Functional and Molecular Characterization of C91S Mutation in the Second Epidermal Growth Factor-like Domain of Factor VII

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Background: Coagulation Factor VII is a vitamin K-dependent serine protease which has a pivotal role in the initiation of the coagulation cascade. The congenital Factor VII deficiency is a recessive hemorrhagic disorder that occurs due to mutations of F7 gene. In the present study C91S (p.C91S) substitution was detected in a patient with FVII deficiency. This mutation has not been characterized by a functional study.

Objectives: In this study, we aimed to evaluate the impact of C91S substitution on factor VII expression and function.

Materials and Methods: The F7 complete cDNA was isolated from HepG2 cell line and inserted into the pcDNA3.1 mammalian expression vector. The desired mutation was generated by the site-directed mutagenesis and the wild-type and mutated constructs were transfected into CHO-K1 cells. The protein activity and antigen level (antigen concentration) were validated in the culture medium and cell lysate of the transiently transformed cells. An immunocytochemistry procedure was also performed to evaluate the intracellular localization of the mutated and the wild-type FVII, as well.

Results: The present in vitro study has demonstrated that C91S antigen expression was increased in the transfected CHO-K1 cells compared to the wild-type (WT) protein. Despite an increased protein secretion, the factor VII coagulant activity was diminished following C91S substitution when it was assessed by a standard one-stage analysis. In addition, the immunocytochemistry procedure revealed that there was no difference in the intracellular localization of the C91S mutated FVII compared to the WT protein.

Conclusions: Our results present that C91S mutation has an effect on the coagulation activity, secretion, biosynthesis, and probably folding of the FVII leading to the FVII deficiency.

Keywords: Coagulation activity, F7 gene, Functional study, Site-directed mutagenesis

1. Background

The human FVII protein is a vitamin-k dependent glycoprotein which circulates in the plasma with a normal concentration of 500 ng.mL⁻¹. It is synthesized and secreted into the blood by the liver. The mature FVII molecule is a single chain protein composed of 406 amino acids and has a molecular weight of 50 kD. This molecule is comprised of several discrete domains including the Gla domain (gamma-carboxy glutamic acid domain), two epidermal growth factor (EGF)-like domains, and a catalytic domain (serine protease) (1). FVII initiates extrinsic blood coagulation pathway. Upon vascular injury, FVII forms a complex with its receptor and cofactor; the tissue factor (TF) in the presence of calcium ion. Then, FVII in the complex is rapidly cleaved to its two-chained active form (FVIIa) and its catalytic activity multiplies so that FVIIa converts factor IX and X zymogens into active enzymes (1, 2). The complete F7 gene (NM 000131) has been cloned and sequenced by O’Hara and colleagues in 1987 (3). It is localized on the 13q34 and comprises of the 9 exons spanning about 13 kb (3). The hereditary FVII deficiency (MIM 227500) is a rare autosomal recessive bleeding disorder with
the variable clinical expressions that range from life-threatening to the very mild hemorrhages (4). This deficiency has an estimated incidence rate of 1 per 300,000-500,000 individuals in different populations (5, 6). There is a considerable molecular and phenotypic heterogeneity in the congenital FVII deficiency (7). A large number of molecular defects have been described in FVII-deficient patients that impair FVII biosynthesis and/or function (7, 8). The therapeutic strategies for the FVII deficiency are currently based on the frequent administration of the fresh frozen plasma or recombinant activated FVII. Therefore, elucidation of the molecular mechanisms of FVII deficiency by studying the effects of molecular defects in different functions of the gene products would help us to design new therapeutic strategies and protein engineering procedures (9).

The C91S (p.C91S) substitution was first reported in 2000 in a British patient with FVII deficiency (10). This substitution occurs in the exon 5 of F7 gene and converts Cysteine 91 residue to a Serine in EGF2 (EGF-like 2) domain of the FVII protein. The EGF-like domains have been found to mediate protein-protein interactions. Experimental studies have shown that both the EGF-like and the serine protease domains are essential for the interaction between the tissue factor and FVII (11).

