

In vitro expression of mutant factor VII proteins and characterization of their clinical significance

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Abstract. Factor VII (FVII) serves an essential role in the initiation of blood coagulation. Mutations in conserved residues within its serine protease domain may lead to dysregulated coagulation activity. The objective of the present study was to elucidate the impact of altering two conserved residues, H348R and S282R, on the functional properties of the FVII protein. The mutation-harboring fragments were derived from genomic DNA of a FVII deficient patient. The fragments were integrated into a pcDNA vector containing FVII cDNA of HepG2 cells. The wild-type and mutated FVII constructs were transfected into CHO-K1 cells as a mammalian cell model. The coagulation activity, antigen levels and intracellular localization of the recombinant proteins were studied in association with their pathological importance. Results indicated that FVII activity was not detectable in conditioned media of the cells transfected with the mutated constructs. The H348R mutation reduced the expression of intracellular and secreted forms of the FVII protein. Following S282R transfection, intracellular FVII expression showed no significant variation; however, extracellular protein was reduced. The pattern of intracellular localization of mutated FVII remained unaltered in comparison to the wild-type protein. In conclusion, the present study suggested that missense mutations within the serine protease domain of FVII affect extracellular levels in addition to the coagulation activity of FVII. These results may contribute to further understanding of the molecular pathogenesis of FVII deficiency and the development of pharmaceutical candidates with improved therapeutic properties.

Introduction

Factor VII (FVII) is a vitamin K dependent clotting factor that participates in the early phases of blood coagulation. It is synthesized by the liver as a single chain 50 kDa zymogen with 406 amino acids and is secreted into blood at a concentration of 500 ng/ml (1). FVII initiates the extrinsic pathway in an activated two-chain form called FVIIa, which is generated from the proteolysis of FVII at Arg152-Ile153. It has a light chain containing a γ -carboxy glutamic acid (Gla) domain and two epidermal growth factor-like domains and a heavy chain comprised of a catalytic domain. The two chains are held together by a disulfide bond formed between cysteines 135 and 262 (2). Subsequent to vascular injury and in the presence of calcium ions, plasma FVIIa binds to tissue factor (TF) exposed on extravascular cells and produces a TF-FVIIa complex. This complex is involved in proteolytic activation of coagulation factors IX and X and thrombin production (3).

The *F7* gene is located on chromosome 13 (13q34), 2.8 kb upstream of the gene encoding factor X and comprises 9 exons spanning ~12.5 kb (4). Hereditary FVII deficiency is commonly inherited as an autosomal recessive disorder and has an incidence of 1 per 500,000 in the general population (5). Clinical manifestations of FVII deficiency range from mild bruising to life threatening hemorrhages (6). The hemorrhagic diathesis in affected patients is highly variable and does not necessarily associate with plasma FVII procoagulant activity (FVII:C) levels (7).

A large number of different *F7* gene variants, including missense, nonsense, promoter and splice site mutations, have been reported. In addition, the functional impact of certain variants has been investigated in order to provide evidence for the underlying molecular mechanisms of FVII deficiency (8,9).

The present study investigated the functional characteristics of H348R and S282R mutations within the serine protease domain of FVII, which was detected in compound heterozygous status in an Iranian patient with FVII deficiency. Ser282 and His348 residues are located on exon 8 of the *F7* gene and are highly conserved across different species. These mutations were previously reported (10,11); however, this patient appears to be the first case harboring the two mutations simultaneously. In the present study, the mutated *F7* gene was expressed in mammalian cells to determine different functional aspects

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of FVII biosynthesis including RNA expression, protein secretion, coagulation activity and intracellular localization of the protein. The confirmation of pathogenicity of these mutations paves the way for the management of the disease and genetic counseling for prenatal diagnosis in affected families.

