Gene analysis of inherited factor X deficiency and functional consequences of G114R and G223V mutations a human factor X ｖariant

Original Article

Journal of the Tsuruma Health Science Society Kanazawa University Vol. 30 (2)
145～151 2006

Gene analysis of inherited factor X deficiency and functional consequences of G114R and G223V mutations in a human factor X variant

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Abstract

Factor X (FX) deficiency is a rare coagulation disorder, inherited as an autosomal recessive trait. A severe bleeding phenotype is usually associated with homozygous or doubly heterozygous conditions. Heterozygous FX deficiency is generally asymptomatic, and in most cases is identified incidentally during pre-operative screening.

In this study, we describe two patients with FX deficiency. In patient 1, the plasma levels of FX activity (FX:C) and FX antigen (FX:Ag) were 45 and 50% of normal control levels, respectively. DNA sequencing of the FX gene revealed that the subjects was heterozygous for glycine (GGG) to arginin (AGG) substitution at position 114 (G114R mutation). In patient 2, the plasma levels of FX:C and FX:Ag were 35 and 61% of normal control levels, respectively. DNA sequencing of the FX gene revealed that the subjects was heterozygous for glycine (GGA) to valine (GTA) substitution at position 223 (G223V mutation). This mutation has never been reported. Both her mother and brother carried a heterozygote for this mutation.

To assess the effect of the G114R or G223V mutations on FX function, the G114R-FX and G223V-FX variants were expressed in BHK 21 cells and studied. Construction of the expression vector, transfection of BHK 21 cells, determination of antigen levels and functional assays of the recombinant G114R-FX (rG114R-FX) and recombinant G223V-FX (rG223V-FX) variants in conditioned medium were conducted. FX:C and FX:Ag levels of rG114A-FX in medium were reduced to 37% and 30% of those of recombinant wild type-FX (rWT-FX), respectively. On the other hand, both FX:C and FX:Ag levels of rG223V-FX in conditioned medium were reduce to below 1% of rWT-FX, The intracellular distribution of rG114R-FX and rWT-FX antigen did not differ according to an immunohistochemical examination. On the other hand, the cytoplasm of cells transformed with rG223V-FX was brighter than that of cells transformed with rWT-FX.

Our expression studies showed that although both rG114R-FX and rG223V-FX were synthesized in BHK transfected cells, rG223V-FX was not secreted and accumulated intracellularly. On the other hand, rG114R-FX was partially secreted. These results demonstrate that Patient 1 is heterozygous of G114R mutation in the second EGF-like domain of FX. G114R mutation is partially secreted. On the other hand, Patient 2 is heterozygous of G223V mutation in the catalytic domain of FX. G223V mutation is not secreted and accumulated intracellularly.

Key words

factor X deficiency, second EGF-like domain, catalytic domain

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Introduction

Human coagulation factor X (FX) is a vitamin K dependent serine protease that plays a central role in the blood coagulation cascade[1]. It is activated by factor VIIa/tissue factor (FVIIa/TF) and by factor IXa/factor VIIIa (FIXa/FVIIIa) in the extrinsic and intrinsic pathways, respectively[2]. Activated FX (FXa) then associates with the cofactor, factor Va (FVa) and this complex activates prothrombin to thrombin[3,4].

Factor X is a glycoprotein composed of a 16.9 kDa light chain bound to a 42.1 kDa heavy chain that contains a serine protease domain[5,6,7]. The light chain contains a γ-carboxyglutamic acid (GLA) domain and two epidermal growth factor (EGF) domains[8,9]. A FX deficiency is a rare, inherited autosomal recessive disorder[10,11]. A hemorrhagic phenotype is usually associated with homozygous or double heterozygous conditions. A heterozygous FX deficiency is generally asymptomatic, and is often incidentally identified during pre-operative screening. Here, we describe gene analysis of two patients with a FX deficiency due to different gene mutations.