Unlike other coagulation factor deficiencies, the FVII deficiency has more complex phenotypic properties. In FVII deficiency there is not a direct association between coagulation tests findings and the clinical manifestations, therefore predicting clinical severity based on laboratory findings is almost impossible (7). Traditionally, functional studies have been conducted on plasma and with recombinant variants to provide molecular elements useful for defining and characterizing genetic alterations that lead to the FVII deficiency. Since there was a lack of previous functional studies to prove C91S pathogenicity and elucidating the effects of the mentioned substitution on the FVII properties, in the present study we cloned the wild-type and mutated FVII genes in pcDNA3.1/neo vector and expressed them in mammalian cells in order to be able to investigate the effects of C91S substitution on the functional properties of the FVII protein.

2. Objectives
The aim of the present study was to study the effect of the C91S substitution on the expression, secretion, intracellular localization, and coagulative activity of the FVII protein (FVIIIC) and to obtain the functional evidence about C91S pathogenicity.

3. Materials and Methods

3.1. Patient
An informed consent was obtained from a patient with FVII deficiency and the blood sample was collected. The patient was a 20 years old male with mild bleeding symptoms (cutaneous symptoms, epistaxis, oral cavity bleeding) and FVII:C (IU.dL⁻¹) 2%. We detected C91S (c.7807G>C) homozygous mutation upon sequencing of the patient’s F7 gene.

3.2. Cell Culture and Construction of Expression Vectors
As the source of normal human FVII mRNA, HepG2 cells were cultured in DMEM-high glucose (Gibco, United States) with 10% FBS, 1% penicillin/streptomycin (Biosera, France) in a 5% CO₂ atmosphere at 37 °C until reaching to the confluency. The total mRNA of the HepG2 cells was isolated using Trizol reagent (Life Technologies, United States) and cDNA was synthesized using RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, United States). Isolation of FVII coding region was performed by PCR using a pair of specified primers with XhoI and EcoRI recognition sites: forward primer: AAGATTTCTTCTACATGTTCTCCAGG and reverse primer: TCTCGAGGCTAGGGAAATGGGGCTCG, respectively. The PCR product and pcCDNA3.1/neo mammalian expression vector were doubly digested with EcoRI and XhoI and purified using GENEJET PCR purification kit (Thermo Fisher Scientific, United States). The concentration of the purified F7 gene and the linearized vector were measured and the fragment was inserted into the vector using Fast DNA ligation kit (Biobasic, Canada).

The resulting recombinant construct (pcDNA/WT), was transformed into DH5α competent bacterial cells. The DH5α competent cells were prepared using the CaCl₂ method (12). After transformation, cells were cultured overnight on LB agar medium containing 100 µg.mL⁻¹ ampicillin at 37 °C. The transformed cells formed single colonies on agar medium. Several of these single colonies were amplified in LB broth medium (containing 100 µg.mL⁻¹ ampicillin) and plasmid DNA of each amplified colony was extracted using GeneJET plasmid miniprep kit (Thermo Fisher Scientific, United States) and sequenced using 3 single primers.

3.3. Mutagenesis
The C91S mutation was introduced into the pcDNA/WT vector using SOEing PCR site-directed mutagenesis. As described in Table 1, two pairs of overlapping primers...
were designed and three independent PCR reactions were performed using pfu polymerase. The first reaction used Rbou and Fmut as primers and pcDNA/WT as the template to achieve a 1031 bp product. The second reaction used Rmut and Fbou primers and pcDNA/WT as the template to achieve a 457 bp product. In the third reaction, we used Fbou and Rbou primers and the product of the two previous reactions as the template. This reaction rejoined the 457 bp and 1031 bp products and resulted in a 1455 bp fragment that was comprised of the mutated F7 gene and some parts of the pcDNA3.1 vector. The 1455 bp fragment was doubly digested applying EcoRI and XhoI to achieve a 1348 bp fragment containing F7 gene. The 1348 bp fragment was then ligated into the double digested pcDNA3.1/neo vector using fast DNA ligation kit. Further sequencing was conducted to verify the presence of the C91S alteration in the resulting recombinant construct (pcCDNA/C91S).

