Original Article

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Factor Ⅶ Deficiency Due to Compound Heterozygosity for the p.Leu13Pro Mutation and a Novel Mutation in the HNF4 Binding Region (–58G>C) in the *F7* Promoter

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Summary: We investigated the molecular basis of factor Ⅶ (FⅦ) deficiency in a Japanese patient and identified compound heterozygous mutations. Factor Ⅶ activity and antigen levels in the patient were less than 5.0% and 6.5% of controls, respectively. All exons, exon–intron boundaries, and the 5' promoter region of *F7* from genomic DNA were amplified using polymerase chain reaction (PCR). Sequencing analysis of PCR fragments revealed that the patient was heterozygous for a known T to C substitution at nucleotide position 38, which resulted in the p.Leu13Pro missense mutation (Factor Ⅶ Morioka) in the signal peptide region, and a novel mutation in the 5' promoter region (–58G>C). An electrophoretic mobility shift assay showed that the mutation in the promoter region reduced the binding of hepatocyte nuclear factor (HNF). It is presumed that the reduced binding of HNF-4 to the *F7* promoter region reduces *F7* transcription and thus reduces the synthesis and expression of FⅦ.

Keywords factor Ⅶ deficiency, factor Ⅶ Morioka, bleeding tendency, genetic analysis, promoter

INTRODUCTION

Coagulation factor Ⅶ (FⅦ) is a vitamin K-dependent serine protease that is synthesized by hepatocytes and secreted into the blood as a single-chain molecule. It then circulates in blood either as an inactive zymogen or an enzyme with negligible activities. Although the mechanism of FⅦ activation is unclear, several coagulation enzymes, including FXa, FIXa, and even FⅦa itself, can activate FⅦ [1,2]. Among these, FXa seems to be the most potent activator of FⅦ [3]. The binding of small amounts of active FⅦ to tissue factor (TF) initiates and augments the blood coagulation system by activating FIX and FX [4,5].

The human FⅦ gene (*F7*) is located on chromosome 13q34 [6]; it contains nine exons and eight introns and spans approximately 12.8 kb [7]. The *F7* promoter is unique because it does not contain a TATA or a CAAT box, which are classically found in 32% and 64% of the promoters of class Ⅱ eukaryotic genes, respectively [8]. The lack of a CAAT box is unique because this region is a critical transcription element for the genes of the other clotting vitamin K-dependent factors such as FIX and FX. The major transcriptional start site of *F7* is localized at position –51, which is close to the binding sites of the transcription factors Sp1, which spans nucleotides 100 and 94, and hepatocyte nuclear factor-4 (HNF-4), which spans nu-

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Abbreviations: EMSA, Electrophoretic mobility shift assay; *F7*, The human FⅦ gene; FⅦ, Coagulation factor Ⅶ; HNF-4, hepatocyte nuclear factor-4; PCR, Polymerase chain reaction; RBD, rare bleeding disorder; TF, tissue factor; Ⅶ:Ag, FⅦ antigen levels; Ⅶ:C, FⅦ coagulant activity

cleotides –63 to –58 [9].

FVII deficiency is an autosomal recessive inherited bleeding disorder resulting from defects in the genes coding for clotting FVII. Historically referred to as hypoproconvertinemia, it is recognized as the most frequently presenting rare bleeding disorder (RBD) [10], estimated to affect 1 in 500,000 people [11]. Males and females are affected equally; however, females are more likely to be symptomatic as a result of gynecological or mucocutaneous bleeding [12,13]. To date, more than 800 mutations in *F7* have been reported, and up to 250 mutations and six polymorphisms associated with increased or decreased plasma FVII levels have been reported in the human FVII mutation databases [14,15]. Most of these are missense mutations, but some nonsense and splice site mutations have also been reported [16]. Both the genetic basis and the biological or clinical aspects of FVII deficiency are highly heterogeneous [17].