Materials and methods

Cell culture and construction of expression vectors. HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin and 5.5 mM D-glucose (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). These cells are known to express high levels of human FVII; however, the HepG2 cell line is misidentified, according to http://web.expasy.org/cellosaurus/CVCL_0027. Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Inc.) and used for cDNA synthesis (1 µg), using a reverse and first strand cDNA synthesis kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Specific primers with *XhoI* and *EcoRI* recognition sites were used to isolate the *F7* coding region. The resulting polymerase chain reaction (PCR) product had an *XhoI* recognition site on one end and an *EcoRI* site on the other end. The sequence of oligonucleotides used to amplify *F7* cDNA were as follows: Forward, 5'-AGA ATTCTTCATCATGGTCTCCAGG-3' and reverse, 5'-TCT CGAGGCTAGGGAAATGGGGCTCG-3'. The purified PCR product and pcDNA3.1/neo plasmid were doubly digested with *XhoI* and *EcoRI* enzymes (Thermo Fisher Scientific, Inc.) and the digestion products were purified using a Genjet PCR purification kit (Thermo Fisher Scientific, Inc.). Using a fast DNA ligation kit (Bio Basic, Inc., Markham, ON, Canada), purified *F7* cDNA was inserted into the pcDNA3.1/neo vector (Invitrogen; Thermo Fisher Scientific, Inc.). The resulting recombinant vector [pcDNA/wild-type (WT)] was amplified in competent DH5α bacterial cells. Amplified plasmids were isolated and sequenced to verify the cloning process.

The desired mutations were introduced into pcDNA/WT by the insertion of mutation- harboring fragments using suitable restriction enzymes. In order to insert the S282R substitution, a PCR reaction was performed on a patient's genomic DNA to amplify a fragment containing the S282R mutation. The fragment had two *Van9II* recognition sites on both sides of the S282R substitution. The PCR product was digested by the *Van9II* restriction enzyme (Thermo Fisher Scientific, Inc.). The linearized pcDNA/WT vector was also prepared by *Van9II* digestion. Then, a mutation containing fragment was inserted into the vector and the recombinant vector (pcDNA/S282R) was amplified in competent DH5α cells and sequenced.

In the same way, the gene fragment containing the H348R substitution on a patient's DNA was amplified by PCR. The reverse primer comprised an *XhoI* site at its 5'-end. First, the PCR product was partially digested by *Van9II* and then completely digested by *XhoI*. The digestion product was inserted into the linearized pcDNA/WT and doubly digested by *XhoI* and *Van9II*. The resulting recombinant vector (pcDNA/H348R) was amplified and verified by sequencing.

CHO-K1 cell culture and transfection. CHO-K1 cells were grown in Glutamax DMEM-F12 (Gibco, KBC, Iran) medium

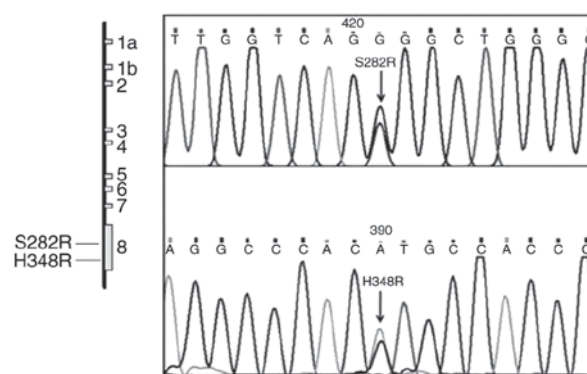


Figure 1. Sequencing of mutated FVII proteins. A schematic presentation of *F7* exons and the location of S282R and H348R mutations on exon 8 (left). H348R and S282R mutations detected in compound heterozygous status in a FVII deficient patient (right). FVII, factor VII.

