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Novel mutation in coagulation factor VII (Carmel mutation): Identification and characterization

Aliza Cassel PhD[1] | Nurit Rosenberg PhD[2,3] | Emad Muhammad PhD[1] | Tami Livnat PhD[2,3] | Rima Dardik PhD[2,3] | Miriam Berl BSc[1] | Meir Preis MD, PhD[1,4]

[1]Institute of Hematology, Carmel Medical Center, Haifa, Israel

[2]The Israeli National Hemophilia, Sheba Medical Center, Tel Hashomer, Israel

[3]Sackler School of Medicine, Tel-Aviv University, Tel-Aviv, Israel

[4]Ruth and Bruce Rappaport Faculty of Medicine, Israel Institute of Technology, Technion, Haifa, Israel

Correspondence
Meir Preis, MD, PhD, Lady Davis Carmel Medical Center, Academic Medical Center, Ruth and Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel
Email: MeirPr@clalit.org.il

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Abstract

Background: Measurement of factor VII (FVII) activity does not enable prediction of bleeding tendency in individuals with inherited FVII deficiency.

Objective: To characterize the molecular and functional features of FVII in a family with FVII deficiency and correlate them with the bleeding tendency.

Patients/Methods: We studied 7 family members with very low FVII activity using prothrombin time (PT), activated factor VII (FVIIa), FVII activity level, and thrombin generation. The factor 7 gene was sequenced and the mutation was analyzed by prediction software.

Results: The proband has very low FVII activity (0%–4%), with PT ranging between 5% to 18% depending on the tissue factor (TF) origin. Direct sequencing demonstrated a single homozygous nucleotide substitution G > A in exon 6, predicting a novel missense mutation Cys164Tyr. Three members of the family were found to be heterozygous carriers of this mutation. One of them was a compound heterozygote, carrying both the Cys164Tyr and Ala244Val mutation (linked to Arg353Gln polymorphism). Her FVII activity and antigen levels were 3%–7% and 8%, respectively. The other heterozygous carriers demonstrated FVII activity of 41%–54%, FVII antigen of 46%–66%, and FVIIa activity of 30%. FVIIa was undetectable in the homozygous and compound heterozygous subjects. Thrombin generation was normal in the presence of calcium, but no response to TF addition was observed in the homozygous proband, and a reduced response was observed in the compound heterozygous subject.

Conclusion: The patient homozygous for the "Carmel" mutation has mild clinical manifestations despite very low FVII activity, which correlates with thrombin generation results.

KEYWORDS

bleeding disorders, factor VII, mutation, thrombin generation

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Essentials

- The bleeding tendency of carriers of inherited factor VII defects is difficult to predict.
- We studied three generations of a family with inherited very low factor VII activity.
- "Carmel mutation" carriers had mild clinical manifestations.
- Thrombin generation is a highly sensitive assay for monitoring patients with factor VII deficiency.

1 | INTRODUCTION

Inherited factor VII (FVII) deficiency is a rare autosomal recessive disorder manifested by bleeding tendency, mainly in homozygotes or compound heterozygotes. The bleeding tendency varies from mild to severe and does not always correlate with the measured levels of FVII.[1] According to the reported data (http://www.factorvii.org), more than 220 mutations causing FVII deficiency have been identified, most of them unique to the affected families. However, one missense mutation (Ala244Val) has been found to be frequent in Iranian and Moroccan Jewish populations (prevalence of 0.024). All homozygotes for Ala244Val are also homozygotes for the Arg353Gln polymorphism, due to linkage disequilibrium between the two DNA alterations.[2-3] In vitro expression of Arg353Gln revealed reduced secretion of functionally normal FVII; similar results were obtained in cell expression of Ala244Val mutant. Coexpression of both Arg353Gln and Ala244Val yielded an additive effect.[2] Since patients carrying the Ala244Val mutation always also carry the Arg353Gln polymorphism, their FVII is lower than in individuals carrying only the Arg353Gln polymorphism, in agreement with the in vitro studies.[2]

