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ORIGINAL ARTICLE

Extensive genetic screening of Iranian Factor FVII-deficient individuals unraveled several novel mutations and postulated founder effects in some cases

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Abstract

Background: As the most frequent congenital rare bleeding disorder that transmits in an autosomal recessive manner, factor VII (FVII) deficiency is a serious bleeding complication in populations with high rate of in-marriages. While diagnosis mainly relies on clinical and laboratory phenotypes, plasma FVII antigen and activity levels do not often correlate with symptoms' severity.

Objectives: Genetic profiling of the affected individuals potentially improves our biological understanding of this complicated rare disorder.

Methods: Conventional polymerase chain reaction-Sanger sequencing and whole-exome sequencing were applied for genetic profiling of *F7* gene in 66 symptomatic FVII-deficient individuals from 62 independent pedigrees. Thirty-nine asymptomatic relatives of the patients were also studied.

Results: Thirty different *F7* pathogenic variations were identified in the studied cases of which 11 have not been reported before. The novel mutations include 5 missenses (c.715G>A, c.794T>C, c.1090C>G, c.1222C>A, c.1265T>C), 3 splicing (c.316+1G>T, c.682-2A>G, c.572-16C>G), 2 nonsenses (c.790delC, c.1248G>A), and 1 frameshift (c.1346delA). A founder effect is proposed for c.790delC that was detected in 8 independent pedigrees who were all from similar geographical regions and ethnic backgrounds. Homozygous c.790delC reduces plasma FVII activity to <1% and causes spontaneous intracranial hemorrhage in early infancy.

Conclusion: From the 66 studied symptomatic FVII-deficient individuals, 58 were homozygous carriers of the identified variations. Identification of homozygotes clarifies the potential role of nucleotide variations in reducing FVII activity and their contributions to a certain phenotype. Some of those variations, such as c.1A>G, c.509G>A, c.634C>T, and c.1285G>A have only been previously reported as heterozygous.

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KEYWORDS
bleeding, coagulation Factor VII, FVII inhibitor, FVII phenotype, founder effect, novel mutation, intracranial hemorrhage, Exome sequencing

Essentials

- Coagulation factor VII (FVII) mutations but not plasma levels predict phenotypic severity in FVII deficiency.
- 105 individuals from 62 independent pedigrees of various ethnicities are genetically analyzed.
- 11 novel mutations in addition to 19 previously reported pathogenic variants are identified.
- The novel c.790 del C mutation is introduced as an Iranian factor VII variant.

1 | INTRODUCTION

Coagulation factor VII (FVII) deficiency (OMIM: 227500) is the most frequent congenital rare bleeding disorder characterized by low or undetectable plasma levels of FVII, a key player in the initiation of blood coagulation. The disease is a single-gene disorder caused by mutations in *F7* gene [1]. Mutations that result in the complete absence of active protein are associated with early onset pathologies such as neonatal mortality or irreversible organ damage such as intracranial hemorrhage (ICH), defined as a severe phenotype [2]. However, trace amounts of active plasma FVII that are sufficient to initiate coagulation significantly affect the phenotype changing it from severe to moderate [3]. Numerous studies in the past 3 decades have failed to fully correlate certain genotypes to observed phenotypes in FVII-deficient individuals mainly because laboratory assays are not accurate enough to differentiate trace amounts of active plasma FVII (FVII:C) from its complete absence [4,5]. Pharmacokinetics studies have shown that in patients under replacement therapy, clotting potential persists even when FVII:C is undetectable [6]. Therefore, several clinically severe to moderate patients are reported with similar levels of FVII:C [7]. Higher plasma FVII:C levels have also been reported in patients manifesting moderate to mild or even asymptomatic phenotypes [8]. In other words, a wide range of phenotype severity is assigned to certain levels of plasma factor VII activity [9]. In this situation, more genetic profiling could potentially deepen our understanding of genotype–phenotype correlations in this rare disorder of coagulation that common genetic polymorphisms significantly affect the patient's phenotype in addition to the disease-causing mutations [10,11].