supplemented with 10% FBS and 1% penicillin and streptomycin. The adherent cells were suspended using trypsin and 4×10^4 cells were transferred to 60-mm dishes for each transfection reaction. According to the manufacturer's instructions, turbofect (Thermo Fisher Scientific, Inc.) was used to transiently transfect the cells with pcDNA/WT, pcDNA/S282R and pcDNA/H348R vectors. To estimate transfection efficiency, a vector containing green fluorescence protein (GFP) reporter gene (pcDNA/GFP) and the empty vector (pcDNA) were used as transfection controls. Three microscopic fields (x40) of each cell culture dishes were randomly observed 24 h following transfection and GFP positive as well as GFP negative cells were counted. The transfection efficiency was calculated using the formula:

$$\text{Transfection efficiency} = \frac{\text{number of GFP positive cells}}{\text{total number of counted cells}}$$

Transfected cells were incubated for 48 and 72 h at 37°C in 5% CO₂ prior to harvesting. Conditioned medium of each dish was collected for FVII protein measurement. Total RNA content of the cells was extracted using TRIzol reagent (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and cell lysates were prepared using freeze-thaw method (3 cycles of 70°C/37°C). All samples were stored at -70°C until further analysis.

Reverse transcription-PCR (RT-PCR) and protein expression assays. To confirm *F7* RNA expression by the transfected cells, RT-PCR analysis was performed on the RNA extracted from transfected CHO-K1 cells using the QIAzol reagent (cat. no. 79306; Qiagen GmbH, Hilden, Germany) as recommended by the manufacturer's instructions. The concentration and quality of the extracted RNA was determined using a NanoDrop spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). The extracted RNAs were used directly for cDNA synthesis by PrimeScript™ RT kit (cat. no. RR014A; Takara Biotechnology Co., Ltd., Dalian, China) using the following temperature program; RNA denaturation: 65°C for 5 min, reverse transcription: 42°C for 15 min followed by 85°C for 5 min to inactivate the reverse transcriptase enzyme. The primers for *F7* CD2 and β-actin were as follows, respectively; *F7* CD2 forward, TGTGTG

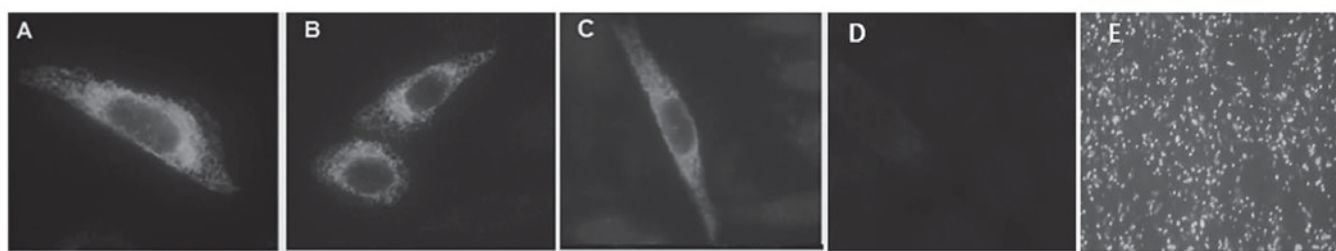


Figure 2. Immunostaining of CHO-K1 cells transfected with recombinant FVII variants using an anti-FVII antibody. Perinuclear staining was observed using fluorescence microscopy (magnification, x100) in cells transfected with (A) WT, (B) H348R and (C) S282R FVII. (D) CHO-K1 cells transfected with the empty vector were used as controls and did not react with the antibody. (E) A representative view (magnification, x40) of CHO-K1 cells transfected with a GFP-expressing vector, suggesting high efficiency ($63\pm 4.1\%$) of the transfection process. FVII, factor VII; WT, wild-type; GFP, green fluorescent protein.

AACGAGAACGGCG and reverse, ACCTTCCGTGACTGCTGC; and β -actin forward, AGAGCTACGAGCTGCCTGAC and reverse, AGCACTGTGTTGGCGTACAG, which were used to amplify the *F7* CD2 and β -actin transcript cDNA with PCR master mix (cat. no. K0171; Thermo Fisher Scientific, Inc.) under the following conditions; denaturation at 95°C for 45 sec, annealing for 40 sec at 56°C , elongation at 72°C for 50 sec, for a total of 30 cycles. The PCR products on electrophoresis agarose gel (2%) were stained with SYBR Green I dye (1:10,000; Thermo Fisher Scientific, Inc.). Relative mRNA ratio was calculated using ImageJ software 2.0 (National Institutes of Health, Bethesda, MD, USA) with β -actin as an internal control (12).