Few mutations demonstrate a discrepancy between the FVII activity and antigen levels[4] and occasionally demonstrate various activity levels dependent on the tissue factor (TF) origin: bovine, rabbit, or human.[5,6] For example: FVII Padua (Arg304Glu) showed low activity (about 10%) with rabbit brain TF but intermediate activity (about 30%) using human (placenta or recombinant) TF[6]; similarly, FVII Nagoya (Arg304Trp) and FVII Arg79Gln showed FVII activity dependent on the origin of TF used in the reagent.[7] Interestingly, these three variants have positive cross-reactive material (CRM+), and all subjects homozygous for these mutations had only mild bleeding tendency or were asymptomatic.[6] Since the measurement of FVII activity depends on proper interaction with TF and factor X (FX), mutations that affect the domains interacting with these proteins may cause ambiguity in measuring mutant FVII activity and, therefore, lack of correlation with the clinical outcome.[8] At least two domains of FVII are involved in TF binding: the catalytic domain encoded by exon 8 and the first epidermal growth factor (EGF)-like domain encoded by exon 4.[9]

The use of thrombin generation assay as a tool for monitoring FVII-deficient patients is debatable. Whereas some studies claimed that thrombin generation is not suitable for predicting the clinical phenotype in FVII-deficient subjects,[10-11] our group has previously showed that thrombin generation was able to predict the impact of recombinant activated factor VII (FVIIa) given to FVII-deficient patients undergoing major surgery or as prophylaxis.[12-13]

The factor 7 (F7) gene is located on chromosome 13q34, and the protein is a single chain that is cleaved at the Arg153Ile bond upon activation, yielding a heavy chain containing the catalytic domain and a light chain containing the Gla domain and the EGF-like domains. Both chains are joined by a disulfide bond (Cys135-Cys262).[9]

The aim of this study was to identify the molecular defect in a family with FVII abnormality, to characterize the functional defect and to find an experimental way to predict its clinical significance.

2 | MATERIALS AND METHODS

Patients

Very low FVII activity was detected in two family members: an 80-year-old woman with a very mild bleeding disorder, who suffered from major bleeding only during labor and minor bleeding episodes during her life, and a pregnant young woman who was her granddaughter (Figure 1), admitted for evaluation due to abnormal coagulation tests and mild bleeding during childbirth. We analyzed four additional family members (II1, II2, II3, and III1) and three healthy unrelated controls with no known coagulation defect or routine coagulation tests abnormalities (one male, two females) (Figure 1). No formal bleeding score was performed. The present study evaluated the novel mutation phenotype and bleeding risk in the proband and her granddaughter prior to labor. All the subjects provided verbal consent prior to blood draw, and each subject provided only one sample.

Collection and processing of blood samples

Blood for coagulation tests was collected in a nonbleeding state into plastic tubes containing 1/10th volume buffered trisodium citrate. Plasma was collected after two consecutive centrifugations at 2500 *g* for 15 minutes and stored at −80°C. For genetic analysis, peripheral blood was collected in potassium–ethylenediaminetetraacetic acid tubes. DNA was extracted from whole blood by using the MagNa Pure Compact Nucleic Acid Isolation kit I (Roche, Basel, Swiss) according to the manufacturer's instructions. DNA samples were stored at −20°C.

Coagulation tests

Prothrombin time (PT) assays were performed with the following PT reagents: RecombiPlasTin 2G (Instrumentation Laboratory, Milan, Italy) containing human recombinant-TF, PT – Fibrinogen HS plus (Instrumentation Laboratory) containing rabbit brain TF,

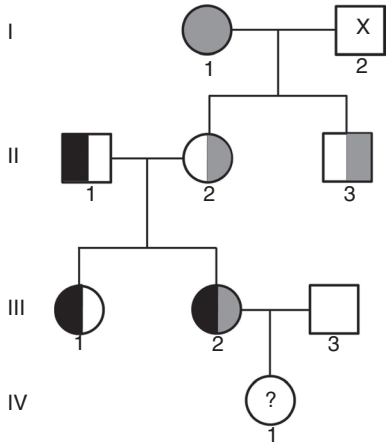

FIGURE 1 Family pedigree. Gray represents the Cys164Tyr; black represents Ala244Val. ?, not determined; X, not available

and Thromborel S containing human placenta TF (Siemens, Berlin, Germany).

Factor VII activity was measured by a PT-based assay using factor VII–depleted plasma (Instrumentation Laboratory) and each of the above-mentioned PT reagents. Factor VII antigen was determined using the AssayMax human factor VII ELISA kit (Assaypro, St. Charles, MO, USA). Factor VIIa activity was determined using the STACLOT VIIa-rTF kit (Diagnostica Stago, Gennevilliers, France). Both assays were conducted according to the manufacturer's instructions.