Genetic analysis is not part of the medical standard of care in Iran, as well as many other developing countries where the costs for laboratory genetic analysis is quite high and not often covered by insurance. At the Iranian Hemophilia and Thrombophilia Association, a charity-based organization, we have tried not only to provide medical assistance for patients affected by bleeding disorders but also to perform population-based genetic studies to better understand the genetic causes of bleeding disorders in this highly populated and understudied patients. In the current study, which is performed over a decade, the entire *F7* gene is sequenced to understand the genetic cause of FVII-deficiency in affected pedigrees of various ethnicities in Iran.

2 | METHODS

2.1 | Patients

A total of 66 symptomatic FVII-deficient individuals from 62 independent pedigrees who had been presented to Iranian Hemophilia and Thrombophilia Association (MAHTA) from March, 2014 to April, 2022 were recruited in this study. Thirty-nine asymptomatic relatives of the patients were also recruited through segregation analysis. In addition to demographic and phenotypic information registration and drawing family pedigrees, prothrombin time (PT), activated partial thromboplastin time (APTT) and FVII:C levels were assessed in blood samples of all individuals, after obtaining informed consent. The study was conducted in accordance with the Helsinki Declaration and approved by the Zanjan University of Medical Sciences Ethics Committee (IR.ZUMS.REC.1398.280 and IR.ZUMS.REC.1398.132).

2.2 | Genetic analysis

After obtaining informed consent from all individuals recruited in the study, genomic DNA was isolated from their peripheral blood. The 9 *F7* exons, their intronic boundaries, and untranslated regions of *F7* gene were polymerase chain reaction (PCR)-amplified, followed by Sanger sequencing with the use of previously published primer sequences [2]. Sequence analyses were performed with Geneious Pro (version 10.0.6). Identified genomic variations were reported according to Human Genomic Variation Society nomenclature as presented in EAHAD *F7* gene variant database (https://f7-db.eahad.org/).

Through PCR-Sanger sequencing, no causative mutation was identified in the entire *F7* coding sequence of 3 out of 66 FVII-deficient patients. Whole-exome sequencing (WES) was therefore planned for those 3 cases. Exome enrichment was done by SureSelect library preparation kit (Agilent Technologies) and sequencing was performed on the HiSeq 4000 genome analyzer (Illumina), both at Macrogen The data were then analyzed as previously described [12]. Briefly, the reads were mapped to the human genome reference sequence (b37) using BWA v0.7.16 and further processed by Genome Analysis ToolKit v4.0.8 [13] and ANNOVAR [14]. Our in-house pipeline was used to filter and prioritize the variants.

2.3 | Ethics approval

The study was conducted in accordance with the Helsinki Declaration and approved by the Zanjan University of Medical Sciences Ethics Committee (IR.ZUMS.REC.1398.280 and IR.ZUMS.REC.1398.132)

3 | RESULTS

Sixty-two independent pedigrees with at least one symptomatic FVII-deficient individual were recruited in this study. Clinical examinations identified 48 individuals with a history of gastrointestinal and central nervous system bleedings, hematoma, and/or hemarthrosis, classified as severe clinical phenotypes. Clinically moderate patients were 9 individuals with a history of prolonged menorrhagia, epistaxis, and frequent bruising. Mild cases (7 females) only presented prolonged PT and menorrhagia without any spontaneous serious bleeding. Genetic analysis of 39 asymptomatic individuals from the studied pedigrees revealed several heterozygous carriers of the identified mutations (Figure 1).

Patients were from various ethnicities including Fars, Arabs, Turks, Kurds, and Lors who had been referred to our center from different regions of the country. Through genetic analysis, 30 different disease-causing mutations that are presented in Figure 1, were identified in 65 out of 66 studied symptomatic FVII-deficient individuals. In one case, no pathogenic variant related to the expressed phenotype was identified in *F7* gene through either Sanger sequencing or WES analysis.

As shown in (Figure 1), mutational spectrum varies from 21 missense to 4 nonsense, 3 splice sites (one of which is deep intronic, likely to affect splicing), 1 frameshift mutation, and 1 indel of which 11 mutations were not previously reported (https://f7-db.eahad.org/). The identified mutations were mostly distributed throughout the entire *F7* coding sequence.