Materials and Methods

1. Sample preparation and FX measurement

Two unrelated individuals with heterozygous FX deficiency were studied. Citrated blood samples were obtained from two patients (Cases 1 and 2) and family members (Case 2) following provision of informed consent. FX activity (FX:C) was determined in a one-stage prothrombin time (PT)-based assay. Sample (100μl) and FX-depleted plasma (100μl) (Dade-Behring, Marburg, Germany) were incubated for 3 minutes at 37℃. Thereafter, 200μl of thromboplastin preparation (Kokusai-Shiyaku, Kobe, Japan) was added and coagulation time was measured.

FX antigen (FX:Ag) levels were measured under enzyme-linked immunosorbent assay (Matched-Pair Antibody Set for ELISA of human Factor X antigen, Affinity Biologicals, Ontario, Canada). We coated overnight at 4℃ with affinity purified goat anti FX IgG. The wells were emptied and then incubated with blocking buffer for 1h at room temperature. After washing, test samples and conditioned medium containing serial dilutions of recombinant wild type FX (100% to 3.13%; 1: 1-1/32 dilution) for a quantitative curve was added to each well. Test samples were not diluted. The plates were incubated at room temperature for 90 minutes, washed, incubated with peroxidase conjugated rabbit anti FX IgG at room temperature for 90 minutes, washed again and then OPD substrate was added. Fifteen minutes later, stop solution was added and the samples were read at a wavelength of 492 nm.

2. Gene analysis

1) Polymerase chain reaction (PCR) and DNA sequencing

To identify the mutation site in the FX gene, we analyzed samples of genomic DNA from the patients, their families and 50 unrelated healthy Japanese control individuals. Written, informed consents were obtained from all participants. Genomic DNA was isolated from peripheral leukocytes. Exons and flanking intron regions of the FX gene were amplified using the polymerase chain reaction (PCR). The nucleotide sequences of all primers and the PCR conditions were essentially as same as described by Miyata *et al*[12]. PCR products of FX were subcloned into pCR2.1 plasmid vector using a TA cloning kit (Invitrogen Co, Carlsbad, CA, USA). Sequencing analysis was performed by the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems).

2) Restriction enzyme analysis

A PCR product from exon 7 was incubated with a restriction enzyme *Mae* III (Roche Diagnostics, Basel, Switzerland) for 3 hours at 55℃ and the bands sizes were visualized on 6% agarose with SYBR Green I.

3. Mutagenesis and expression of FX recombinant protein *in vitro*

The Gly114→Arg (G114R) and Gly223→Val (G223V) mutations were introduced into FX cDNA cloned into pCI-neo (Promega, Madison, WI, USA)

using the TaKaRa Ligation kit. Ver. 2 (TaKaRa, Ohtsu, Japan). The forward primers for mutagenesis were : 5'-ccgcaggtacaccctggctg-3' (G114R) and 5'-ctgtggtgtaactattctgag-3' (G223V) and the reverse primers were: 5'-gcgcaggagcacaccacag-3' (G114R) and 5'-aaaccctcgttttcctcattg-3' (G223V). The entire FX cDNA was sequenced to confirm the presence of a mutation and exclude to DNA polymerase-induced errors.

Syrian hamster fibroblast cell line, BHK21 cells were transfected with the wild-type FX (wt-FX), G114R-FX and G223V-FX expression vectors using the Plus™ Reagent (Invitrogen Co., Carlsbad, CA, USA) and Lipofectamine™ (Invitrogen Co.) according to the manufacturer's protocol. One day after transfection, cells were selected in growth medium containing 700 μg/ml G418.

4. Immunostaining microscopy

The human astrocytoma cell line U373MG transfected with wt-FX, G114R-FX and G223V-FX were cultured on cover slips. The cover slips were washed with PBS, and incubated with BODIPY® FL C_5-ceramide complexed to BSA (Invitrogen Co.) for 30 minutes at 4℃, incubated with PBS for 30 minutes at 37℃, and washed with PBS. The cells fixed with 4% paraformaldehyde for 20 minutes at room temperature. The cells were permeabilized with 99.5% ethanol for 5minutes, washed with PBS, and incubated with monoclonal anti-human FX clone HX-1 purified mouse immunoglobulin (Sigma-Aldrich Co., St. Louis, MO, USA) for 1h at 37℃. The cells were washed with PBS twice and incubated with anti-mouse IgG TRITC conjugate (Sigma-Aldrich Co.) for 30 minutes at 37℃. The cells were washed, mounted and examined using a Fluophot fluorescence microscope (OLYMPUS BX50; Olympus Co., Tokyo, Japan).

