Novel factor VII gene mutations in six families with hereditary coagulation factor VII deficiency

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Abstract
Introduction: Hereditary human coagulation factor VII (FVII) deficiency is an inherited autosomal recessive hemorrhagic disease involving mutations in the F7 gene. The sites and types of F7 mutations may influence the coagulation activities of plasma FVII (FVII:C) and severity of hemorrhage symptoms. However, the specific mutations that impact FVII activity are not completely known.

Methods: We tested the coagulation functions and plasma activities of FVII in seven patients recruited from six families with hereditary FVII deficiency and sequenced the F7 gene of the patients and their families. Then, we analyzed the genetic information from the six families and predicted the structures of the mutated proteins.

Results: In this study, we detected 11 F7 mutations, including four novel mutations, in which the mutations p.Phe84Ser and p.Gly156Cys encoded the Gla and EGF domains of FVII, respectively, while the mutation p.Ser339Leu encoded the recognition site of the enzymatic protein and maintained the conformation of the catalytic domain structure. Meanwhile, the mutation in the 5′ untranslated region (UTR) was closely associated with the mRNA regulatory sequence.

Conclusion: We have identified novel genetic mutations and performed pedigree analysis that shed light on the pathogenesis of hereditary human coagulation FVII deficiency and may contribute to the development of treatments for this disease.

KEYWORDS
factor VII, gene mutation, hereditary FVII deficiency, pedigree analysis, protein structure
1 | INTRODUCTION

Hereditary human coagulation factor VII (FVII) deficiency is an inherited autosomal recessive hemorrhagic disease with extended prothrombin time (PT) and normal activated partial thrombin time (APTT). The pathogenesis of hereditary FVII deficiency involves mutation of the F7 gene, which exists in the autosome 13q34. There is considerable heterogeneity in the clinical manifestations of hereditary FVII deficiency that heterozygotes usually exhibit no bleeding symptoms, but multiple forms of bleeding are observed in homozygotes or compound heterozygotes such as those with rhinorrhea, dermal ecchymosis, hemarthroses, menometrorrhagia, and life-threatening hemorrhage, including visceral hemorrhage and intracranial hemorrhage.

Factor VII is a vitamin-K-dependent glycoprotein that plays an important role in the initiation of the endogenous coagulation pathway. Mature FVII in the plasma is composed of 406 amino acid residues and has a relative molecular weight of 50 kDa. In circulation, FVII is activated to form factor VIIa (FVIIa), which consists of two chains connected by a disulfide bridge between Cys135 and Cys262. Although FVIIa has almost no catalytic activity, it can bind to cell surface receptor tissue factor (TF) in the presence of calcium ions and trigger coagulation at the injury site, which further activates factor IX (FIX) or factor X (FX) and eventually leads thrombin generation and fibrin clot formation.

The gene encoding FVII spans approximately 12.8 kb and consists of nine exons, with a precursor sequence containing a hydrophobic translocation signal and a presequence that is important for γ-carboxylation. Exon 2 encodes the prepeptide and gamma-carboxyglutamic acid (Gla) domain, while exon 3 encodes a short hydrophobic region called aromatic stack helix. Exons 4 and 5 encode the first and second epidermal growth factor-like (EGF) domains while Exons 6–8 provide genetic information for the serine protease catalytic domain. It has been reported that most single nucleotide missense mutations in F7 reduce plasma FVII level, while short deletions (2–30 nucleotides) in this gene are rare.

Previous studies have revealed that the severity of hemorrhage symptoms has no significant correlation with blood coagulation factor activity and plasma antigen level. Large number of amino acid changes caused by genetic mutations have been confirmed to be associated with FVII deficiency, although the specific mutations that influence FVII activity and function remain to be fully elucidated. Therefore, investigating F7 gene mutations in patients may further reveal the pathogenesis and development of hereditary FVII deficiency, and facilitate the development of effective therapeutic approaches. In this study, we performed coagulation index tests and gene sequencing on seven hereditary FVII deficiency patients from six families and some of their family members. Furthermore, we explained the pathogenesis of the disease based on an analysis of genetic information of these families.