In addition to disease-causing mutations, 9 different common variants of *F7* gene were identified in studied cases (Table 1) [4,11,15–17]. Among them, c.159C>G (Gly53Gly) has not been reported to our knowledge. While c.159C>G is absent in F7 EAHAD, Iranome (an exome database of 700 healthy samples from Iranian population, http://www.iranome.ir/) and gnomAD databases, here we report 8 homozygous and 16 heterozygous individuals carrying this variation. Another synonymous variant, the c.285G>A (Glu95Glu) is previously reported as heterozygous in 4 individuals while we identified 7 homo- and 15 heterozygotes carrying this variant. Among the patients, 2 with severe FVII phenotypes (<1% FVII:C) had c.159C>G and c.285G>A common variations together with a common mutation; Cys370Phe, all in homozygous. More information on how these 2 synonymous variations might affect FVII function requires further functional studies.

The number of homo- or heterozygous incidences of each common variant in subjects enrolled in the current study is also presented in (Table 1). Interestingly, one patient with less than 1% FVII:C and severe FVII phenotypes most likely caused by a homozygous c.1A>G (Met1Val) start loss mutation was carrier of all 9 common variants that are listed in (Table 1). Due to the severity of phenotype in this individual, the effect of common variants was not distinguishable.

3.1 | c.790 del C: an Iranian factor VII variant possibly resulted from the founder effect

Through genetic analysis, a homozygous c.790delC was identified in 7 probands from 8 independent pedigrees. All 7 patients had suffered from spontaneous ICH in early infancy. In one case, ICH caused irreversible brain damage. Proband of one pedigree had expired due to severe ICH but heterozygous c.790delC was identified in both his parents. Upon treatment with recombinant FVII, 4 of the patients with the same "C" deletion developed anti-FVII inhibitors at ages 40 days, 1 month, 3 and 8 years (36, 17, 191, and 170 BU $mL^{1,}$ respectively).

In heterozygotes carrying c.790delC (Figure 1) that were all asymptomatic, a wide range of FVII:C levels from 15% to 68% was observed. Origin of all families carrying this variant was from west of Iran and mainly from Lor and Kurd ethnicity. One family was a Kurd immigrant from Iraq. Presence of a founder effect is therefore suggested for this gene alteration according to common geographical origin (Figure 2) and ethnic background.

4 | DISCUSSION

Coagulation factor VII (FVII) deficiency is known as a rare bleeding disorder with an estimated prevalence of 1:500,000 individuals. The actual prevalence in Iran is at least 5 times higher, partially due to the high rate of consanguineous and in-marriages. The present number of registered patients based on the 2020 annual global survey released by the World Federation of Hemophilia on October 2021 is 915 patients, comprising 24% of all Iranian patients with rare bleeding disorders. However, the actual number of FVII-deficient patients should be much higher, considering the fact that the majority of mild cases remain unidentified [18]. Here, we report a considerable number of pathogenic variations detected in 65 symptomatic registered cases of whom mild phenotype was only observed in 7 females. In females, prolonged menorrhagia helps with diagnosis. One of the symptomatic cases (No. 66) remained unidentified.

The molecular and clinical aspects of FVII deficiency are extensively reviewed in a recent article by Bernardi and Mariani [19]. The molecular genetics of this frequent rare bleeding disorder is also nicely summarized by Giansily-Blaizot et al. [20], where they introduced the EAHAD F7 database. The mutations we report here will add to our current understanding of FVII deficiency-causing genetic variations by identifying novel mutations as well as mutations previously reported in one or a few cases. For instance, an A to G transition that results in the replacement of Met with Val at the first amino acid of the FVII protein (Figure 1) is only reported in 2 mild cases that were both heterozygous carriers of the mutation with 2% FVII:C [9,21]. Here, we report, notably 5 independent pedigrees in which all 5 probands carry homozygous c.1A>G (Met1Val) (Figure 1 and Table 2). All cases presented severe