Here, we report a case of FVII deficiency that was caused by a known heterozygous mutation (p.L13P) in the signal peptide region and a novel single nucleotide substitution in the HNF-4 binding site of the 5' promoter region.

PATIENT AND METHODS

Patient

The patient was a 50-year-old Japanese male with a thyroid tumor. He was admitted to our hospital for tumor resection. Preoperative examination incidentally revealed a prolonged prothrombin time (31.4 seconds; PT-INR = 2.65). Further examination of his plasma showed a marked reduction in FVII activity (<5%) and antigen levels (6.5%) compared to controls, but normal coagulant activities of FII, FV, and FX. Thus, the diagnosis of FVII deficiency was confirmed. He had no history of bleeding and had undergone surgery twice at the ages of 28 years and 30 years without abnormal bleeding. His family also had no tendency toward bleeding. His parents had no consanguinity.

Plasma FVII assays

FVII coagulant activity (VII:C) was measured on citrated plasma using a one-stage clotting assay. FVII antigen levels (VII:Ag) were assayed using an enzyme-linked immunosorbent assay kit (Asserachrom VII:Ag, Diagnostica Stago, Asnières sur Seine, Cedex, France). Calibration curves were made using dilutions of human plasma containing FVII from 100% to a 21-fold dilution. The levels of VII:C and VII:Ag are expressed as a percentage of the levels in normal plasma.

Polymerase chain reaction (PCR) and the sequencing of F7

Genomic DNA was extracted from peripheral blood leukocytes using standard techniques with informed consent. All exons, exon-intron boundaries, and the 5' promoter region of *F7* were amplified using PCR. The primer sets were designed as described by Giansily-Blaizot et al. [18]. All PCR products were sequenced using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) as described elsewhere. For nucleotide numbering, position +1 was based on the translational start site, whereas amino acid numbering position +1 corresponded to the first translated amino acid.

Two heterozygous mutations were identified in the *F7* gene of the patient. Therefore, we investigated whether these two mutations were present in different alleles using cloning and sequencing. PCR fragments containing both the 5'-flanking region and exon 1a were subcloned into the pCR4-TOPO vector plasmid using a TOPO TA Cloning kit (Invitrogen, Carlsbad, CA, USA), and each subcloned fragment was sequenced directly as described above.

Electrophoretic mobility shift assay (EMSA)

Thirty base pair complementary single-stranded oligonucleotides from position –76 to –47 of the HNF-4 binding site in *F7* were annealed at 94°C for 10 minutes. The oligonucleotide sequences used in the reactions were as follows [19]: wild-type, 5'-TTGGAGGCAGAGAACTTTGCCCGTCAGTCC-3'; and mutant, 5'-TTGGAGGCAGAGAACTTTCCCCGTCAGTCC-3'. The sense strand of the oligonucleotide was 3'-labeled with biotin-TEG, and the antisense strand of the oligonucleotide was not labeled. EMSA was performed using a Panomics EMSA Gel Shift kit (Panomics, Fremont, CA, USA) following the manufacturer's instructions with minor modifications. First, the protein–DNA complexes were formed by pre-incubating 6 μg of human liver nuclear extract (Active Motif, Carlsbad, CA, USA) on ice for 10 minutes with 1 μg poly d(I-C), 50 ng poly L-lysine, and 0.5 μg salmon sperm DNA in 10 μl of binding buffer (provided in the kit). Next, 4 ng biotin-labeled double-stranded oligonucleotide was added to the mixture, and the reactions were incubated on ice for an additional 30 minutes. In competition studies, 40 ng or 400 ng of unlabeled competitor oligonucleotide was added to the mixture after the pre-incubation step and incubated on ice for 10 minutes. Following incubation, the

DNA–protein complexes were electrophoresed on a 6% non-denaturing polyacrylamide gels in 0.5X TBE for 1 hour at 4°C after pre-running the gel for 10 minutes at 120 V. The polyacrylamide gel was then transferred to a positively charged nylon membrane (Pall, Port Washington, NY, USA) for 30 minutes at 300 mA in 0.5X TBE. After transfer, the oligonucleotides bound to the membrane were fixed by baking for 1 hour in a dry oven at 80°C and were then detected using streptavidin-HRP and a chemiluminescent substrate. The bands were visualized after exposure to an ImageQuant LAS chemiluminescent-imaging system for 15 minutes (GE Healthcare Japan, Tokyo, Japan).