Thrombin generation was measured in plasma prepared as described previously.[14] Briefly, 20 μL of working buffer containing 4 μM phospholipid with 0, 1, or 5 pM of TF (Diagnostica Stago) were placed in 96-well plates, and 80 μL of plasma was added to each well. The initiation of thrombin generation was by adding 20 μL of fluorogenic substrate/$CaCl_2$ buffer (FluCa kit, Diagnostica Stago). Fluorescence was measured using a fluorometer (Fluoroskan Ascent, Lab System, Helsinki, Finland) with an excitation filter at 390 nm and an emission filter at 460 nm. The lag time was calculated by dedicated computer software attached to the fluorimeter (version 3.0.0.29, Thrombinoscope BV, Maastricht, The Netherlands). All plasma samples with the different TF concentrations were tested simultaneously. All experiments were carried out in duplicate.

Molecular methods

Polymerase chain reaction (PCR) amplification was performed in a 15-μL mixture containing 50 ng of DNA, Taq polymerase master mix (Ampliqon, Copenhagen, Denmark), 500 nM of forward and reverse primers (Table 1), according to the following protocol: 95°C for 2 minutes, 35 X (95°C for 30 seconds, annealing temperature for each set according to Table 1 for 15 seconds, 72°C for 1 minute) 72°C for 5 minutes; 3 μL of PCR products were size checked on a 2% agarose gel and 5 μL were purified with 1 μL of ExoSap (Life Technologies, Grand Island, NY, USA).

Primers were designed using the Primer3 software.

Sequencing of PCR-amplified FVII exons: Sequencing reactions (10 μL) were performed using 1 μL of ExoSap-purified PCR amplicons, 1 μL of BigDye Terminator v.1.1 kit (Life Technologies), 300 nM of sequencing primer, and the following protocol: 96°C for 1 minute, 25 X (96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes). The sequencing reactions were purified using the BigDye XTerminator purification kit and run on an ABI prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequencing Analysis Software v5.4 and Chromas Lite Version 2.4.4 was used for data analysis. The nomenclature for nucleotide and amino acid changes are according to Human Genome Variation Society recommendation (http://varnomen.hgvs.org/).

Evolutionary conservation and structural analyses

The evolutionary conservation analysis was conducted using the Bayesian methods[15] implemented in the ConSeq Web server.[16] The conservation scores range from 1 to 9, indicating variable to conserved positions. The solvent accessible surface area was calculated, and a residue with <5% surface accessible to the solvent was considered to be buried.[17] The prediction of the mutation consequences was based on the PolyPhen-2 (Polymorphism Phenotyping v2) tool, which predicts the possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations.[18]

TABLE 1 Primers used for PCR and sequencing

Exon	Forward	Reverse	Annealing temperature °C
Promotor	5′-GGCTCACCTAAGAAACCAGCCT-3′	5′-GTTGACATTCCCCATGGGAC-3′	62
1	5′-AGCTGGGGTGTTCAGAGGAC-3′	5′-TGCCTGGATGCTGGTTTCTA-3′	55
2	5′-GCTTCACGGAACTCGCATT-3′	5′-TGCAAATCGTATTTTCTGATGTGA-3′	65
3 & 4	5′- AACCCCAGTTCATGGTGTG-3′	5′-TACACACCCCACCAGGTTGT-3′	55
5	5′-CTCCAGGCAGAACACCACT-3′	5′-ACCTCACAATTGGTCAGTGC-3′	58
6	5′-TCAAGGCCTCTCAGAGGATG-3′	5′-CTGACTTGGAGCCTGGTGGG-3′	60
7	5′-AGGGCGAGTCATCAGAGAAA-3′	5′-TGAGACACTTGAGAGCTGCG-3′	60
8	5′-GACCTAGAAATGGCCACAGC-3′	5′-TGTGGAAGTGACAGCACGAA –3′	58

3 | RESULTS

3.1 | FVII activity and antigen

Plasma samples were taken from the proband, six family members (Figure 1), and three normal controls and were characterized using various PT reagents containing TF from rabbit brain (PT-FIB HS plus), human recombinant TF (RecombiPlasTin) and human placenta TF (Thromborel S). Table 2 demonstrates that PT values of the proband[19] and her granddaughter (III2) were less prolonged with rabbit TF compared to human TF.