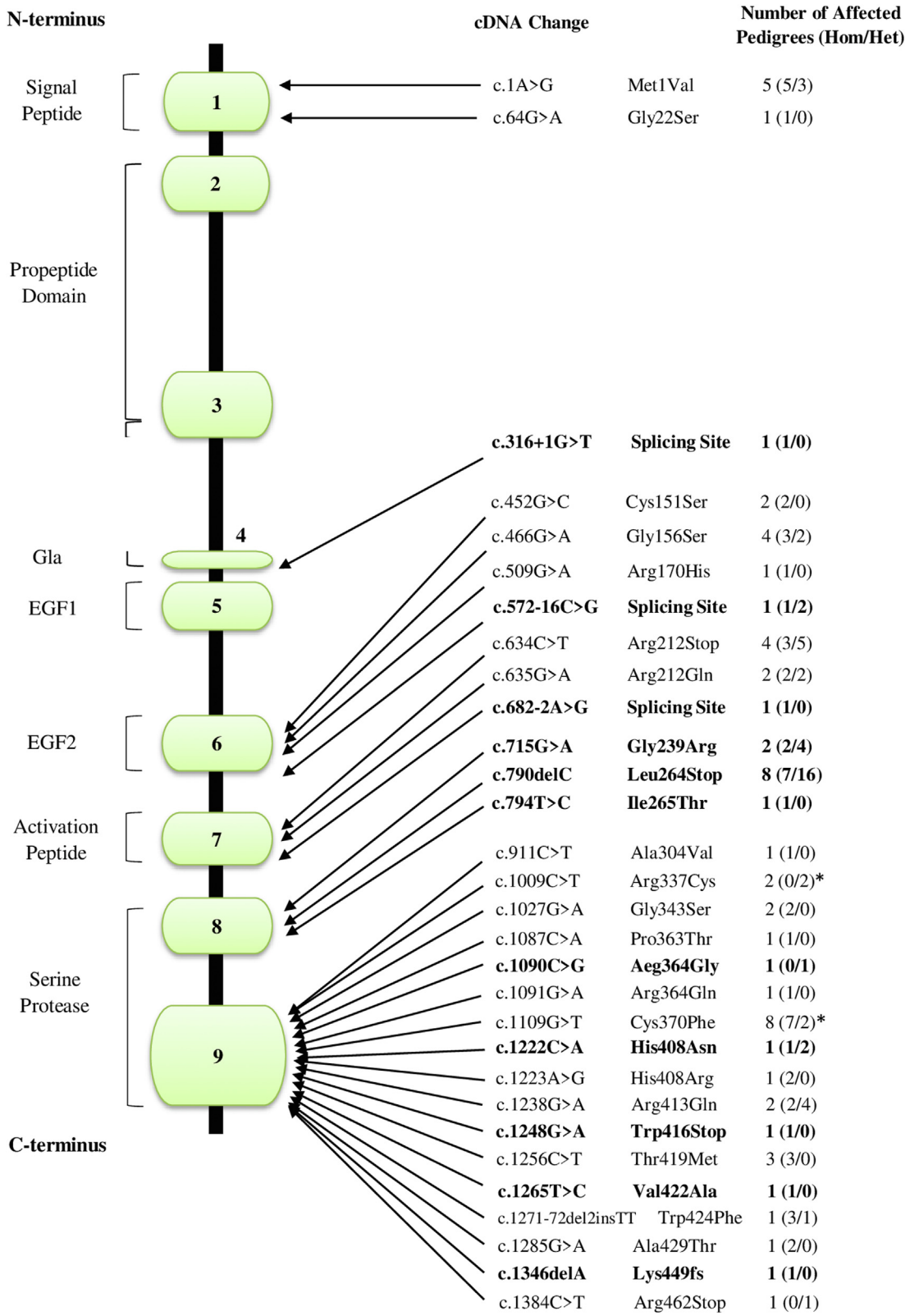

FIGURE 1 Distribution of pathogenic variants throughout the *F7* gene and factor VII (FVII) protein domains. Schematic representation of F7 nine exons and their corresponding protein domain are depicted on the left. For every exon or exon/intron junction, identified nucleotide changes and their position in F7 cDNA sequence as well as the related amino acid change (if applicable) are presented in the middle of the figure. Bolded variants were not previously reported. The number of affected pedigrees and the number of homo- or hetero-zygotes carrying identified mutations are presented on the right side. In agreement with the EAHAD FVII gene (*F7*) variant database (https://f7-db.eahad.org/), amino-acids are numbered according to the Human Genome Variant Society (HGVS) numbering and codons are numbered with codon +1 coding for the first residue (Met) of the 60-residue signal peptide/propeptide of FVII protein. (*): One of the pedigrees is shared between the 2 mutations because the proband is a compound heterozygous carrier of both mutations.