RESULTS

Plasma FVII levels

The FVII:C and the FVII:Ag levels were less than 5.0% (reference range, 75 to 140%) and 6.5% (reference range, 50 to 150%), respectively.

DNA sequencing

Sequencing analysis revealed two heterozygous nucleotide substitutions. A T to C substitution at nucleotide 38 in exon 1a in the hydrophobic core of the signal peptide was identified, which resulted in the substitution of leucine (CTT) to proline (CCT) at codon 13 (Fig. 1a). This mutation was described previously as Factor VII Morioka [20]. We also identified a second novel mutation: a –58G to C substitution in the HNF-4 binding site of the 5' promoter region (Fig. 1b). No other mutations were found in the amplified PCR fragment of *F7*. To investigate whether these mutations were on the same allele or different alleles, DNA fragments encompassing both mutated regions were amplified using PCR and cloned. Three cloned fragments were sequenced, which revealed that each mutation was present on a different allele.

EMSA

A biotin-labeled wild-type oligonucleotide encompassing the HNF-4 binding site of *F7* bound specifically to a protein present in human liver cell nuclear extracts (Fig. 2, lane 1). This binding was reduced

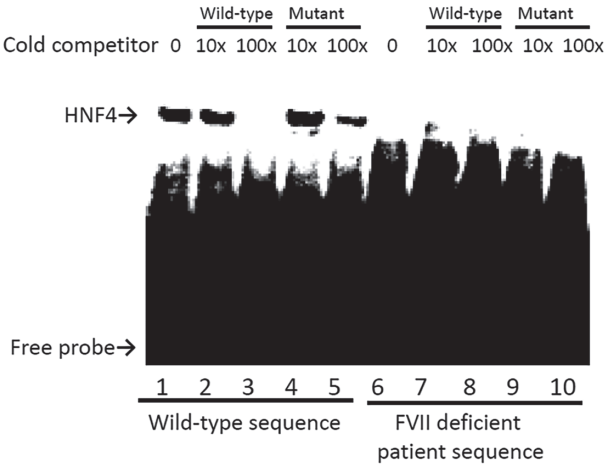

Fig. 2. EMSA using wild-type and mutant oligonucleotide sequences. A biotin-labeled wild-type oligonucleotide encompassing the HNF-4 binding site in the *F7* gene bound specifically to a protein present in human liver nuclear extract (lane 1). The reactions performed with incubation with an unlabeled wild-type (lanes 2 and 3) and mutant (lanes 4 and 5) competitor oligonucleotide at 10× and 100× concentrations are shown. Lanes 6 to 10 shows the absence of binding using a 30-bp biotin-labeled oligonucleotide containing the G to C mutation at position –58 in the absence of cold competitor (lane 6), and in the presence of 10× and 100× concentrations of wild-type (lanes 7 and 8) or mutant (lanes 9 and 10) cold competitor.