2 | MATERIALS AND METHODS

2.1 | Patients

We recruited seven patients (five females and two males) with hereditary FVII deficiency from six families between January 2012 and September 2020 from the Department of Hematology, Qilu Hospital, Shandong University and Shandong Hemophilia Treatment Center. The patients were diagnosed based on phenotypic examination and genetic detection. Patients who had a history of tumor, liver and kidney dysfunction, HIV, HPV, HBV, or other viral infections were excluded. Ethical approval was obtained from The Medical Ethics Committee of Qilu Hospital, Shandong University. Written consent was obtained from all patients and their family for participation in the study according to the Declaration of Helsinki.

2.2 | Collection and processing of peripheral blood samples

We collected blood samples from seven patients and fifteen related family members by venipuncture into plastic tubes containing 1/9th volume of 0.109 mol/L buffered trisodium citrate, then centrifuged at 1500 × g for 15 min at room temperature. The upper platelet-poor plasma (PPP) was used for the detection of various blood coagulation factors, and the substratum blood cells were cryopreserved at ~80°C until used for extracting genomic DNA.

2.3 | Coagulation tests and factor VII assays

Activated partial thrombin time, PT, thrombin time (TT), plasma fibrinogen (Fg), and other indicators suggestive of coagulation function were detected on STA-R automated coagulation analyzer (STAGO STA-R MAX) with corresponding reagents. Coagulation activities of the plasma FVII (FVII:C) were determined using recombinant human tissue factor (Stago) and corresponding factor-deficient plasma (Stago).

2.4 | DNA isolation and PCR amplification

We extracted genomic DNA from peripheral blood cells using the phenol-chloroform method with a commercial genomic DNA extraction kit (Tiangen) according to the manufacturer’s instructions. Then, 11 pairs of primers were designed for the 9 exons, 5′ and 3′ UTRs, and flanking sequence of the F7 gene using Primer Premier 5.0 software (GenBank J02933.1).

2.5 | PCR product sequencing

PCR products were purified and sequenced by Shanghai Biosune Biotechnology Company, using the Sanger dideoxy chain-termination
method with an ABI3730XL sequencer. The sequenced PCR products were compared with the F7 gene sequence (NCBI NM_000131.4 and NM_001255.2) published by NCBI Gene Bank of the United States using Blaster software to identify the mutation site and then confirmed by reverse sequencing.

2.6 | Bioinformatics analysis

Conservation of the amino acid mutation sites was analyzed using ClustalW software, while the changes in local space configurations and molecular inter-atomic forces between wild-type and mutant FVII protein models were analyzed using PyMOL2.4 software and Swiss-PDB Viewer 4.1.0 (https://spdbv.vital-it.ch/). The pathogenicity of missense variants identified was evaluated using Sorting Intolerant From Tolerant (SIFT, http://sift.jcvi.org/www/SIFT_enst_submit.html) and Polymorphism Phenotyping Version2.2.2 (POLYPHEN-2, http://genetics.bwh.harvard.edu/pph2/index.shtml) software programs.

3 | RESULTS

3.1 | Clinical information and coagulation indexes of probands

Clinical manifestations and relevant coagulation indicators of seven probands with hereditary FVII deficiency, including two males and five females, are summarized in Table 1. None of the patients had a history of consanguineous marriage. Four (57.1%) patients had obvious bleeding symptoms, including one case with short-time inguinal hematoma; one case with anaphylactic purpura, nephritis hematuria, and skin purpura; and two cases with pregnancy hemorrhage. All the probands had prolonged PT compared with the normal range (32.6 ± 8.4s vs. 11-13s), with the highest being 41s, which was 3.15-fold higher than the normal value. All probands had normal APTT (31-43s). Moreover, all the probands exhibited reduced FVII: C levels compared with the normal range (4.4% ± 2.4% vs. 30%), in which five of them were <5%, indicating an increased risk of hemorrhage in these patients, including four patients with bleeding symptoms.