Results

1. Case history and coagulation studies

Case 1 is a 70-year-old Japanese man who hemorrhaged after nasal polypectomy. A PT and an activated partial thromboplastin time (APTT) were both prolonged. Plasma levels of FX:C and

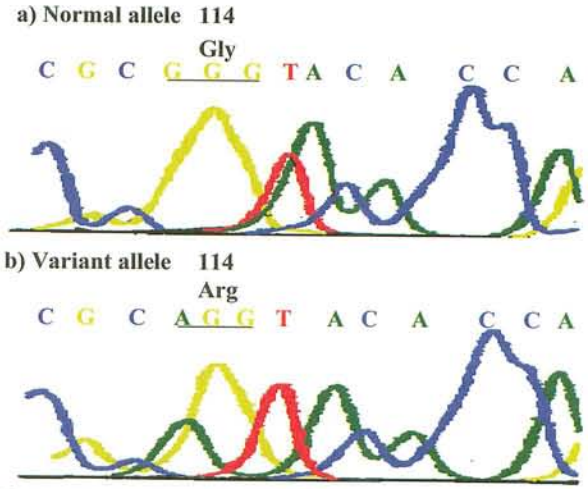

Fig. 1 Sequencing analysis of the PCR products of exon 5. The sequences of the sense strands of exon 5 from a healthy control subject and a proband with FX G114R mutation were shown. The position of the G→A mutation is shown by an arrow. The underline shows the codon for the amino acid substitution.

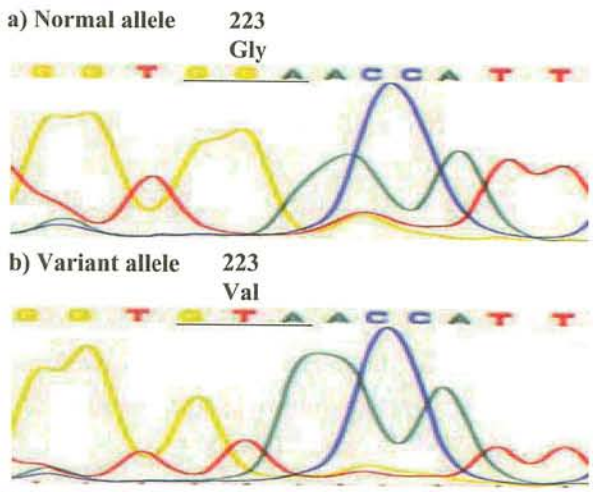

Fig. 2 Sequencing analysis of the PCR products of exon 7. The sequences of the sense strands of exon 7 from a healthy control subject and a proband with FX G223V mutation were shown. The position of the G→T mutation is shown . The underline shows the codon for the amino acid substitution.

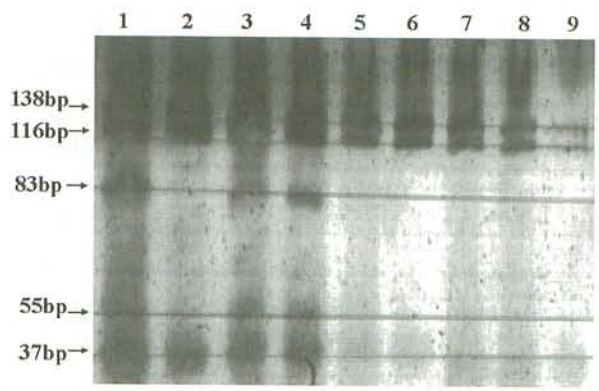

Fig. 3 PCR-RFLP analysis of exon 7. Presence of the G223V mutation creates the restriction site of Mae III, resulting in two bands of 83 bp and 55 bp. In the absence of the mutation, a single band of 138 bp is produced. Lane 1, patient 2, lane 2, father, lane 3, mother, lane 4, brother, lane 5-9, normal control.