FVII activity was measured by PT-based assays using FVII-depleted plasma and the same PT reagents (Table 3). Based on the level of FVII, the family members could be segregated into two groups: FVII activity in the range of 40%–50% (II1, II2, II3, III1 putative heterozygotes) and FVII activity <5% (I1 and III2 putative homozygotes). Measurement of FVII antigen by ELISA revealed a good correlation between activity and antigen levels of FVII (CRM negative or reduced): FVII antigen of 1% (I1) and 8% (III2) for the subjects with severe FVII deficiency and 44%–66% for the subjects with mild FVII deficiency. The clotting potential of the mutant FVII was measured by the activity of innate FVIIa in plasma samples of all the family members using recombinant soluble TF, which can no longer activate FVII by autoactivation.[20] It binds only to FVIIa and facilitates the activation of factor X (FX) to activated factor X (FXa). Similar activity (32-39 mIU/mL) was measured in control plasma and in plasma samples of subjects II2, II3, and III3. However, in both severe FVII-deficient family members (I1 and III2), no FVIIa activity could be detected (<2 mIU/ml), and in II2 and II3, the FVIIa activity detected was about 12 mIU/mL.

3.2 | Factor 7 molecular diagnosis

Sequencing of factor 7 exons identified a new, single base change G > A in exon 6 at position c.659G > A (NM019616), resulting in the substitution of p.Cys164Tyr (NP_062562) in the mature protein (224 in the inactive precursor preprotein) (Figure 2). This mutation was named "Carmel mutation." In addition, we detected an additional mutation, Ala244Val along with the Arg353Gln polymorphism (both in exon 8) in the pregnant woman III2, originating from her paternal side of the family: The Ala244Val mutation, previously characterized in the Israeli population, is known to be linked to the Arg353Gln polymorphism,[2] as well as to a silent polymorphism at position NM-019616:c (513C > T) His115 in exon 5.[21] The genetic status of all the family members is summarized in Table 4.

Taken together, the molecular analysis and the FVII assays revealed mild FVII deficiency (activity and antigen) in subjects heterozygous for either the Carmel mutation or the known mutation NP_062562:p.(Ala244Val) (linked to the NP_062562:p.[Arg353Gln] polymorphism), moderate FVII deficiency in the compound heterozygous subject (III2), and severe FVII deficiency for subjects homozygous for the Carmel mutation. Interestingly, heterozygotes for Carmel mutation have low levels of FVIIa (~12 mIU/mL), while heterozygotes for the known mutation Ala244Val have normal levels (>32 mIU/mL).

3.3 | Thrombin generation assay

Since the extent of PT prolongation was reagent dependent, and those reagents contain TF from various origins in unknown concentrations, we explored the direct influence of human TF on thrombin generation in plasma collected from all the family members. The results are summarized in Table 4. In control subjects, the TF concentration affects the lag time of thrombin generation but not the maximal amount of thrombin generated. In control subjects and in subject heterozygous for each mutation (Carmel or Ala244Val), we observed a three- to fourfold shortening of lag time upon adding 5 pM of human TF. In the homozygous proband, there was absolutely no response to the addition of TF, while the compound heterozygote

TABLE 2 Prothrombin time results with different reagents

PT Thromborel S Human placenta		PT Recombiplastin Human recombinant		PT HS+ Rabbit brain			
%	sec	%	sec	%	sec	Relation	Subject
9	**64.4**	**5**	**142.2**	**18**	**59.4**	**Proband**	**I1**
82	12.8	82	13	87	15	Father of granddaughter 2	II1
93	12.1	82	13	91	14.6	Daughter	II2
81	12.9	78	13.4	83	15.5	Son	II3
81	12.9	77	13.5	78	16.1	Granddaughter 1	III1
27	**25.6**	**26**	**31.5**	**35**	**28.8**	**Granddaughter 2**	**III2**
126	10.6	102	11.6	107	13.3	Spouse of granddaughter 2	III3
88	12.4	96	11.7	107	13.3	control	control

Note: Bold text: the proband and granddaughter 2 exhibited prolonged PT.

TABLE 3 Factor VII activity and antigenicity

FVIIa activity (mIU/mL)	FVII antigen (%)	FVII activity (%) TF origin			Subject
		Human placenta	Human recombinant	Rabbit brain	
<2	**1**	**3.6**	**<1**	**<1**	**I1**
38.2	54	76.1	41.2	43	II1
11.8	66	53.3	46.9	53.5	II2
11.9	46	50.1	40.8	51.8	II3
37.2	44	41.5	38.7	31.8	III1
<2	**8**	**5.5**	**3.8**	**3.4**	**III2**
38.8	95	108.9	102	105	III3
32.5	98	120	102	108	control

Note: Bold text: the proband and granddaughter 2 exhibited low level of factor VII.

