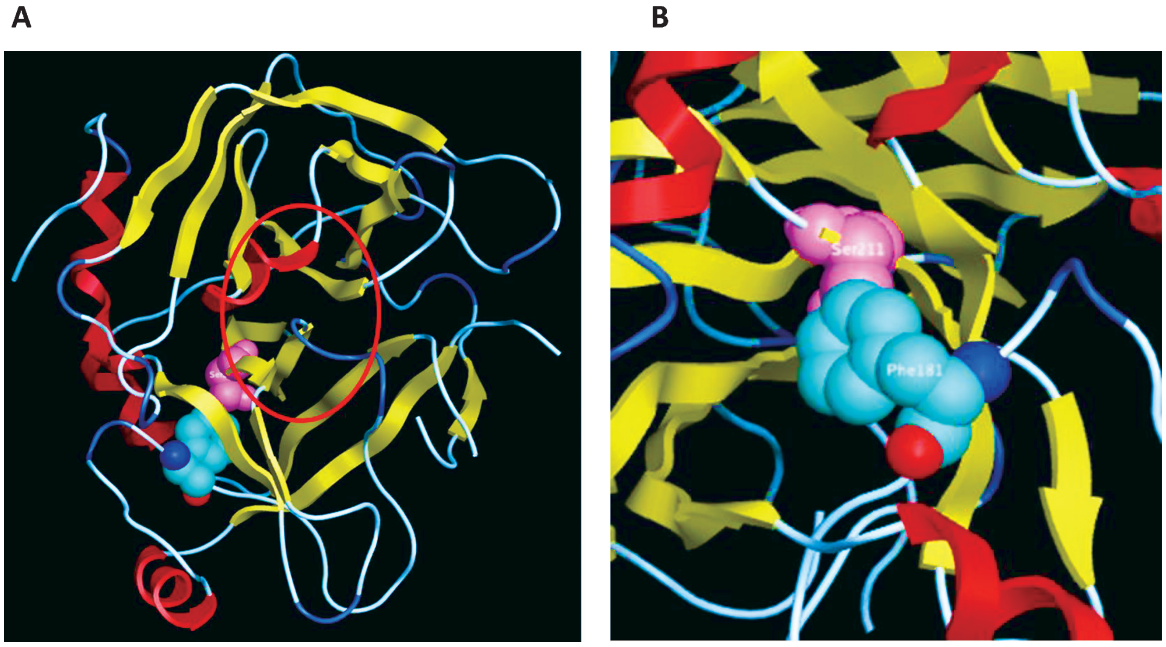

Fig. 5. Three-dimensional structure of FX p.G435S
'A' shows the catalytic pocket (S1 site) of FX p.G435S. The position of mutated serine (pink) was located at the back of the S1 site. The red circle indicates the S1 site.
'B' shows the reverse sides of 'A'. Mutated serine 435 (pink) appeared in the close vicinity of phenylalanine 403 (light blue).

DISCUSSION

We herein described a patient with inherited type 1 FX deficiency who presented with severe hemorrhagic diathesis. A genetic analysis of F10 revealed compound heterozygous missense mutations. One mutation was previously reported, p.G244R in exon 6, while another was a novel p.G435S in exon 8.

The molecular analysis of the p.G244R mutation was described in detail by Bereczky Z, et al. from Hungary. They showed that the homozygous G244R transition caused a structural change in the FX molecule and a secretion defect due to retention at the trans-Golgi-late endosome level, subsequently leading to type 1 FX deficiency [17].

p.G435S FX was produced in our expression study, but like p.G244R FX it was non-secretable. A three-dimensional structure analysis of this mutated FX revealed a change in the fundamental structure in the vicinity of the S1 catalytic site. Moreover, glycine at 435 was broadly and strictly conserved among mammalian trypsin-like serine proteases. These results suggested that p.G435S was the mutation responsible for type 1 FX deficiency.

This patient developed a severe bleeding tendency soon after birth. Considering the increase in his physical activity with growth, he may need to receive regular replacements with prothrombin complex concentrate preparations at appropriate intervals to prevent massive bleeding and maintain his quality of life.

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