Thrombosis Research 138 (2016) 108–113

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

A novel Ala275Val mutation in factor X gene influences its structural compatibility and impairs intracellular trafficking and coagulant activity

Nannan Sun [a], Yongheng Chen [b], Hongling Peng [a], Yujiao Luo [a], Guangsen Zhang [a,*]

[a] Department of Haematology, Institute of Molecular Haematology, The Second Xiang-Ya Hospital, Central South University, Changsha, Hunan, PR China
[b] Laboratory of Structural Biology, Key Laboratory of Cancer Proteomics of Chinese Ministry of Health, XiangYa Hospital & State Key Laboratory of Medical Genetics, Central South University, Changsha, Hunan, PR China

ARTICLE INFO

Article history:
Received 8 September 2015
Received in revised form 16 November 2015
Accepted 12 December 2015
Available online 15 December 2015

Keywords:
FX deficiency
FX Ala275Val mutation
In vitro expression
Subcellular localization
Molecular modeling

ABSTRACT

Factor X (FX) deficiency is an autosomal recessive severe bleeding disorder. Here, we identified a novel homozygous missense mutation (p.Ala275Val) in the *F10* gene in a patient with severe FX deficiency. The novel mutation was analyzed by *in vitro* expression and modeling. Site-directed mutagenesis of FX cDNA was used to introduce the FX Ala275Val mutation, wild-type as well as mutant FX proteins were expressed in HEK293 cells, and subcellular localization experiments were performed. Expression experiments showed that the FX Ala275Val mutation led to a significant reduction in antigen and activity levels in the culture medium. Moreover, compared to the wild-type, mutant FX-Ala275Val was mainly distributed in the endoplasmic reticulum and rarely entered the Golgi apparatus, suggesting a transportation defect for FX from the endoplasmic reticulum to the Golgi apparatus. Molecular modeling analysis indicated that the Ala275 is spatially located to the catalytic triad of FXa, which is composed of His276, Asp322, and Ser419. The Ala to Val substitution may change the conformation of the catalytic pocket and alter protein folding and enzymatic activity. Our findings demonstrated that the Ala275Val substitution is a pathogenic mutation that causes the inherited FX deficiency.

1. Introduction

Human coagulation factor X (FX) is a vitamin K-dependent plasma serine protease that plays a key role in the coagulation cascade, acting as the first enzyme in the common pathway of thrombin generation [1]. Following activation to FXa, the protease is complexed with activated factor V, a negatively charged membrane surface, and Ca^{2+} to form the prothrombin complex, which then cleaves prothrombin and subsequently generates thrombin. FX can be physiologically activated by either the tissue-factor/FVIIa complex in the extrinsic pathway or the FIXa/FVIIIa complex in the intrinsic pathway. It is also activated *in vitro* by a specific enzyme isolated from Russell's viper venom [2]. FX is synthesized in the liver as a precursor with a 40-residue prepropeptide that targets the protein for secretion [3] and is proteolytically cleaved before secretion. The mature FX is then secreted into the plasma as a two-chain protein consisting of a 17-kDa light chain and a 45-kDa heavy chain that are linked together by a disulfide bond [4]. The protein structure of FX consists of a Gla domain, two epidermal growth factor (EGF)-like domains, an activation peptide, and a catalytic serine protease domain; it is structurally homologous to other vitamin K-dependent proteins such as prothrombin (FII), FVII, FIX, protein C, and protein S [5]. In the serine protease domain of FX, residues His276, Asp322, and Ser419 (His57, Asp102, and Ser195 in the chymotrypsinogen numbering, respectively) constitute the catalytic site [6].