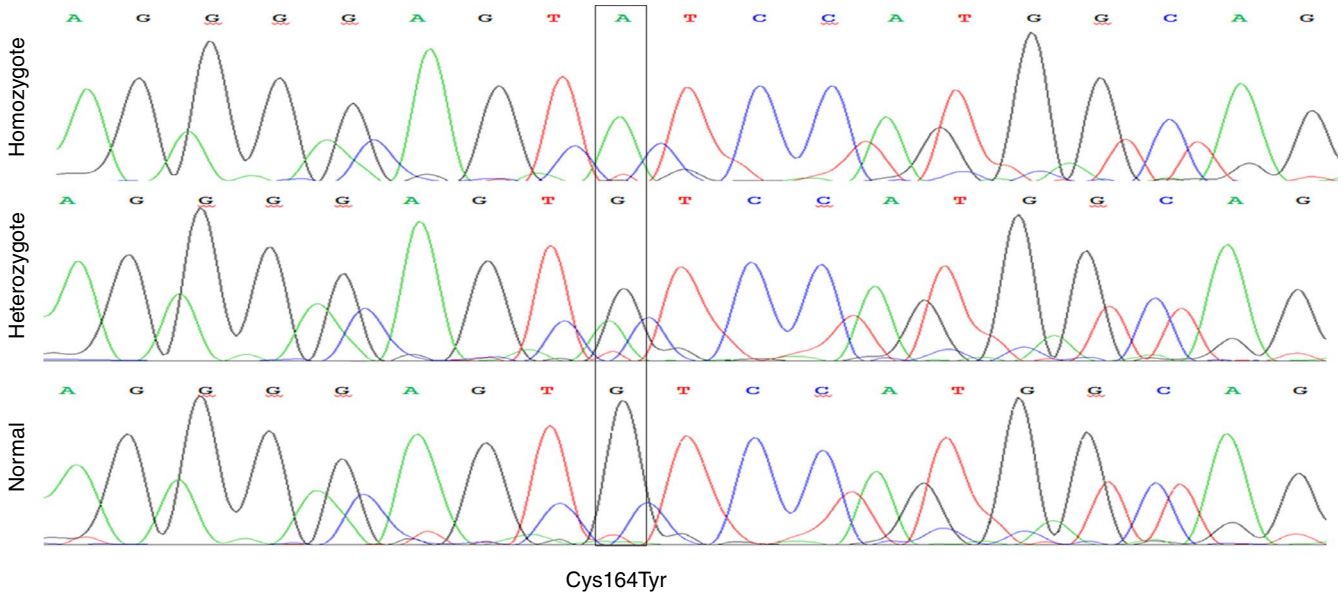

FIGURE 2FACTORVII(FVII) sequencing results. Sanger sequencing results of the new site of mutation in exon 6. Shown are homozygote, heterozygote, and normal control. The G659A exchange is boxed

(III2) demonstrated a lower but significant shortening of the lag time in response to TF (2.28-fold). Thrombin generation plots in plasma taken from subjects homozygous and heterozygous for Carmel mutation, in the presence and absence of external TF, are shown in Figure 3.

3.4 | Structural analyses

Evolutionary conservation analysis of the mutant coagulation FVII by ConSeq reveals that the mutated site is highly conserved in various species (degree of conservation = 8/9), supporting the importance of this position for the structure or the function of FVII protein. The surface of Cys 164 accessible to solvent is <5%, and therefore it is defined as buried residue (Figure 4). A residue that is highly conserved and buried is predicted to be structurally important. Moreover, this mutation is also predicted to be "probably damaging" with a score of 1 according to the Poly-Phen prediction (The same result was obtained from HumDiv and HumVar data models).[18] The direct damage of this mutation is the abrogation of the Cys159-Cys164 disulfide bond, positioned on a loop between two β-strands. Abrogation of this S-S bond probably weakens the H-bonds between the β-strands and probably has an impact on the structure of the protein and on the distance between Cys164 and the catalytic triads (~12Å), as well as the distance to Arg152 at the end of the light chain (~11Å),[22,23] both of which are very important for the integrity and function of FVII.