3.2 | Genetic analysis of probands and their family members

The seven probands were analyzed for mutations in the F7 gene, and the results are summarized in Table 2. In total, 11 F7 mutations were detected in six families with hereditary FVII deficiency. These mutations included c.-16T>G in the 5’UTR, p. Gly222Ser (c.64G>A) in exon 1, p. Phe84Ser (c.251T>C) in exon 3, p. Gly156Cys (c.466G>A) in exon 5, and p. Arg364Gln (c.1091G>A), p. Cys389Gly (c.1165T>G), p. His408Gln (c.1224T>G), p. Ser423Ile (c.1268G>T), p. Gln426* (c.1384C>T), and p. Arg462* (c.1276C>T) in exon 9. Among these, p. Ser339Leu in proband 1, c.-16T>G in proband 3, p. Phe84Ser in proband 4, and p. Gly156Cys in proband 5 were novel mutations that have not been reported previously. Most of the detected mutations (63.6%) were located in exon 9, which encoded the catalytic domain and played a key role in the activation of serine protease. One mutation was in exon 1, which encodes the leading presequence of FVII protein, and two mutations distributed in exons 3 and 5 that are involved in encoding the Gla and EGF domains of the protein. Another mutation was present in the 5’ UTR, which is closely associated with the regulatory sequence of the transcribed mRNA (Figure 1). Then, the three newly discovered missense mutations were compared with the predictions of Polyphen-2 and SIFT software and were defined as pathogenic mutations. Meanwhile, the sites of these three mutations were highly conserved among other species, according to ClustalW (Figure 2).

The results of pedigree analysis were in accordance with Mendel’s laws, as shown in Figure 3. Probands 1, 2, 3, 5, 6, and 7 were all compound heterozygous. The double heterozygous mutations in proband 5 included a paternally inherited mutation and a de novo point mutation. The other five compound heterozygous mutations were inherited from the parents. Proband 4 harbored a homozygous mutation.

| Proband | Gender/age | Manifestations | APTT(s) | PT(s) | TT(s) | Fib(g/L) | FVII: C (%) |
|---------|------------|----------------|---------|-------|-------|----------|-------------|
| 1       | Male/18 years | Inguinal hematoma | 35.7    | 32.2  | 15    | 4.45     | 2.00        |
| 2       | Female/42 years | Puerperal hematoma | 28.7    | 24.2  | 14.5  | 3.19     | 5.00        |
| 3       | Male/10 months | –              | 44.7    | 41    | 17.5  | 2.74     | 3.41        |
| 4       | Female/11 years | Anaphylactic purpura, nephritis, hematuria, skin purpura | 33.4    | 38.4  | 15.9  | 2.45     | 2.00        |
| 5       | Female/5 years | –              | 34.7    | 36.4  | 18.1  | 1.99     | 2.00        |
| 6       | Female/34 years | Puerperal hematoma | 34.9    | 38.1  | 16.4  | 2.21     | 2.60        |
| 7       | Female/31 years | –              | 36.6    | 36.3  | 18.1  | 1.98     | 6.80        |

Table 1: Clinical manifestations and relevant coagulation indicators of the seven probands
TABLE 2 Examination of FVII mutations in the seven probands

| Probands | Mutation site | Codon changes | Amino acid changes |
|----------|--------------|---------------|-------------------|
| 1        | Exon 9       | c.1165T>G     | p. Cys389Gly      |
|          | Exon 9       | c.1016C>T     | p. Ser339Leu      |
| 2        | Exon 1       | c.64G>A       | p. Gly225Ser      |
|          | Exon 9       | c.1091G>A     | p. Arg364Gln      |
| 3        | 5′UTR        | c.-16T>G      | —                 |
|          | Exon 9       | c.1276C>T     | p. Gln426*        |
| 4        | Exon 3       | c.251T>C      | p. Phe84Ser       |
|          | Exon 9       | c.1224T>G     | p. His408Gln      |
| 5        | Exon 5       | c.466G>A      | p. Gly156Cys      |
| 6        | Exon 9       | c.1384C>T     | p. Arg462*        |
|          | Exon 9       | c.1268G>T     | p. Ser423lle      |
| 7        | Exon 9       | c.1384C>T     | p. Arg462*        |
|          | Exon 9       | c.1268G>T     | p. Ser423lle      |

3.3 | Protein structures of FVII with novel mutations

We predicted the crystal structures of three newly detected mutant FVII proteins (Figure 4). In proband 1, p. Ser339Leu mutation was detected in exon 9, which encodes the recognition site of the enzymatic protein that plays an important role in maintaining the conformation of the catalytic domain structure. The hydrophobic leucine residue usually forms rotamers and is always in the interior of the protein. When forming β-sheets, leucine often constitutes continuous hydrophobic sheets, while in α-helices, leucine preferentially forms long, unbranched side chains. The conformational changes in the protein may lead to corresponding changes in the catalytic domain and thus affect FVII activation. In proband 4, a phenylalanine was replaced by serine in the Gla domain. Serine is a common phosphorylated amino acid residue that can be a nucleophilic active site, facilitating the formation of turning structures in the protein. The replacement of phenylalanine results in the loss of its benzene ring structure, which changed the structure of side chain and may influence the stability of FVII protein. Proband 5 harbored a homozygous mutation in exon 5, which encodes the second EGF domain, thus resulting in the replacement of Gly156 by cysteine. The sulfur atoms in tyrosine form a polar sulfhydryl that participates in the formation of disulfide bonds, which confer certain structural characteristics to the protein. Random introduction of disulfide bonds at mutation sites may cause adverse tension in the molecule, thus reducing the stability of the protein. Moreover, due to the lack of β-carbon atoms, glycine has higher conformational flexibility compared with other amino acids, and it is easier to achieve the spatial junctions required for protein function. The substitution of glycine at this site may have deleterious effects on the stability of the whole molecule.