3.4. CHO-K1 Cell Culture and Transfection

Mammalian cells of choice to express F7 gene were CHO-K1 cells. These cells do not secrete blood coagulation proteins but are able to efficiently carry out the normal processing events and post-translational modifications occurring in the liver (13-15). CHO-K1 cells were cultured in DMEM-F12 medium (Gibco, United States) supplemented with the 10% FBS plus 1% penicillin/streptomycin (Biosera, France) in a 5% CO2 atmosphere at 37 °C in a T25 flask to proliferate. 400000 cells were counted and subcultured in 60 mm plates 24 h prior to transfection. The cells were transfected with the pcDNA/C91S and pcDNA/WT vectors in the 60 mm plates using Turbofect transfection reagent (Thermo Fisher Scientific, United States) based on the manufacturer’s protocol. To estimate the fidelity of transfection, a third vector carrying GFP reporter was also applied. 48 h after transfection, the conditioned medium of each dish were collected. Then, the cells of each transfection were trypsinized and lysed using a freeze-thaw protocol. The collected conditioned media and lysates were kept at -70 °C until analysis.

3.5. mRNA Study

To confirm the successful transfection and FVII expression in CHO-K1 cells, total RNA of transfected cells was also extracted using Trizol reagent (Life Technologies, United States). The cDNA was synthesized (RevertAid First Strand cDNA Synthesis kit, Thermo Fisher Scientific, United States) and an RT-PCR was performed to amplify human F7 mRNA. The following primers were used in RT-PCR reaction: FVIICD2-F: TGTGTGAACGAGAACGGCG and FVIICD2-R: ACCTTCCGTGACTGCTGC.

3.6. Protein Expression and Coagulant Activity Measurement

FVIIc was determined by a one-stage PT-based method on the collected conditioned media and cell lysates. This procedure was performed using an automated Sysmex CA-1500 Coagulation Analyzer System. The FVII secretion and its intracellular level were evaluated by the measurement of FVII antigen in the collected conditioned media and cell lysates using ELISA method (Factor VII human ELISA kit Abcam Cat#ab108829, United Kingdom). The standard curve was constructed applying a serial dilution of the standard recombinant human FVII. This assay was performed in duplicate for all standard samples and test samples. The conditioned medium and cell lysate of untransfected CHO-K1 cells were used as negative control for this test.

3.7. Immunocytochemistry

In order to study intracellular localization of the wild-type and mutated FVII protein, we conducted an immunocytochemistry test on CHO-K1 cells transfected with the wild-type and the mutated FVII, respectively. To this purpose, CHO-K1 cells were grown overnight on the glass coverslips and transfected as described earlier. The coverslips were washed once in PBS and fixed in the 3% paraformaldehyde in PBS for 1 h. The cells were washed 3 more times and permeabilized in 0.1% triton X100 (Sigma, United States) for 5 min. After further washing, we blocked the coverslips in 1% BSA in PBS for 30 min. Then,
rabbit anti human factor 7 antibody (5 µg.mL⁻¹ in PBS containing 1% BSA, Abcam Cat# ab97614, United Kingdom) was added to each coverslip and incubated for 1 h at room temperature. The cells were again washed 3 times with the PBS and then incubated with DyLight® 488 goat anti-rabbit IgG (H+L, at a dilution of 1/300 in PBS containing 1% BSA; Abcam cat# ab96899, United Kingdom) for 30 minutes. After further washing, the coverslips were mounted onto glass slides and were studied under a fluorescent microscope (Olympus).

4. Results

4.1. Constructs and Mutagenesis
pcDNA/WT and pcDNA/C91S sequencing analysis showed the desired direction of the F7 gene with no unwanted changes in the sequence of the gene and vector. The C91S (c.7807G>C) mutation was successfully created on pcDNA/C91S (Fig. 1). The parallel CHO-K1 transfection by pcDNA/GFP showed an appropriate rate of transfection.