FVII procoagulant activity (FVII:C) and antigen levels (FVII:Ag) were measured in conditioned media and cell lysate samples, 48 and 72 h following transfection. FVII:C was determined by the one-stage prothrombin time (PT)-based method (1) using FVII-deficient plasma as a substrate and commercial human thromboplastin preparation. This procedure was performed by an automated Sysmex CA-1500 coagulation analyzer system. The concentration of FVII protein in conditioned media and cell lysates was analyzed using a commercial ELISA assay (cat. no. ab108829, human factor VII ELISA kit; Abcam, Cambridge, UK).

Immunocytochemistry. In order to study intracellular localization of WT and mutated FVII protein, immunocytochemistry was performed on CHO cells. Transfected cells were seeded onto glass coverslips and fixed with 3% paraformaldehyde for 1 h at room temperature. Permeabilization was performed with 0.1% Triton X-100 (Sigma-Aldrich; Merck KGaA) for 10 min at room temperature and the coverslips were blocked with 1% bovine serum albumin in PBS (Sigma-Aldrich; Merck KGaA). A rabbit anti-human FVII antibody (1:150, cat. no. ab97614; Abcam) was added to each coverslip and incubated for 1 h at 37°C . Following incubation and PBS washing steps, the cells were incubated with Dylight 488 goat anti-rabbit IgG (1:250, cat. no. ab96899; Abcam) for 30 min at 37°C and 10 fields of each slide were analyzed under a fluorescent microscope (Olympus Corporation, Tokyo, Japan).

Statistical analysis. The comparison between mean levels of protein expression in different study groups was performed using a one-way analysis of variance followed by a Tukey's multiple comparison test using GraphPad Prism V.7.03

(GraphPad software, Inc., La Jolla, CA, USA). Data are expressed as the mean \pm standard deviation. $P < 0.05$ was determined to indicate a statistically significant difference.

Results

Mutagenesis and transfection of CHO cells. The sequencing of pcDNA/WT, pcDNA/S282R and pcDNA/H348R vectors indicated that the 2 mutations were successfully created at the desired positions on *F7* cDNA (Fig. 1). The control CHO cells transfected with pcDNA/GFP in parallel with the mutated *F7* expression vectors indicated appropriate transfection efficiency with $63\pm 4.1\%$ GFP-positive cells (Fig. 2).

Expression of the mutated transcripts and proteins. RT-qPCR demonstrated that transfected cells expressed the WT, S282R and H348R transcripts, indicating that the mutations had no effect on *F7* mRNA expression (data not shown). Ser282 and His348 residues on exon 8 are highly conserved amino acids across different species, which have remained unaltered through evolution (Table I). To investigate the effects of mutations on procoagulant activity of FVII (FVII:C), conditioned media was analyzed using a one-stage PT-based method. No FVII procoagulant activity was detected in conditioned media of the cells transfected with the mutated constructs compared with cells expressing the WT construct (Table II). Also, an ELISA assay was used to determine the concentration of FVII antigen (FVII:Ag) in the conditioned media as well as in the lysate of transfected cells (Fig. 3). The mean concentration of the FVII antigen in conditioned media of the cells harboring WT vector was 23.84 ng/ml, whereas in the pcDNA/S282R or pcDNA/H348R groups, it was 4.3-fold (5.5 ng/ml; Table II and Fig. 3). In addition, conditioned media of the cells that were co-transfected with pcDNA/S282R and pcDNA/H348R had reduced extracellular FVII levels (Fig. 3).