4 | DISCUSSION

Hereditary FVII deficiency is an autosomal recessive hemorrhagic disease caused by mutations of the gene encoding FVII protein. It has been revealed that F7 gene mutations could lead to the deficiency of the activation and secretion of FVII protein. To further analyze the possible effects of F7 mutations on FVII activity, we sequenced the F7 gene of seven hereditary FVII deficiency patients and their family members and found 11 mutations including four that have not been reported previously. Studies have revealed that the severity of hemorrhage symptoms has no significant correlation with blood coagulation factor activity and plasma antigen level, but the sites and types of F7 mutations may influence the coagulation activities of plasma FVII and hemorrhage symptoms. We summarized the clinical manifestations related to the mutation sites identified in this study with that in previous studies (Table 3). The clinical symptoms associated with the same mutation site were not consistent, but mutation p. Cys389Gly (c.1165T>G), p. Gly225Ser (c.64G>A), and p. His408Gln (c.1224T>G), and p. Ser423lle (c.1268G>T) were all related to significant bleeding symptoms. Among them, mutation c.64G>A was shown to be associated with puerperal hematoma in both cases. Meanwhile, the nonsense mutation p. Gln426* (c.1276C>T) showed no symptom in both studies. In this study, all the identified mutations were single-point mutations, of which two were nonsense mutations while the remainder were missense mutations. Mutations are distributed throughout the F7 gene, suggesting that all parts of the protein are needed for the maintenance of its overall structure and specific functions. Notably, four patients with FVII deficiency had obvious hemorrhage symptoms, and all of them harbored compound heterozygous mutations in F7 gene. It is considered that the severe mutations which damage synthetic functions of FVII protein are associated with life-threatening bleeding. Moreover, mice with F7 gene mutations die of fatal bleeding on the first day after birth. In our study, the genotype of most patients with severe bleeding symptoms appears to include mutations that reduce the level of functional FVII protein. Proband 1 in our study was a patient with inguinal hematoma, whose plasma FVII activity was <2% of the wild type and had heterozygous p. Cys389Gly and p. Ser339Leu mutations in exon 9, which encodes the important catalytic domain of FVII. Another clinically severe patient, proband 4, was an 11-year-old female with anaphylactic purpura nephritis, hematuria, and skin purpura, who exhibited plasma FVII activity <2% of the wild type and had heterozygous p. Phe84Ser and p. His408Gln mutations in exons 3 and 9, respectively.

The stability of the protein structure is key to maintaining normal protein function. The mutation p. Gly156Cys, detected in proband 4, was a novel mutation. Notably, Gly156 plays an important role in maintaining the protein structure because glycine is always located at spatial angles of the protein or at sites where macromolecular side chains produce steric hindrance to influence the helix structure and enable binding with substrates. Due to the lack of the side chain, glycine has stronger chemical reactivity in the adjacent lateral face and...
mutations at this site could affect both the EGF domain and structural stability of the FVII protein.