FX: Ag were 45% and 50% of normal control values, respectively. No other coagulation abnormalities were detected. The patient had no history of abnormal bleeding tendencies. His family members also had no apparent history of bleeding tendencies, but were unavailable for this study. His parents were unrelated.

Case 2 is a 27-year old Japanese woman who experienced paraplegia of her legs. However, CT and MRI finding ruled out cerebral hemorrhage. Her plasma PT and APTT were prolonged and the plasma levels of FX: C and FX: Ag were 35% and 61% of normal control values, respectively. She had no history of abnormal bleeding tendencies. Other members of her family were investigated: her mother and brother had FX: C levels of 41 and 40 % respectively. No one in her family had a history of bleeding.

2. Identification of the mutations in the FX gene

Case 1 harbored a G to A mutation at nucleotide 10909 of the FX gene, which changed the codon for Gly114 (GGG) to Arg (AGG) (Figure 1). Among 10 clones, 5 each presented the mutated and normal FX sequences, respectively, which confirmed heterozygosity of this mutation. None of 50 healthy controls carried this mutation.

Case 2 carried a G to T mutation at nucleotide number 17537 of the FX gene, which changed the codon for Gly223 (GGA) to Val (GTA) (Figure 2). Among 7 clones, 4 and 3 harbored the mutated and normal FX sequences, respectively, which confirmed the heterozygosity of this mutation. This point mutation caused a *Mae* III restriction endonuclease recognition site in the FX gene sequence. A normal allele from amplified DNA of FX gene exon 7 shows four fragments (138 bp, 116 bp, 37 bp and 24 bp), whereas a mutant allele of this mutation causes a *Mae* III recognition site in the 138 bp fragment, resulting in producing other two fragments of 83 bp and 55 bp. Her mother and brother carried the heterozygous mutation (Figure 3). None of 50 healthy controls carried this mutation.

Table 1. Stable expression assays of wild type, G114R and G223V FX in BHK 21 cells

Recombinant FX	FX:C (%)	FX:Ag (%)
Wild type	100	100
G114R	37±13	30±8
G223V	<1	<1

3. Recombinant FX expression

The contribution of the G114R-FX and G223V-FX substitution to the phenotypes of the patients was investigated through functional assays of the recombinant molecule. We estimated the FX-specific activity of variants. Factor X variants in medium from stable clones were further functionally characterized. Compared with rwt-FX, the clotting activities of the rG114R-FX and rG223V-FX variants tested in triplicate at various concentrations, were about 37% and below 1%, respectively (Table 1). The FX: Ag levels of rG114R-FX and rG223V-FX in conditioned medium were reduced to 30% and below 1% as compared with the cells transfected with rwt-FX, respectively (Table 1).

The intracellular distribution of rG114R-FX and rwt-FX antigen did not differ according to an immunostaining examination (Figure 4 (A), (D)). On the other hand, the cytoplasm of cells transformed with rG223V-FX was brighter than that of cells transformed with rwt-FX (Figure 4 (G)). In addition, according to composite photograph of FX and Golgi complex, both rG114R-FX and rG223V-FX were synthesized in the cells (Figure 4 (C), (F), (I)).

Discussion

Here, we characterized genetic defects responsible for an FX deficiency in two families, which we designated as FX G114R and FX G223V.

In Case 1, we found a G→A transition at nucleotide 10909, resulting in a single amino acid substitution of Arg (AGG) for Gly-114 (GGG) in the second EGF-like domain. Gly-114, a conserved amino acid among humans and bovines, is located in the second EGF-like domain. Both EGF-like domain contain 1 major and 1 minor β-sheet cross-connected by 3 disulfide bridges. The second EGF-