TABLE 1 *F7* gene (2155/NM_000131.4) common variants identified in studied patients.

FVII protein domain	Nucleotide substitution	AA change	Variant type	Variant effect	Homozygous	Heterozygous
Flanking 5′	c.-401g>t	-	SNP	Promoter	15	23
Flanking 5′	c.-325 cctatatcct	-	Insertion of 10-bp	Promoter	15	23
Flanking 5′	c.-122c>t	-	SNP	Promoter	15	23
IVS 1	c.64+9g>a	-	SNP	Intronic DS	10	18
Gla	**c.159C>G**	**Gly53Gly**	**SNP**	**Silent**	**8**	**16**
Gla	c.285G>A	Glu95Glu	SNP	Silent	7	15
EGF2	c.525C>T	His175His	SNP	Silent	15	23
IVS 7	37-bp repeat	-	VNTR	Intronic DS	22	14
Serine protease	c.1238G>A	Arg413Gln	SNP	Missense	15	23

Notes: Nucleotide substitutions that are identified in our patients while previously reported as genetic polymorphisms [15,16] of *F7* coding sequence and their corresponding FVII protein domain are presented. The bolded nucleotide substitution is novel and not reported in EAHAD F7 database. Nucleotide substitutions that result in an amino acid codon change are indicated. In-frame sequences are given in upper case and off-frame sequences in lower case.
AA, amino acid; DS, donor splice site; EGF, epidermal growth factor-like domain; Gla, a γ-carboxyglutamic acid domain; IVS, intron; SNP, single nucleotide polymorphism; VNTR, variable number tandem repeats.