4.2. mRNA Expression
RT-PCR showed a 680 bp fragment, suggesting that the transfected cells expressed wild-type and mutated human F7 genes (Fig. 2) and the transfection had been performed successfully.

4.3. Antigen Assay
Upon sketching the standard curve, FVII antigen assay of the conditioned medium and the cell lysates revealed an elevated expression of the mutated FVII compared to the WT transfected cells. The conditioned medium of the C91S transfected cells showed 3 folds increase in the level of FVII antigen. The level of antigen in the cell lysate of the C91S substitution transfected cells was 1.5 fold more than that of WT (Table 2).

4.4. Coagulation Activity
Another functional property of FVII that may be affected by the mutation is its coagulative activity. We studied this function by running a PT-based test on supernatants and lysates collected from transfected cells. The results of this test revealed that in spite of an increased antigen level, FVII with the C91S mutation had undetectable coagulative activity in both cell supernatant and lysate compared to the coagulative activity of the WT transfected cells (as considered 100%) (Table 2).

4.5. Immunocytochemistry
Using immunofluorescent staining to detect intracellular FVII antigens, we found no difference between intracellular localization of the wild-type and mutated FVII (Fig. 3). The staining pattern of the WT and C91S FVII was mostly perinuclear, with some diffuse signal throughout the cell cytoplasm, suggesting that the C91S substitution does not cause the FVII protein to retain in any cellular compartments.

Figure 1. The sequencing results showing the successful introduction of the C91S mutation against the wild-type construct. Arrow indicates the substituted nucleotides.

Figure 2. RT-PCR performed on the cDNA obtained from the transfected cells. Agarose gel electrophoresis shows a 680 bp band.
5. Discussion
The most severe cases of the FVII deficiency are either homozygous or compound heterozygous for the deleterious mutations resulting in the FVII:C level to an amount of less than 2% of the normal condition (7). Here, we have reported a patient homozygous for the Cys91Ser substitution which is caused by the c.7807G>C mutation in the exon 5. The C91S residue is a highly conserved amino acid that has been unchanged in the course of evolution between different species (Fig. 4). There are several conserved cysteine residues in the structure of FVII as well as other vitamin–K dependent factors which play essential roles in the activity, secretion, and 3D structure of these proteins by forming disulfide bonds. Mutations in either of these amino acids will have a major effect on the protein structure. The EGF2 domain contains 6 cysteines that are arranged to form 3 disulfide bonds. One of these disulfide bonds is located between cysteine 91 and cysteine 102 that could be disrupted due to C91S substitution and severely impair the structural integrity of the FVII protein (16, 17). Therefore, this mutation could be considered as a basis for the severe dysfunction of the enzyme. However, the patient did not exhibit severe complications such as hemarthrosis or gastrointestinal bleeding. So, the main purpose of the present study was to demonstrate whether or not the C91S is a disease-causing mutation.

By FVII antigen measurement in the conditioned medium, we showed that the C91S mutation affects the secretion and causes the mutated FVII to be secreted in higher levels compared to the wild-type FVII protein. Evaluation of the FVII antigen in the cell lysate also revealed that the mutated FVII has a higher intracellular level than that of wild-type FVII. The increased intracellular level of FVII has been previously reported

Table 2. Transient expression assays of the FVII WT and FVII C91S in CHO-K1 cells. The table illustrates the results of FVII antigen and coagulant activity measurements in the cell lysates and cell supernatants of the transiently transfected cells. The FVII level measurement in the cell lysates and in the conditioned media 48 hours after transfection of the cells with each construct.

|                | FVII WT | FVII C91S |
|----------------|---------|-----------|
| Supernatant (VII:Ag) | 23.84 ng.mL⁻¹ | 73.3 ng.mL⁻¹ |
| Cell lysate (VII:Ag)   | 142.65 ng.mL⁻¹ | 218.28 ng.mL⁻¹ |
| Supernatant (VII:C)    | 100%    | ND        |
| Cell lysate (VII:C)    | 100%    | ND        |