Inherited FX deficiency is a rare and severe autosomal recessive bleeding disorder. The prevalence of the disease in the general population is about 1:1,000,000 and the carrier frequency is about 1:500 [7]. The *F10* gene, located on chromosome 13q34 and approximately 2.8 kb downstream of the *F7* gene [8], consists of eight exons and seven introns. Each exon encodes a specific domain within the protein [9]. Molecular defects in the *F10* gene are the main causative factors that result in FX deficiency. To date, approximately 130 *F10* gene mutations have been identified, including missense, deletions, insertions, frame shift, and splice site mutations [10], of which missense mutations are predominant. Based on the results of both functional activity and immunological antigen level, FX deficiency can be classified into two types: those in which both functional activity and antigen level are concomitantly decreased are defined as type I, whereas those with low activity and normal or a near-normal antigen level are defined as type II [1]. The common clinical manifestations of FX deficiency include easy bruising, hematomas, epistaxis, hemarthrosis, umbilical cord bleeding, gastrointestinal and central nervous system (CNS) bleeding [11], which has a poor correlation with laboratory phenotypes [12].

In the present study, we report a novel homozygous Ala275Val mutation in the catalytic domain of the *F10* gene in a Chinese patient with

* Corresponding author at: Department of Haematology, Institute of Molecular Haematology, The Second Xiang-Ya Hospital, Central South University, No. 139 Renminzhong Road, Changsha, Hunan, 410011, PR China.
E-mail address: zgsllzy@163.com (G. Zhang).

http://dx.doi.org/10.1016/j.thromres.2015.12.009

type-1 FX deficiency, and the molecular characteristics of the mutation and abnormal subcellular localization of mutated FX protein were analyzed. The mechanism of bleeding in the patient and molecular modeling analysis were explored.

2. Materials and methods

2.1. Patient

The proband was a 28-year-old Chinese female born from non-consanguineous parents. She had experienced repeated gum bleeding, nose bleeding, and skin ecchymosis after a slight collision during childhood. Since her menarche, the patient has suffered from increased menstrual bleeding and significantly longer menstrual period (about 15 days for every menstrual period). She had five pregnancies in total, including three natural labors and two miscarriages. Postpartum hemorrhage was observed in all pregnancies, and blood transfusion therapy was needed every time. The mother of the proband had a similar but milder ecchymosis after collision, and other family members had no history of bleeding.

2.2. Coagulation assays

The written informed consent was obtained from the patient and her mother, who participated in the present study. The study was approved by the Ethics Committee of the Second Xiang-Ya Hospital, Central South University. After receiving their informed consent, venous blood from the proband and her mother were collected. FX clotting activity assay was performed using a one-stage clotting method based on prothrombin time (PT) or aPTT using FX-deficient plasma on ACL-TOP 700 automatic coagulometer (HemosILTM, IL, MA, USA). FX antigen level was measured using a commercially available AssayMax Human Factor X (FX) ELISA Kit (Assay Pro, St. Charles, MO, USA). FX activity and antigen levels were expressed as the percentage of normal pooled plasma obtained from 30 healthy individuals, which was set as 100%. And all of these participants provide their written informed consent to participate in this study.

2.3. Genetic analysis

Genomic DNA of the proband and her mother were extracted from peripheral blood leucocytes using a standard protocol (the TIANamp Genomic DNA Kit, Beijing, China). DNA sequence analysis of the *F10* gene was performed as previously described [13], and the mutations were confirmed by reverse sequencing. To rule out the possibility that the detected sequence variant of the patient was a common gene polymorphism, 110 healthy individuals were screened for this particular variant as well. Mutation was named according to the standard international nomenclature guidelines recommended by the Human Genome Variation Society (HGVS at http://www.hgvs.org/mutnomen/recs.html), with the methionine encoded by the translation initiation site (start codon) being numbered as residue 1. The genomic DNA (GenBank accession number: 12738260) and cDNA (GenBank accession number: M57285) sequences of the *F10* gene were used as reference sequences. The sequences of the PCR primers for amplifying all exons and exon/intron boundaries of the *F10* gene are listed in Table S1.

2.4. In silico analysis of amino acid change

The possible impact of the novel missense mutation on the structure and function of FX was assessed by using two bioinformatics tools:

Sorting Intolerant from Tolerant (SIFT, http://sift.jcvi.org) and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/). The protein ID"P00742"was used. The conservation of the affected amino acids were further checked by multiple sequence alignment (HomoloGene, http://www.ncbi.nlm.nih.gov/sites/entrez) with sequences from *Pan troglodytes*, *Macaca mulatta*, *Canis lupus familiaris*, *Bos taurus*, *Mus musculus*, *Rattus norvegicus*, *Gallus gallus*, *Danio rerio*, and *Xenopus tropicalis*.