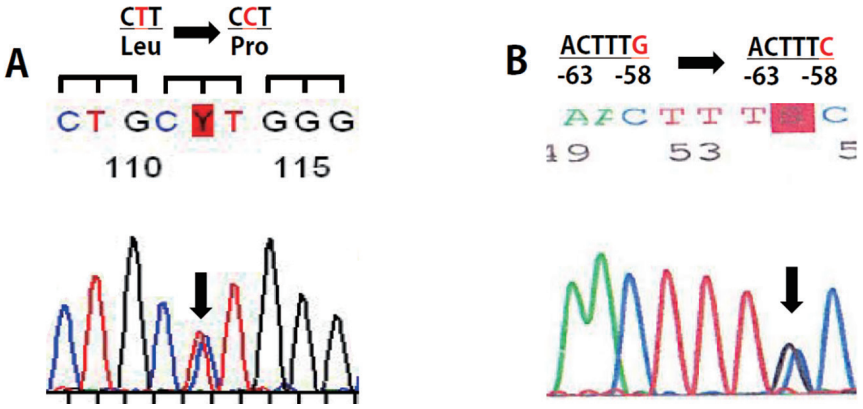

Fig. 1. Sequence analysis revealed that the patient was a compound heterozygote.
A. One allele carried a missense mutation (p.Leu13Pro) in the central hydrophobic core of the signal peptide.
B. The other allele had a point mutation of a –58G to C substitution before the translation start site in the hepatocyte nuclear factor 4 (HNF-4) binding site (b).

competitively when the reactions were performed using unlabeled wild-type oligonucleotides at 10× and 100× concentrations (Fig. 2, lanes 2 and 3), indicating specific binding. However, unlabeled mutated oligonucleotide did not inhibit binding completely at 100× concentration (Fig. 2, lane 5). In contrast, no protein binding was found when biotin-labeled mutated oligonucleotide was used (Fig. 2, lane 6).

Predicting the secondary structure of mutant FVII and the signal peptide cleavage site

The secondary structure of FVII carrying the p.Leu13Pro mutation was predicted using the Chou–Fasman and Robson method (GENETYX ver10). The mutation caused loosening of the helical structure, causing it to change into a coiled structure (Fig. 3). Hydrophobicity was also decreased (data not shown). In addition, hydrophilicity/hydrophobicity and cleavage site of the FVII signal peptide were analyzed using SignalP 4.1 software [21]. Data revealed that the mutant peptide was slightly more hydrophilic than the wild-type peptide (Fig. 4). The raw C-score (cleavage score) of the mutant peptide was also slightly higher; however, the position of the highest peak of the Y-score (combined cleavage site score) curve, which affords better cleavage site prediction than does the raw C-score alone, did not change in the mutant peptide (Fig. 5), suggesting that the cleavage site was not affected by the mutation.

DISCUSSION

In this study, we identified two mutations in *F7*: a previously known p.Leu13Pro mutation in the signal peptide and a novel –58 G to C substitution in the *F7* promoter site. Both the FVII:C and FVII:Ag levels were extremely low in the patient; however, he was asymptomatic with no bleeding tendency. We investigated the molecular mechanism of FVII deficiency in this patient.

The p.Leu13Pro mutation, reported previously as Factor VII Morioka by Ozawa et al. [20], impairs the secretion of FVII. Suzuki et al. performed an expression study to demonstrate that with the p.Leu13Pro mutation, FVII is synthesized in the cell but is secreted poorly into the culture medium compared with wild-type [22]. Rizzotto et al. investigated the intracellular localization of the mutant FVII using a FVII-GFP chimera protein [23], which suggested that it had impaired translocation into the endoplasmic reticulum (ER). This is consistent with the reduced FVII:C and FVII:Ag observed in this case. Ozawa et al. reported a case with a homozygous p.Leu13Pro mutation, whose Factor VII activity and antigen level were 10.7% and 11% compared to controls, respectively, without hemorrhagic episodes [20]. Our heterozygous case had no bleeding tendency, and FVII:C and FVII:Ag levels were less than 5.0% and 6.5% of normal, respectively. Therefore, the small amounts of plasma FVII in our patient might be derived from the p.Leu13Pro allele. The p.Leu13Pro mutation has only been reported in Japanese individuals, suggesting that there might be a founder effect.

The predicted secondary structure of p.Leu13Pro mutant FVII exhibits decreased hydrophobicity; however, the cleavage position was not affected. The conformational change caused by p.Leu13Pro might not affect the signal peptide cleavage. However, it might induce the early degradation of the propeptide and inappropriate secretion of the mature protein.