4 | DISCUSSION

In the present study, we identified a novel mutation [24] in an Israeli Jewish family of Moroccan origin, in addition to the already

TABLE 4 The lag time in "Carmel mutation" carriers during thrombin generation assay (with or without addition of tissue factor)

Subjects	Exon	Nucleotide Change	Amino Acid Change	genotype	no TF (sec)	+1 pM TF (sec)	+5 pM	Ratio = no TF/5pM TF
I1	**6**	**(G659A)**	**Cys164Tyr (Cys224Tyr)**	**Homozygote**	**9.3**	**8.7**	**9.1**	**1**
II1	6	(C999T)	Ala244Val (Ala304Val)	Heterozygote	8.3	6	2.3	3.6
II2	6	(G659A)	Cys164Tyr (Cys224Tyr)	Heterozygote	12.3	11.7	4	3
II3	8	(G659A)	Cys164Tyr (Cys224Tyr)	Heterozygote	8.7	6.3	2.2	4
III1	8	(C999T)	Ala244Val (Ala304Val)	Heterozygote	9.3	6.7	2.2	4.3
III2	**6 + 8**	**(G659A) (C999T)**	**Cys164Tyr (Cys224Tyr) + Ala244Val (Ala304Val)**	**Compound Heterozygote**	**8**	**7.3**	**3.5**	**2.3**
III3		Control		WT	9.3	7.3	2.3	4

Note: Bold text, the proband and granddaughter 2 exhibited low level of factor VII.

Accesion numbers of NM_019616 and NP_062562.1 were used for cDNA and protein template.

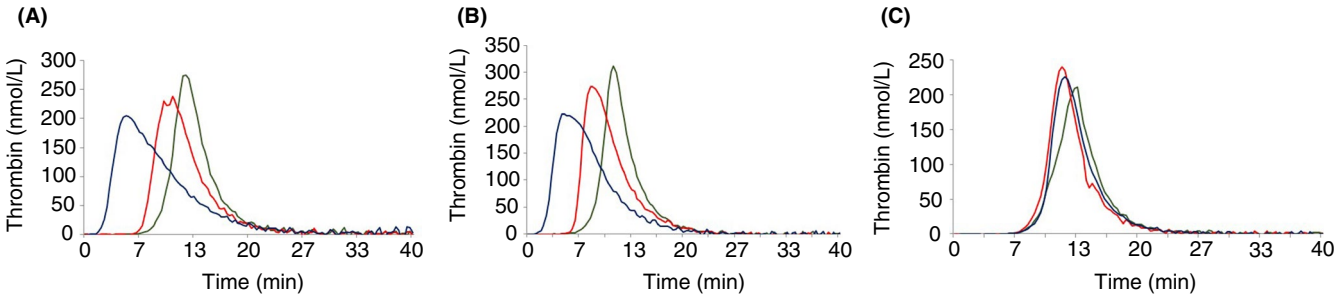

FIGURE 3 Thrombin generation was measured in plasma of two members of the family, in the absence (in green) or presence of TF (1 pM in red, 5 pM in blue). A, demonstrates plasma taken from normal control; B, from heterozygote to Carmel mutation with normal TG; and C, demonstrates the TG in plasma taken from homozygotes to Carmel mutation

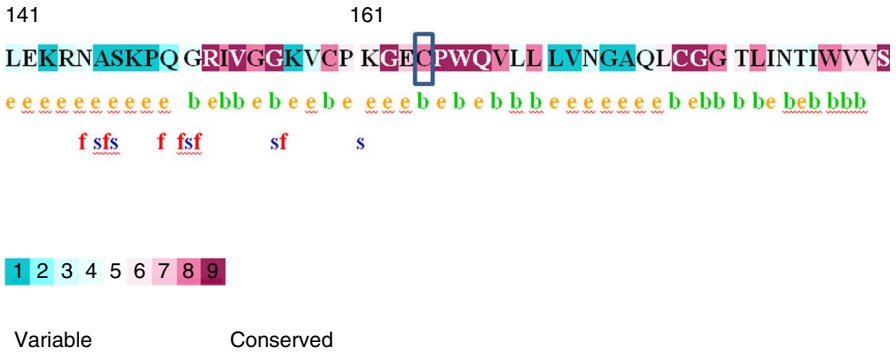

FIGURE 4 ConSeq evolutionary conservation analysis of Cys164 FVII protein. Prediction conservation of residues 141-184 in the mature protein, colored according to evolutionary conservation score. Scale bar for the evolutionary conservation scores is displayed in the bottom. Position 164 marked in square demonstrate the missense mutation. The nature of the residues designated as detailed: e - An exposed residue according to the neural-network algorithm.b - A buried residue according to the neural-network algorithm.f - A predicted functional residue (highly conserved and exposed).s - A predicted structural residue (highly conserved and buried)