2.5. Construction of expression vectors and site-directed mutagenesis

A 1507-bp gene fragment encompassing the full-length human FX cDNA was obtained from the entry clone pDONR223-FX (Genechem, Shanghai, China) by PCR using the forward primer, 5′-ACGGGCCCTCTAGA***CTCGAG***ATGGGGCGCCCACTGCACCTC-3′, and introducing a *XhoI* site, and the reverse primer, 5′-TTAAACTTAAGCTT***GGTACC***TCACTTTAATGGAGAGGACGTTATG-3′, and introducing a *KpnI* site. This objective gene was cloned into multiple cloning sites of pcDNA3.1 (−) (*XhoI*/*KpnI* enzyme, Genechem, Shanghai, China) to generate the vector, pcDNA/FX-WT.

PCR-mediated site-directed mutagenesis was performed to introduce the Ala275Val mutation. Except for the conventional primers of the 5′-end and 3′-end (mentioned above), a pair of partially complementary oligonucleotide primers containing the mutation loci were synthesized. The forward primer sequence, was 5′-CTAACGGCAGTCCACTGTCTCTACCAAGCCAAG-3′, and the reverse primer sequence, was 5′-GAGACAGTGGACTGCCGTTAGGATGTAGAACTC-3′ (underlined letter indicates the mutated base pair). Both the wild-type and variant cDNA vector constructs were transformed into DH5a competent cells. DNA from the resulting colonies was used for sequence confirmation.

2.6. Cell culture and transfection assays

Human embryonic kidney (HEK293) cell culture and transient transfection with the wild-type, mutant and vehicle plasmid were performed as previously described [13]. The experiment was conducted three times independently. Supernatants of the media were collected in pre-chilled tubes and centrifuged at 3000 *g* for 10 min at 4 °C to remove cell debris, and then stored at −80 °C until analysis. Proteins were extracted with RIPA Lysis Buffer (Beyotime, Shanghai, China), supplemented with a protease inhibitor cocktail (Roche AG, Basel, Switzerland) and PMSF (Beyotime, Shanghai, China). As described above, FX activity of the wild-type and mutant FX was evaluated by a one-stage clotting method based on PT or aPTT on supernatants, and the FX antigen was detected by ELISA both in the supernatant and cell lysate.

2.7. Quantitative real-time PCR (qRT-PCR) for FX mRNA expression

Total RNA was isolated from HEK293 cells transfected with plasmid constructs [pcDNA3.1 (−), pcDNA/FX-WT, and pcDNA/FX-A275V] using TRIzol reagent (TaKaRa Bio, Otsu, Japan) according to the manufacturer's protocol. Then, 1 μg of total RNA was used to synthesize cDNA. QRT-PCR was performed using SYBR Green qPCR Master Mix (TaKaRa Bio, Otsu, Japan). The housekeeping gene β-actin was used as an internal control for the cDNA quantification in each sample, and results were derived from three independent transfection assays with triplicate RNA samples. The primer pairs used in qRT-PCR were as follows: FX: forward, 5′-GAAACAGACCCTGGAACGCA-3′ and reverse, 5′-GGTGAGGTTGTTGTCGCCC-3′; and β-actin: forward, 5′-TTCCAGCCTTCCTTCCTGGG-3′ and reverse, 5′-TTGCGCTCAGGAGGAGCAAT-3′.

2.8. Expression of recombinant factor X

The recombinant protein secreted by HEK293 cells into the culture medium was concentrated 10-fold by using an Amicon Ultra-4 10K device (Millipore, Billerica, MA, USA). FX-WT and mutant FX-A275V proteins from both the culture medium and cell lysate were electrophoresed on 10% SDS-PAGE gel (Beyotime, Shanghai, China) under non-reducing conditions, and transferred onto polyvinylidene difluoride membranes (Millipore, MA, USA). The polyvinylidene difluoride