HNF-4, a member of the steroid hormone receptor superfamily [24], regulates the *F7* gene [25]. To date, six mutations in the HNF-4 binding site of the *F7* promoter region have been reported [19,26,27,28,29] (Table 1). Five of the six are single-nucleotide substitutions (–61 T to G, –60 T to C, –59 T to G, –55 C to

TABLE 1.
Previously reported factor VII mutations in the HNF-4 binding site

	Mutation site	Location	Domain	Type	Genotype	FVII activity (%)	FVII Ag level (%)	Reported by
1	-61T → G	promoter	promoter	regulatory	homozygote	<1	<1	Arbini et al. 19)
2	-55C → T -61T → G	promoter promoter	promoter promoter	regulatory regulatory	comp. hetero	<2	<2	Carew et al. 26)
3	-59T → G A294V	promoter Exon8	promoter catalytic	regulatory missense	comp. hetero	8	24	Kavlie et al. 27)
4	-60T → C	promoter	promoter	regulatory	comp. hetero	<1	<1	Zheng et al. 28)
5	-65G → C -60_-59delTT	promoter promoter	promoter promoter	regulatory regulatory	homozygote	<1	<1	Giansily-Blaizot et al. 29)
our case	-58G → C	promoter	promoter	regulatory	comp. hetero	<5	6.5	

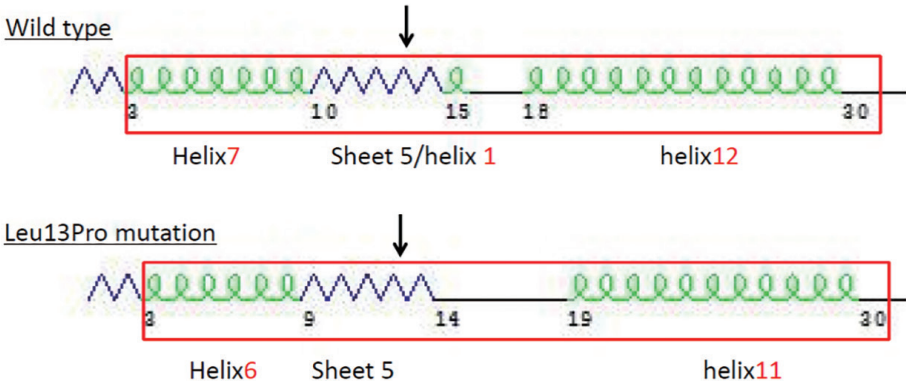

Fig. 3. Chou–Fasman and Robson protein secondary prediction using GENETYX ver10. The Leu13Pro mutation loosened the helical structure and converted it into the coiled structure. Hydrophobicity was decreased with the mutation (data not shown). Arrows indicate the mutated sites.