In the lysate of the cells expressing the H348R allele, FVII antigen level was reduced by $\sim 32\%$ (96.85 ng/ml) compared with the cells expressing the WT allele (142.65 ng/ml). FVII antigen in the lysate of the cells expressing S282R allele was comparable to that of the WT allele. However, in co-transfected cells, the protein expression levels were enhanced (195.8 ng/ml) compared with the WT group (Table II).

Immunocytochemistry. Intracellular localization of WT, S282R and H348R FVII proteins was determined by fluorescent microscopy. Perinuclear weak staining was observed in

Table I. Conservation of S282 and H348 residues across different species.

Protein ID species	Protein sequence (S282R)	Protein sequence (H348R)
spIP22457IFA7_BOVIN	AFVRFSAVSGWGQLLERGV	SKDACKGDSGGPHATRFRGTWFL
spIP08709IFA7_HUMAN	AFVRFSLVSGWGQLLDRGAT	SKDSCKGDSGGPHATHYRGTWYL
spIQ2F9P2IFA7_PANTR	AFVRFSLVSGWGQLLDRGAT	SKDSCKGDSGGPHATHYRGTWYL
spIQ2F9P4IFA7_PANPA	AFVRFSLVSGWGQLLDRGAT	SKDSCKGDSGGPHATHYRGTWYL
spIQ8K3U6IFA7_RAT	ASIRFSRVSGWGQLLDRGAT	FKDACKGDSGGPHATHYHGTWYL
spIP70375IFA7_MOUSE	ARIRFSRVSGWGQLLDRGAT	TKDACKGDSGGPHATHYHGTWYL

Table II. Transient expression of recombinant FVII variants by transfected CHO-K1 cells.

FVII variants	WT	S282R	H348R	Co-transfection
FVII:Ag (conditioned medium, ng/ml)	23.84	5.5	5.5	7.2
FVII:Ag (cell lysate, ng/ml)	142.65	141.22	96.85	195.8
FVII:C (conditioned medium, %)	100	ND	ND	ND

ND, not detectable; WT, wild-type; FVII:Ag, factor VII antigen; FVII:C, factor VII procoagulant activity.

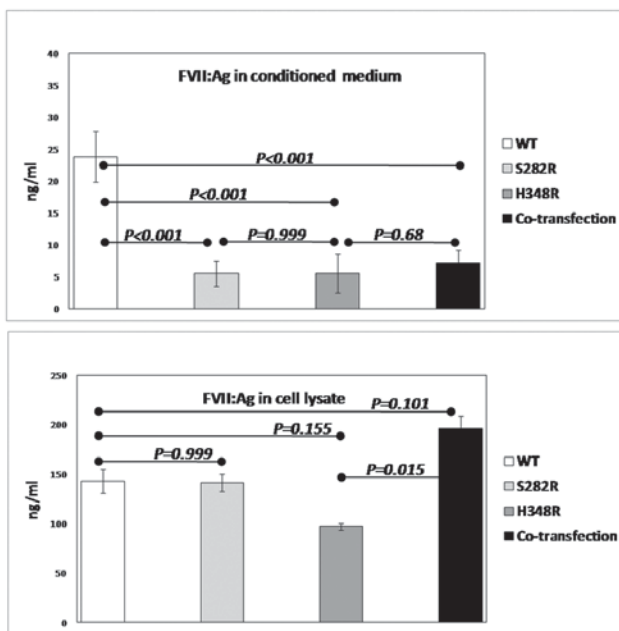


Figure 3. The concentration of different FVII protein variants WT, S282R, H348R were determined by ELISA in cell culture conditioned medium and cell lysate of transfected CHO-K1 cells. The ELISA assay standard curve was generated using serially increased concentrations of standard FVII antigen. Remarkable reduction in secreted FVII protein was observed in cell culture media of the cells transfected with mutant vectors, while intra-cellular FVII protein content was comparable to the cells transfected with WT vector. The mean \pm standard deviation concentration of FVII protein was calculated as the average of values detected in triplicate experiments. FVII:Ag, factor VII antigen; WT, wild-type.

the cytoplasm of the cells expressing WT protein (Fig. 2A). The same observation of perinuclear staining was observed in the cells expressing the mutated FVII (Fig. 2B and C). The mutated proteins exhibited similar immunofluorescence intensity to

that of the WT protein, suggesting that the mutations had no marked effect on the intracellular localization of FVII.