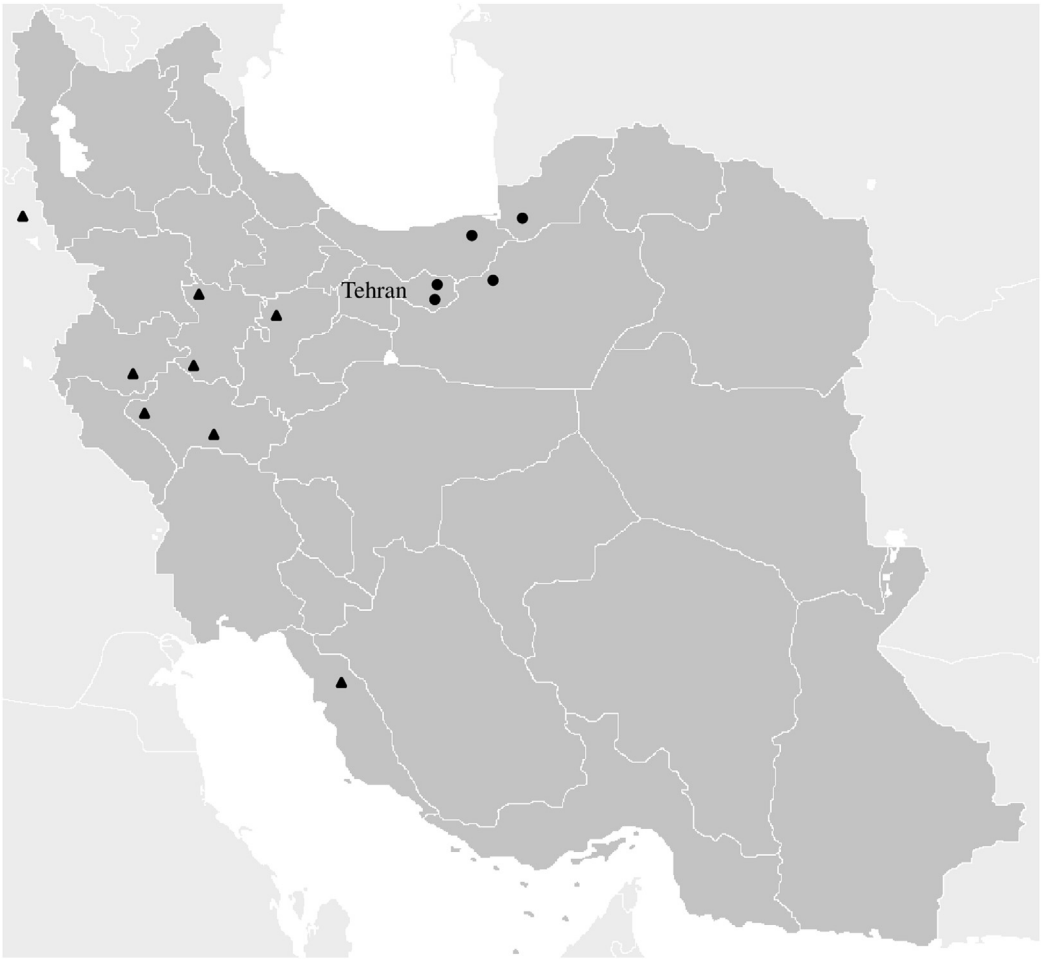

FIGURE 2 Distribution map of c.790delC (Leu264Stop) and c.1A>G (Met1Val) in F7 gene of patients with similar ethnicity. Every independent pedigree is presented by a symbol. Distribution of pedigrees carrying c.790delC (Leu264Stop) in west and southwest of Iran is depicted by filled triangles (▲). As indicated, one pedigree was from Iraqi Kurdistan. Distribution of pedigrees carrying c.1A>G (Met1Val) in the north of Iran is depicted by filled circles (•).

TABLE 2 Genotype, phenotype, and laboratory data of studied patients with FVII-deficiency.

Nucleotide substitution	AA change	Protein domain	Affected Individuals			Genomic coordination (hg19)	Previous reports	Iranome	gnomAD	No. of cases in EAHAD DB
			Affected pedigrees	No. of homozygotes (FVII:C and clinical severity)	No. of heterozygotes (FVII:C and clinical severity)					
c.1A>G	Met1Val	Signal Peptide	5	5 (<1%, Severe)	3 (ND, Asymptomatic)	113760156	Yes	0	7.89E-05	2 het
c.64G>A	Gly22Ser	Propeptide	1	1 (3%, Severe)	-	113760219	Yes	0	4.49E-05	4 (1 hom, 3 het)
c.316+1G>T	Splicing Site	-	1	1 (2%, Severe)	-	113768091	No	0	0	c.316+5G>A is reported in a severe patient (1 het)
c.452G>C	Cys151Ser	EGF2	2	2 (ND, Moderate)	-	113769995	Yes	0	0	3 hom
c.466G>A	Gly156Ser	EGF2	4	2 (ND, Moderate) 1 (5%, mild)	1 (ND, Asymptomatic) 1 compound Het. (c.466 G>A and c.525 C>T) (25%, mild)	113770009	Yes	0.0001	3.49E-05	6 (4 hom, 2 het)
c.509G>A	Arg170His	EGF2	1	1 (ND, Moderate)	-	113770052	Yes	0	7.998E-06	3 het
c.572-16C>G	Splicing site	-	1	1 (4%, Moderate)	2 (ND, Asymptomatic)	113771064	No	0	0	c.572-1G>A, c.572-2A>G, c.572-12T>A and c.572-392C>G are reported.
c.634C>T	Arg212 Stop	Activation Peptide	4	3 (<1%, Severe)	4 (ND, Asymptomatic) 1 (22%, Mild)	113771142	Yes	0	1.66E-05	11 Het
c.635G>A	Arg212Gln	Activation Peptide	2	2 (ND, Severe)	2 (ND, Asymptomatic)	113771143	Yes	0	7.961E-06	13 (5 hom, 8 het)
c.682-2A>G	Splicing site	-	1	1 (<1%, Severe)	-	113771785	No	0	0	c.682-1G>A is reported (4 het)
c.715G>A	Gly239Arg	Serine Protease	2	2 (<1%, Severe)	4 (ND, Asymptomatic)	113771820	No	0	0	c.715G>C is reported (2 het)

(Continues)

TABLE 2 (Continued)