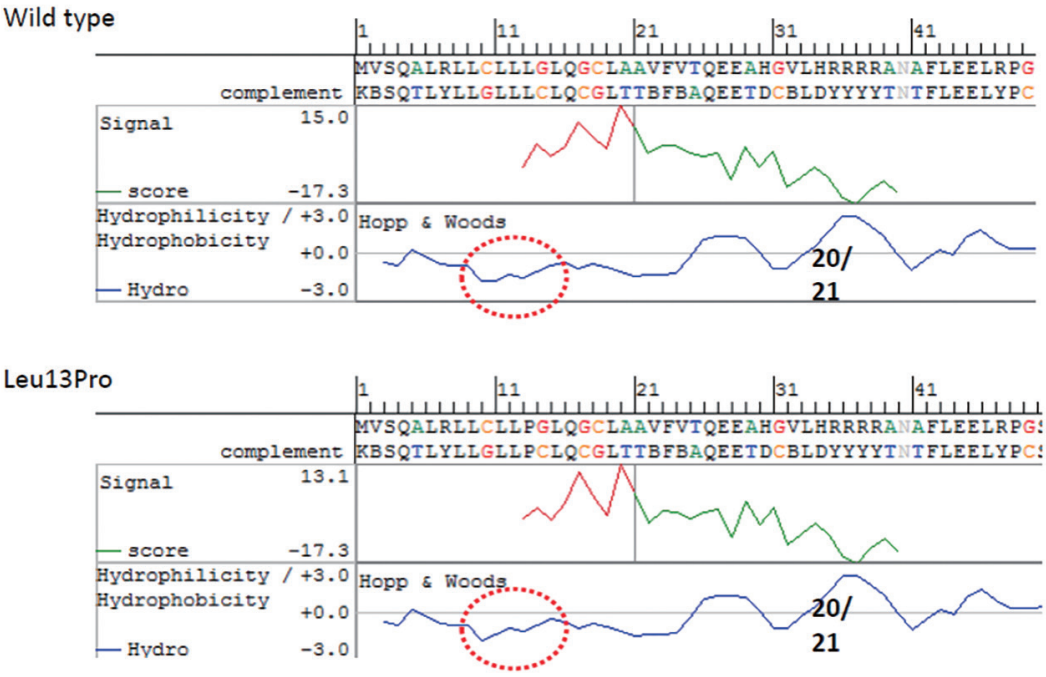

Fig. 4. Hydrophilicity and hydrophobicity of the signal peptide region predicted using SignalP 4.1 software. The hydrophilicity of the mutant peptide was slightly higher than that of the wild-type peptide (circled region).

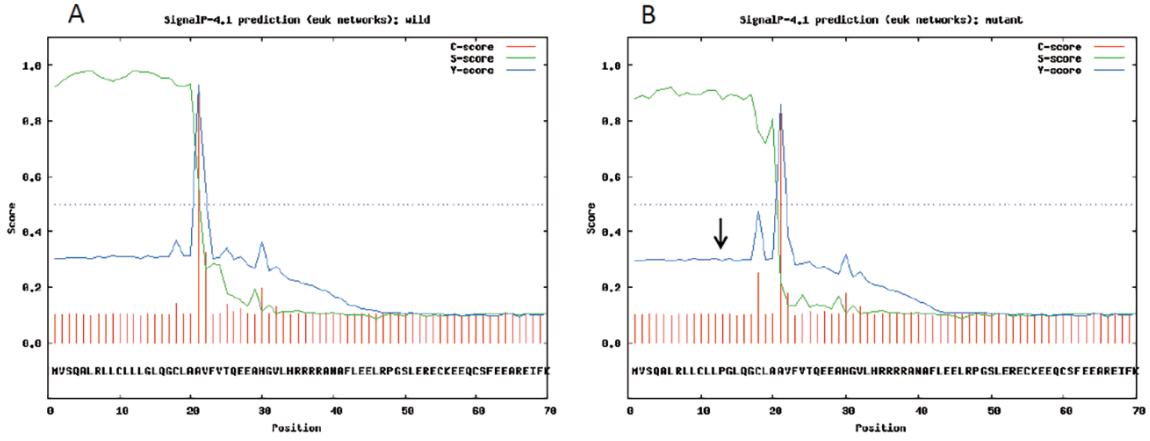

Fig. 5. Sequence and structure analysis of the Leu13 to Pro mutation using SignalP 4.1 software. The curves of several scoring metrics are shown. Arrow indicates the mutation sites. The highest peak of the curve for the Y-score (blue curve) indicates the most likely cleavage site of the signal peptide. According to this prediction, the cleavage position of the wild-type (a) and mutated (b) signal peptide is the same.

T, and −65 G to C), and the other is a two-bp deletion, −60_−59 delTT. Each of these mutations causes either reduced HNF-4 binding activity or reduced transcriptional activity. These mutation sites are very close to each other, and are also close to the novel mutation in the 5' promoter region (−58G>C) identified in this case. An EMSA showed that the mutation in the promoter region reduced the binding of HNF. This suggests that the region at nucleotide −55 to −65 in the promoter region is critical for sufficient transcription.