Factor VII activation is not a stand-alone process. FVII and FVIIa can form chemical complexes with TF and rapidly convert zymogen to active enzymes,\textsuperscript{18,19} which is widely considered as the major initiation step for endogenous blood coagulation.\textsuperscript{20} Point mutations in F7 gene can lead to the replacement of the free radical in the molecular catalytic domain and are likely to affect the interactions with TF, resulting in FVII activation defects.\textsuperscript{12,21,22} In the mutation p. Arg364Gln (p. Arg304Gln), arginine304 is a highly conserved site of FVII and is homologous with FIX Arg333, FX Arg298, prothrombin Arg490, and protein C His326, which are located in the catalytic domain.\textsuperscript{12} Therefore, p. Arg364Gln could result in conformational changes of FVII protein, reducing TF binding affinity at the binding site via a transmission effect. Besides, the mutation could directly occur at the binding site of TF, affecting multiple functional domains of the enzyme. Another mutation, p. Cys389Gly (p. Cys329Gly) in proband 1, resulted in alterations of important proteins located in the catalytic domain of FVII.\textsuperscript{23,24} Pedigrees with Cys329Gly\textsuperscript{25,26} and Cys329Arg\textsuperscript{27} mutations have been reported previously, in which the levels of FVII antigens were reduced in all patients, with even lower levels of FVII activity. In vitro studies indicated that Cys329Gly had
little effect on the synthesis and secretion of FVII, although the coagulation activity of the mutant protein was almost undetectable. 28 Ser339 was another novel mutation site detected in proband 1, which resulted in a primary aliphatic hydroxy in the catalytic domain of FVII protein; thus, mutations in this site could affect the activity of FVII correspondingly.

FIGURE 3 Mutations in six families with hereditary FVII deficiency. Six of the seven patients harbored compound heterozygous mutations, and five of them inherited the mutations from their parents, while one had a combination of paternally inherited mutation and de novo mutation. One patient inherited a homozygous mutation derived from heterozygous parents.

FIGURE 4 Predicted structure of FVII protein. The newly detected mutations p. Ser339Leu, p. Phe84Ser, and p. Gly156Cys are shown in the protein crystal model in red. The specific structural changes due to the amino acids at these sites in the normal protein sequence and after mutation are shown in red and blue, respectively.
The previously described His348Gln mutation has been proven to result in altered protein secretion pathway. The antigen level of mutant FVII was only 11.0% of the wild-type and when CHO cells were transfected with mutant FVII, only 37.3% of the labeled FVII was secreted into the conditioned medium while the rest remained in the cells. The crystal structure of FVII revealed that the residue His348 is located inside the protein between the catalytic serine and COOH-terminal helix. The substitution of H348 may affect the molecular conformation, thus resulting in abnormal secretion of the mutant protein. We also detected a codon CAG→TAG mutation in proband 5, which resulted in the presence of a premature termination codon at the 426th position at the 3’ end of exon 1 that encodes a pre-pro leader sequence and a pro-sequence, which are important for gamma-carboxylation, may result in inefficient splicing and reduced mRNA levels, and accordingly destruct the secretion and synthesis of FVII.

| Mutation | Type   | Reported symptoms                                                                 | Identified symptoms in our research     |
|----------|--------|-----------------------------------------------------------------------------------|----------------------------------------|
| c.1165T>G (p. Cys389Gly) | Missense | FVII:C:4.40% Repeated hematuria, epistaxis, and oral mucosal bleeding. Patient's sister also had bleeding tendency characterized by menorrhagia and easy bruising | FVII:C:2.00% Inguinal hematoma.         |
| c.64G>A (p. Gly22Ser) | Missense | FVII:C:8.00% Puerperal hematoma                                                   | FVII:C:5.00% Puerperal hematoma         |
| c.1091G>A (p. Arg364Gln) | Missense | FVII:C:0.3 U/ml No symptoms                                                     | FVII:C:5.00% Puerperal hematoma         |
| c.1276C>T (p. Gln426*) | Nonsense | FVII:C:3.00% No symptoms                                                              | FVII:C:3.41% No symptoms                   |
| c.1224T>G (p. His408Gln) | Missense | FVII:C:5.20% Recurrent hematemesis and was referred to the hospital                 | FVII:C:2.00% Anaphylactic purpura, nephritis hematuresis and skin purpura |
| c.1384C>T (p. Arg462*) | Nonsense | Unknown                                                                              | FVII:C:2.60% Puerperal hematoma         |
| c.1268G>T (p. Ser423Ile) | Missense | FVII:C:<1.00% Bleeding after trauma or surgery                                       | FVII:C:2.60% Puerperal hematoma         |

5 | CONCLUSIONS

In conclusion, the present study sequenced the DNA of seven patients from six families with FVII deficiency and members of their respective families and detected 11 kinds of mutations changing the protein structure and affecting the stability and function of FVII. The results of the functional experiments and specific analysis of the protein changes caused by the new mutations will be presented in our future work.

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CONFLICT OF INTEREST

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

DATA AVAILABILITY STATEMENT

The data that provided the evidence for the study are available from the corresponding author upon reasonable request.

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