Discussion

In the present study, H348R and S282R mutations within serine protease domain of FVII were investigated, as these mutations were detected in compound heterozygous status in an Iranian patient with an FVII deficiency. Ser282 and His348 residues on exon 8 are highly conserved amino acids across different species, which have remained unaltered through evolution. Exon 8 is the largest exon of the gene and accommodates the majority of missense mutations scattered across the *F7* gene. This exon encodes for the catalytic domain of FVII protein, which has serine protease activity and mediates factor X activation by proteolytic cleavage (13). Mutations in this domain may severely affect the coagulation activity and the secretion of the FVII protein. However, considerable evidence now supports the concept that there is no clear genotype-phenotype association in FVII deficiency (14). Preceded by several publications based on various mutations, it has been demonstrated that the disease manifestations, including epistaxis, hemarthrosis and menorrhagia, do not always associate with FVII:C (1,15). Recently, Quintavalle *et al* (14) demonstrated the variations in clinical and molecular aspects of the FVII deficiency disease. They reported an association between the type of *F7* variant and FVII:C levels, but not for bleeding tendency and FVII:C.

Using immunofluorescent staining, the present study demonstrated that perinuclear localization of both S282R and H348R protein variants was similar to that of the WT protein (Fig. 2). Normally, the FVII protein is predominantly localized to the perinucleus with cytoplasmic expression. This suggests that FVII accumulates in the endoplasmic reticulum (ER) and golgi apparatus in order to achieve correct folding

and modifications prior to secretion (3). Certain mutations may alter this localization due to impaired secretion pathway or degradation of FVII in various cellular compartments (16).

Generally, there is a certain degree of difference between intra- and extra-cellular levels of various secreted proteins. This difference may be due to the accumulation of the protein in the ER or golgi apparatus prior to secretion. In the case of FVII, the protein accumulates inside the cell until it gains the correct post-transcriptional modifications and conformational properties. In order to reveal the effect of the mutations on FVII secretion, FVII:Ag was measured in conditioned media of transiently transfected cells. The present study indicated that S282R and H348R substitutions reduced the secretion of the protein variants into the conditioned media. This result is consistent with the results of previous studies on other *F7* gene mutations (17,18). In the ELISA assay, which was performed on the lysates of the transfected cells, there was a discrepancy regarding the impact of the two mutations on the intra-cellular FVII levels. The quantitative measurement of intracellular FVII in the cells transfected with the H348R variant exhibited reduced levels of the protein compared with the WT protein, whereas the intracellular levels of the mutated S282R FVII was equal to the WT control. The difference in the expression and stability of FVII protein variants has been previously reported indicating the distinct effect of each mutation on the overall intracellular FVII protein content in transfected cells (5,19).

The enzymatic activity (FVII:C) of the recombinant FVII variants in conditioned media was investigated using a PT-based assay, which indicated no detectable coagulation activity in S282R and H348R media. However, the concentration of the secreted proteins (5.5 ng/ml) was very low in the media, which may lead to undetectable FVII:C activity. However, the present study did not study the function of the proteins in the cell lysate samples. Therefore, further functional analysis of the mutations is required to determine the activity of the two protein variants and to explain the discrepancy in their intra-cellular levels.

In conclusion, the results of the present study indicated that S282R and H348R substitutions within the FVII serine protease domain affected the secretion of the enzyme. Although the pattern of intracellular localization of the mutated proteins remained unaltered, there were differences between S282R and H348R intra-cellular levels. The validation of pathogenic effects of these mutations may be implemented for genetic counseling and prenatal diagnosis of FVII deficiency in families with affected children in the future.

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