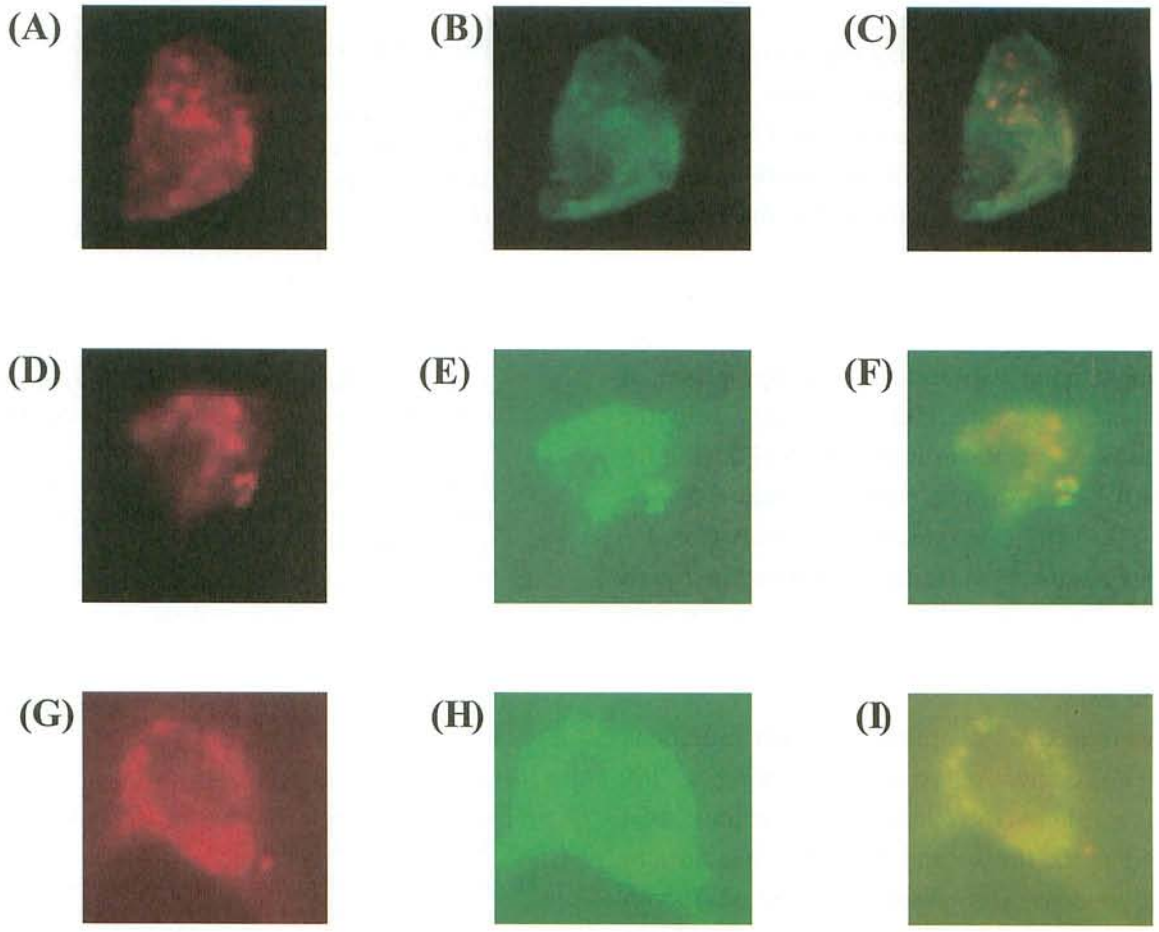

Fig. 4 Immunohistochemical localization of wild type and mutant FX in U373MG cells. (A),(B),(C) FX wild type, (D),(E),(F) FX G114R, (G),(H),(I) FX G223V. (A),(D),(G) FX:Ag, (B),(E),(H) Golgi complex, and (C),(F),(I) FX and Golgi complex.

like domain is positionally ordered and contacts the catalytic domain at numerous sites. Experiments with recombinant chimeric molecules have suggested that the substitution of Arg for Gly-114 should alter interaction with other molecules, particularly with factor VIIIa/Va[13]. Modeling this substitution using the FX crystal structure suggested that this substitution would lead to non-bonded contacts between the Arg side chain and Tyr-130[14].