The clinical phenotype of FVII deficiency is heterogeneous, and there is a poor correlation between coagulant activity and the severity of bleeding symptoms. Benlakhal et al. retrospectively investigated 157 surgical procedures performed on 83 unrelated patients with FVII deficiency but without FVII replacement [30]. They showed that a previous history of deep traumatic hematoma, but not FVII activity, was correlated with bleeding at surgery.

FVII deficiency is not only related to bleeding tendency but is also relevant to thrombosis. Girolami et al. reviewed FVII-deficient cases, and they demonstrated that thrombosis occurred more frequently in cases with cross-reactive material (CRM)-positive variants [31]. This phenomenon may reflect the complexity of FVII deficiency. The function of FVII in coagulation and hemostasis needs further investigation.

REFERENCES

1. Radcliffe R and Nemerson Y. Activation and control of factor VII by activated factor X and thrombin. Isolation and characterization of a single chain form of factor VII. J Biol Chem 1975; 250(2):388-395.
2. Neuenschwander PF, Fiore MM, and Morrissey JH. Factor VII autoactivation proceeds via interaction of distinct protease-cofactor and zymogen-cofactor complexes. Implications of a two-dimensional enzyme kinetic mechanism. J Biol Chem 1993; 268(29):21489-21492.
3. Butenas S and Mann KG. Kinetics of human factor VII activation. Biochemistry 1996; 35(6):1904-1910.
4. Osterud B and Rapaport SI. Activation of factor IX by the reaction product of tissue factor and factor VII: additional pathway for initiating blood coagulation. Proc Natl Acad Sci USA 1977; 74(12):5260-5264.
5. Bauer KA, Kass BL, ten Cate H, Hawiger JJ, and Rosenberg RD. Factor IX is activated in vivo by the tissue factor mechanism. Blood 1990; 76(4):731-736.
6. Pfeiffer RA, Ott R, Gilgenkrantz S, and Alexandre P. Deficiency of coagulation factors VII and X associated with deletion of a chromosome 13 (q34). Evidence from two cases with 46,XY,t(13;Y)(q11;q34). Hum Genet 1982; 62(4):358-360.
7. O'Hara PJ, Grant FJ, Haldeman BA, Gray CL, Insley MY et al. Nucleotide sequence of the gene coding for human factor VII, a vitamin K-dependent protein participating in blood coagulation. Proc Natl Acad Sci USA 1987; 84(15):5158-5162.
8. Suzuki Y, Tsunoda T, Sese J, Taira H, Mizushima-Sugano J et al. Identification and characterization of the potential promoter regions of 1031 kinds of human genes. Genome Res 2001; 11(5):677-684.
9. Pollak ES, Hung HL, Godin W, Overton GC and High KA. Functional characterization of the human factor VII 5'-flanking region. J Biol Chem 1996; 271(3):1738-1747.
10. Peyvandi F, Menegatti M and Palla R. Rare bleeding disorders: worldwide efforts for classification, diagnosis, and management. Semin Thromb Hemost 2013; 39(6):579-584.
11. Mannucci PM and Tuddenham EG. The hemophilias--from royal genes to gene therapy. N Engl J Med 2001; 344(23):1773-1779.
12. Mariani G, Herrmann FH, Dolce A, Batorova A, Etro D et al. Clinical phenotypes and factor VII genotype in congenital factor VII deficiency. Thromb Haemost 2005; 93(3):481-487.
13. Napolitano M, Di Minno MN, Batorova A, Dolce A, Giansily-Blaizot M et al. Women with congenital factor VII deficiency: clinical phenotype and treatment options from two international studies. Haemophilia 2016; 22(5):752-759.
14. Beroud C, Hamroun D, Collod-Beroud G, Boileau C, Soussi T et al. UMD (Universal Mutation Database): 2005 update. Hum Mutat 2005; 26(3):184-191.
15. The F7 mutations database [updated 18/08/18 Available from: http://www.umd.be/F7/W_F7/index.html.
16. Hewitt J, Ballard JN, Nelson TN, Smith VC, Griffiths TA et al. Severe FVII deficiency caused by a new point mutation combined with a previously undetected gene deletion. Br J Haematol 2005; 128(3):380-385.
17. Cooper DN, Millar DS, Wacey A, Banner DW, and Tuddenham EG. Inherited factor VII deficiency: molecular genetics and pathophysiology. Thromb Haemost 1997; 78(1):151-160.
18. Giansily-Blaizot M, Aguilar-Martinez P, Biron-Andreani C, Jeanjean P, Igual H et al. Analysis of the genotypes and phenotypes of 37 unrelated patients with inherited factor VII deficiency. Eur J Hum Genet 2001; 9(2):105-112.
19. Arbini AA, Pollak ES, Bayleran JK, High KA, and Bauer KA. Severe factor VII deficiency due to a mutation disrupting a hepatocyte nuclear factor 4 binding site in the factor VII promoter. Blood 1997; 89(1):176-182.
20. Ozawa T, Takikawa Y, Niiya K, Ejiri N, Suzuki K et al. Factor VII Morioka (FVII L-26P): a homozygous missense mutation in the signal sequence identified in a patient with factor VII deficiency. Br J Haematol 1998; 101(1):47-49.
21. Petersen TN, Brunak S, von Heijne G, and Nielsen H. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods 2011; 8(10):785-786.
22. Suzuki K, Sugawara T, Ishida Y, and Suwabe A. Compound heterozygous mutations (p.Leu13Pro and p. Tyr294*) associated with factor VII deficiency cause impaired secretion through ineffective translocation and extensive intracellular degradation of factor VII. Thromb Res 2013; 131(2):166-172.