ND: not detectable.
for the several mutated variants. It has been shown that the intracellular level of Thr359Met and Cys22Arg increases due to an impaired secretion (18, 19). But, unlike C91S in both cases, the extracellular level of FVII was simultaneously reduced. Therefore, it can be deduced that an increased intracellular level of the C91S variant is not due to an impaired secretion, but, it might be due to the increased biosynthesis or a folding problem. For a number of proteins, it has been shown that mutations which lead to the loss of disulfide bonds can cause the protein to fold with a decreased rate, resulting in accumulation of the protein in the cells (20). On the other hand, binding of secretory proteins (such as coagulation factors) to BiP (Binding Immunoglobulin Protein) chaperon can affect secretion rate, intracellular accumulation, and biosynthesis of these proteins (18, 21, 22). In addition, as C91S substitution occurs in the coding region and the expression of the cloned F7 gene is under control of a strong CMV promoter; it might be suggested that the increased level of mutated FVII protein occurs in the translational or post-translational steps of the expression instead of transcription.

In spite of an increased FVII level in the conditioned medium of the cells transfected with the defective FVII, coagulant activity was undetectable suggesting that C91S substitution does severely affect FVII structure and activity. These results are consistent with the previous studies showing that EGF2 mutations impair FVII coagulant activity. EGF-like domains have an important role in the FVII activity, structure and protein-protein interactions and amino acid substitutions of these domains have destructive effects on the enzyme activity. In a study by D’Andrea et al., it has been revealed that occurrence of Arg110Cys and Asp123Tyr mutations; both of which in the EGF2 domain, cause a severe coagulant activity reduction (23). Similarly, in another study by Hunault et al., functional study of Gly97Cys and Gln100Arg mutations also showed a reduced coagulant activity of the mutated proteins (1). It can be hypothesized that the decreased activity observed in the presence of C91S mutation could be due to severe conformational changes and reduced interaction between FVII and tissue factor (11, 24).

We used immunocytochemistry method to localize intracellular WT and mutated FVII. This method showed that there was no difference in the cellular localization between WT and the mutated FVII. Staining of the FVII was mostly perinuclear with some diffuse staining all over the cell cytoplasm, showing that the wild-type and the mutated FVII are biosynthesized normally and accumulate in the endoplasmic reticulum and Golgi apparatus just prior to the secretion in order to achieve folding and post-translational modifications(1, 17). This pattern has been previously observed for the Gly97Arg mutation in EGF2 (1) and Ser190Phe mutation in the catalytic domain (25). A normal pattern of staining and lack of difference between WT and C91S suggests that the mutation doesn’t impair transportation of mutated FVII through the secretory pathway.

6. Conclusions

From this, we conclude that C91S mutation affects FVII biosynthesis and/or folding that causes a higher intracellular level of the protein. As a result of FVII intracellular accumulation, more defective FVII molecules are secreted into extracellular space and cause an increased level of FVII antigen. This conclusion is consistent with the phenotypic characteristics of the patient in whom C91S substitution was found, meaning that increased level of secreted mutated FVII causes the patient to have the minimal FVII:C, sufficient for the initiation of coagulation cascade leading to the mild bleeding symptoms despite a severe loss of coagulation activity (FVII:C 2%) (26). The unrecognized variants within the FVII gene locus and/or related gene loci which may modulate the FVII gene expression or protein processing in vivo should be considered in the phenotypic manifestations of the patient.

We have found a naturally occurring missense mutation within the second EGF-like domain that affects the coagulation activity, secretion, biosynthesis and probably folding of the FVII that leads to the FVII deficiency. Therefore the present study provides a functional evidence showing that C91S substitution is a disease-causing mutation. This further suggests the importance of the second EGF-like domain in the FVII functions.

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