Nucleotide Substitution	AA Change	Protein Domain	Affected Individuals			Genomic coordination (hg19)	Previous Reports	Iranome	gnomAD	No. of Cases in EAHAD DB
			Affected Pedigrees	No. of Homozygotes (FVII:C and Clinical Severity)	No. of Heterozygotes (FVII:C and Clinical Severity)					
c.790delC	Leu264Stop	Serine Protease	8	7 (<1%, Severe)	11 (32%, asympt) (24%, asympt) (15%, asympt) (59%, asympt) (39%, asympt) (51%, asympt) (53%, asympt) (51%, asympt) (68%, asympt) (42%, asympt) (30%, asympt) 5 (ND, asympt)	113771895	Yes*	0	0	-
c.794T>C	Ile265Thr	Serine Protease	1	1 (20%, Mild)	-	113771899	No	0	0	-
c.911C>T	Ala304Val	Serine Protease	1	1 (4%, Severe)	-	113772832	Yes	0	2E-04	58 (19 hom, 39 het), 8 Iranian Jewish [36]
c.1009C>T	Arg337Cys	Serine Protease	2*	-	1 (ND, Mild) 1 compound Het. (c.1109 G>T and c.1009 C>T) (ND, Severe)	113772930	Yes	0.0002	6.74E-05	13 (4 hom, 9 het)
c.1027G>A	Gly343Ser	Serine Protease	2	2 (<1%, Severe)	-	113772948	Yes	0	7.127E-06	12 (5 hom, 7 het)
c.1087C>A	Pro363Thr	Serine Protease	1	1 (<1%, Severe)	-	113773008	Yes	0	0	3 hom
c.1090C>G	Arg364Gly	Serine Protease	1	-	1 (ND, Moderate)	113773011	No	0	1.206E-05	c.1090C>T is reported 7 (3 hom 4 het)
c.1091G>A	Arg364Gln	Serine Protease	1	1 (ND, Severe)	-	113773012	Yes	0.0001	0.000519	72 (23 hom, 49 het)

Nucleotide Substitution	AA Change	Protein Domain	Affected Individuals			Genomic coordination (hg19)	Previous Reports	Iranome	gnomAD	No. of Cases in EAHAD DB
			Affected Pedigrees	No. of Homozygotes (FVII:C and Clinical Severity)	No. of Heterozygotes (FVII:C and Clinical Severity)					
c.1109G>T	Cys370Phe	Serine Protease	8*	4 probands (<1%, Severe) 1 proband (3%, ND) 2 probands (ND, Severe)	1 (ND, mild) 1 compound Het. (c.1109 G>T and c.1009 C>T) (ND, Severe)	113773030	Yes	0.0005	4.25E-05	45 (18 hom, 27 het)
c.1222C>A	His408Asn	Serine Protease	1	1 (2%, Severe)	2 (ND, Asymptomatic)	113773143	No	0	0	c.1222C>T (His408Tyr) is reported (1 het)
c.1223A>G	His408Arg	Serine Protease	1	2 (ND, Severe)	-	113773144	Yes	0	4.036E-06	7 (3 hom, 4 het)
c.1238G>A	Arg413Gln	Serine Protease	2	1 (25%, Moderate) 1 (<1%, Severe)	4 (ND, Asymptomatic)		Yes	0.239	0.126	130 (64 hom, 66 het)
c.1248G>A	Trp416 Stop	Serine Protease	1	1 (<1%, Severe)	-	113773169	No	0	0	-
c.1256C>T	Thr419Met	Serine Protease	3	3 (ND, Severe)	-	113773177	Yes	0	1.70E-05	29 (15 hom, 14 het)
c.1265T>C	Val422Ala	Serine Protease	1	1 (10%, Moderate)	-	113773186	No	0	0	c.1264G>T (Val422Phe) is reported (1 het) c.1264G>C (Val422Leu) is reported (2 het)
c.1271_1272del2insTT	Trp424Phe	Serine Protease	1	3 (ND, Severe)	1 (ND, Asymptomatic)	113773194_113773195/113773192_113773193	Yes	0	0	1 hom
c.1285G>A	Ala429Thr	Serine Protease	1	2 (ND, Severe)	-	113773206	Yes	0	5.09E-05	1 het
c.1346delA	Lys449Ser	Serine Protease	1	1 (4%, Severe)	-	113773267	No	0	0	-
c.1384C>T	Arg462 Stop	Serine Protease	1	-	1 (30%, Mild)	113773305	Yes	0	2.68E-05	6 (2 hom, 4 het)

Notes: Nucleotide substitutions were identified in *FVII* coding sequence as well as exon-intron boundaries. As a result, various missense, nonsense, frameshift, and splicing mutations are observed. Number of affected pedigrees homo- and heterozygotes are indicated. Where information was available, coagulant FVII activity (FVII:C) and correlated phenotypes (severe, moderate, mild, or asymptomatic) is also reported. Previously reported mutations are differentiated from the novel mutations (bolded) in the 8th column.