23. Rizzotto L, Pinotti M, Pinton P, Rizzuto R, and Bernardi F. Intracellular evaluation of ER targeting elucidates a mild form of inherited coagulation deficiency. Mol Med 2006; 12(7-8):137-142.
24. Sladek FM, Zhong WM, Lai E, and Darnell JE Jr. Liver-enriched transcription factor HNF-4 is a novel member of the steroid hormone receptor superfamily. Genes Dev 1990; 4(12B):2353-2365.
25. Erdmann D and Heim J. Orphan nuclear receptor HNF-4 binds to the human coagulation factor VII promoter. J Biol Chem 1995; 270(39):22988-22996.
26. Carew JA, Pollak ES, Lopaciuk S, and Bauer KA. A new mutation in the HNF4 binding region of the factor VII promoter in a patient with severe factor VII deficiency. Blood 2000; 96(13):4370-4372.
27. Kavlie A, Hiltunen L, Rasi V, and Prydz H. Two novel mutations in the human coagulation factor VII promoter. Thromb Haemost. 2003; 90(2):194-205.
28. Zheng XW, Kudaravalli R, Russell TT, DiMichele DM, Gibb C et al. Mutation in the factor VII hepatocyte nuclear factor 4α-binding site contributes to factor VII deficiency. Blood Coagul Fibrinolysis 2011; 22(7):624-627.
29. Giansily-Blaizot M, Lopez E, Viart V, Chafa O, Tapon-Bretaudiere J et al. Lethal factor VII deficiency due to novel mutations in the F7 promoter: functional analysis reveals disruption of HNF4 binding site. Thromb Haemost 2012; 108(2):277-283.
30. Benlakhal F, Mura T, Schved JF, Giansily-Blaizot M, and French Study Group of Factor VIID. A retrospective analysis of 157 surgical procedures performed without replacement therapy in 83 unrelated factor VII-deficient patients. J Thromb Haemost 2011; 9(6):1149-1156.
31. Girolami A, Sambado L, Bonamigo E, Ferrari S, and Lombardi AM. Pathogenetic role of Factor VII deficiency and thrombosis in cross-reactive material positive patients. Lab Hematol 2013; 19(4):17-21.