described and well-characterized FVII mutations in the Israeli population.[2] The novel mutation, designated the Carmel mutation, is a point mutation in exon 6 of factor 7, c.659G > A (NM-019616), leading to p.Cys164Tyr (NP_062562) substitution in the protein. This change resulted in very low FVII/FVIIa activity and antigen, and lack of response to human TF addition in thrombin generation assay. Interestingly, the extent of PT prolongation varied depending on the TF origin in the reagent. Since the phospholipid composition and the amount of TF present in the PT reagents are unknown,[25,26] we cannot explain the difference detected in the PT-based assays (Tables 1-2).

Structural analysis supports the biological assays, since it predicts a structural defect damaging the protein and resulting in very low amount of FVII antigen or activity in the plasma. Cys164 is a buried and conserved residue, involved in S-S bond formation, which makes it structurally important. Such a mutation usually impairs protein synthesis and secretion in agreement with the low level of FVII or FVIIa in the homozygous patient (I1 in Table 3).

Hereditary FVII deficiency is a rare autosomal recessive bleeding disorder commonly observed in Moroccan and Iranian Jewish patients due to the Ala244Val mutation, also found in the family described in this study. Heterozygotes for mutations in FVII are usually asymptomatic, with about 25%–75% FVII activity, similarly to the heterozygous family members II1, II2, II3, III1 (Table 3). However, the hemorrhagic diathesis in severely affected homozygotes or compound heterozygotes is variable and does not always correlate with the very low FVII activity documented. Therefore, the standard laboratory assays, such as PT and PT-based FVII levels, cannot predict the risk of bleeding. This is also the case in our patients, presenting with very mild clinical manifestations despite the dramatic laboratory findings, suggesting that a low level of FVII, even 1%–2%, is sufficient to prevent bleeding. This is in line with the role of FVII in the coagulation cascade, mostly in the initiation step. However, total absence of FVII have been shown to be associated with life-threatening hemorrhage in a neonate[27] and FVII knockout mice frequently died from fatal hemorrhage soon after birth.[28] It seems that the functional FVII levels determined with human placenta TF correlate better with the mild clinical severity of the proband.

There was no effect of TF concentration on thrombin generation in plasma taken from a homozygote for the Carmel mutation, while a 2-4-fold lag time decrease was demonstrated in plasma of normal, heterozygous, or compound heterozygous individuals (Table 4). These results are in line with our previous study showing a pronounced shortening of thrombin generation lag time by increasing TF concentrations in plasma taken from a patient with 4% factor VII.[12] Interestingly, in plasma taken from a patient with <1% factor VII, no thrombin generation was detected even when the TF concentration was elevated up to 5 pM,[13] suggesting that thrombin generation is a highly sensitive assay as a tool for monitoring patients with factor VII deficiency and correlates with severe bleeding manifestation in patients with <1% functional FVII.

Recently, in a publication of the Ad-Hoc study group, a large cohort of patients with reduced FVII activity was genotyped. One of 123 patients was documented with an identical mutation; however, the phenotype, the functional study and the clinical manifestation are missing.[29]

The inherent difficulty in measuring FVII activity in FVII mutants that affect the interaction with TF poses a significant diagnostic problem with clinical implications. To predict the incidence of bleeding in these patients, we need a reliable laboratory method that can separate between the innate FVII activity and the full activity in the presence of TF. In cases of novel genetic mutations in FVII, we suggest performing a thrombin generation assay to assess the bleeding tendency. This assay provided us with important information that was in concordance with the mild clinical manifestation of this genetic disorder. Given the bleeding tendency, we recommend screening for this mutation in subjects of Mediterranean origin with FVII deficiency.

RELATIONSHIP DISCLOSURE

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

AC and MP designed the research; NR, EM, TL, RD, and MB performed experiments; AC and MP analyzed data; and AC and NR wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

ORCID

Tami Livnat https://orcid.org/0000-0002-1279-2557

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