G114R mutation was not found in 50 healthy Japanese individuals. Our immunostaining study revealed that rG114R-FX was synthesized in the cell and was transported to Golgi complex as same to rwt-FX. Moreover, the measurements of rFX activity and antigen showed that rG114R-FX was secreted from the cells. This substitution was identified previously in a Swedish patient, FX Ockero[15]. This patient was homozygous for a Gly-114→Arg substitution and had a mild FX deficiency (25% FX:C) with mild bleeding diathesis. Herrmann *et al.*[16] also described a Venezuela woman who was homozygous in this mutation. She had FX:C level of 19% and FX:Ag level of 25%. Our expression study *in vitro* showed that rG114R-FX had FX:C and FX:Ag levels of about 30% in conditioned media. These reduction levels (about 30%) in our study were similar to those in the homozygous patient's plasma (19% FX:C and 25% FX:Ag). Accordingly, we concluded that the mutation was probably responsible for the reduction in plasma FX level observed in our patient.

In case 2, the sequencing analysis revealed that the FX gene in this patient contained a G→T substitution at nucleotide position 17537 in exon 7, resulting in a Gly 223 to Val substitution in the catalytic domain of FX. To our knowledge, this is

the first report of G223V mutation in the FX. The Gly223 is highly conserved not only in terms of FX structure, but also in function. This position is conserved in bovine FX and in other vitamin K-dependent glycoproteins of coagulation factors. Thus, this position is important for the catalytic activity of FX.

The expression study showed that rG223V-FX was not secreted from the cells at all. On the basis of immunstaining experiments, the cytoplasm of cells transformed with rG223V-FX was brighter than that of cells transformed with rwt-FX (Figure 4 (G)(H)(I)). These results suggested that although rG223V-FX was synthesized in transfected U373MG cells and was transported from the rough endoplasmic reticulum (RER) to the Golgi, it was retained in Golgi and was gradually degraded inside the cells.

In conclusion, we identified two point mutations in the FX genes of patients carrying a FX deficiency phenotype, leading to amino acid substitutions in the second EGF-like domain and catalytic regions. Our results suggest that both G114R and G223V substitutions are responsible for the deficiency of FX. In the future, pulse-chase and endo-β-*N*-acetylglucosaminidase H digestion experiments are required to understand the functional consequences of these substitutions in FX structure.

Acknowledgements

We thank Dr Masataka Mori (Nagasaki national hospital) for providing us with DNA samples.

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先天性第X因子欠損症の遺伝子解析、および変異タンパクG114RとG223Vの機能解析

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要　　旨

われわれは先天性FX欠損2家系について遺伝子解析および変異タンパクの機能解析を行ったので報告する。発端者1のFX活性（FX:C）は45%、FX抗原量（FX:Ag）は50%と低下しており、遺伝子解析の結果FX Gly-114→Arg（G114R）への一アミノ酸置換を認め、同変異を有するヘテロ接合体であった。発端者2のFX:Cは35%、FX: Agは61%と低下しており、遺伝子解析の結果FX Gly 223→Val（G223V）の一アミノ酸置換を認めた。発端者はこの変異を有するヘテロ接合体であり、発端者の母および弟も同変異のヘテロ接合体であった。次に、野生型およびG114R変異型、G223V変異型FX cDNAを作製し、培養細胞BHK21に安定形質導入した。細胞上清中のFX抗原量をELISA法にて、FX活性を凝固時間法にて測定した。また、蛍光免疫染色法により細胞内のFXの局在を検討した。G114R変異型の細胞上清中のFX:Cは野生型FXを100%とした場合37%、FX:Agは同じく30%に減少していた。一方、G223V変異型の細胞上清ではFX:C、FX:Agともに野生型の1%未満であった。蛍光免疫染色法では、G114R変異型は野生型と同じような局在が認められたが、G223V変異型では細胞質により強いFXの局在が認められた。以上の事より、G114R変異FXはタンパクの分泌障害あるいは分泌後のタンパクの安定性に障害があることが予測されたが、タンパクの安定性の程度は今回の実験では確認できなかった。一方、G223V変異FXはタンパクの分泌障害が、血中FX低下の原因であろうと考えられた。