IVS, intron; ND, not determined; Hom, homozygous; Het, heterozygous; ins, insertion and del, deletion.

phenotypes and had <1% FVII:C. Due to this mutation, the entire transcription is likely impaired as the transcription machinery does not recognize the start point of transcription. Segregation analysis of proband's parents in one pedigree identified them as asymptomatic heterozygous carriers of the mutation. It seems that the heterozygous c.1A>G variant results in FVII deficiency only when accompanied with another disrupting variant on a second allele [21]. The c.1A>G was the third most frequent mutation identified in our study. Patients were all Fars, from the north of Iran (Tehran, Semnan, Gorgan, and Sari) (Figure 2). The prevalence of this mutation in certain parts of Iran should be noted in genetic counseling of Iranian patients.

Among the mutations we are reporting here, a significant number are novel to our knowledge (11 mutations). As presented in Figure 1, 5 novel missense homozygous mutations were found throughout the *F7* coding region including c.715G>A (Gly239Arg) and c.794T>C (Ile265Thr) in exon 8 and c.1090C>G (Arg364Gly), c.1222C>A (His408Asn) and c.1265T>C (Val422Ala) in exon 9. Homozygous splicing site mutations of c.316+1G>T and c.682-2A>G, as well as a deep-intronic mutation, the c.572-16C>G, were also newly identified. The c.316+1G>T and c.682-2A>G resulted in a severe phenotype and <1% FVII:C in studied patients who were probands of 2 independent pedigrees, probably by impairing transcript splicing. One novel homozygous frameshift deletion, the c.1346delA (Lys449fs) on exon 9 was also identified. Two novel nonsense mutations ie, c.790delC (Leu264Stop) and c.1248G>A (Trp416Stop) were also detected in the studied patients. While homozygous c.1248G>A was only detected in one male patient, the c.790delC was the most frequent mutation identified in this study.

The c.790delC was detected in 8 independent pedigrees where homozygous probands all presented a severe phenotype accompanied by <1% FVII:C. One proband had expired due to severe ICH. This mutation has not appeared in F7 database (https://f7-db.eahad.org/) although it seems to have been previously identified in an expired child of an Iranian couple who carried the variant in heterozygous [22]. In that report by Garagiola et al. [22], the codon number and nucleotide position seem to be incorrectly drafted as the correct amino acid codon number is 204 (Legacy number) not as stated, 205). However, the phenotypes are matched with those we observed in our patients. Due to the regional prevalence of the identified mutation and its limitation to certain ethnicities (Kurds and Lors), here, we propose that this mutation is resulted from a founder effect and should be considered in genetic counseling of the corresponding patients, inside and outside Iran, especially in Kurdish populations of Iraq, Syria, and Turkey.

Another most frequent mutation in our study, the substitution of Cys with Phe at position 370 (c.1109 G>T) that affects the serine protease domain of FVII protein was also identified in 8 independent pedigrees. The distribution of families throughout the country from Lorestan, Tehran, and Khorasan including Lor and Fars ethnicities as well as 45 other reported cases worldwide (https://f7-db.eahad.org/) shows that it is a common mutation in various populations [2,9,10,18,23-25,26,27].

Worldwide distribution of *F7* mutations is mainly elusive while in Iran, the picture has become clearer. To our knowledge, this is the largest genotyping study on FVII-deficient patients in Iran which is being gradually performed over a decade. Upon submission of this manuscript, 271 unique variants corresponding to 1058 individual cases had been compiled within the EAHAD F7 variant database (https://f7-db.eahad.org/) [20]. Eleven novels in addition to 19 previously reported pathogenic variants in a total of 104 cases from 61 independent pedigrees we report here are compared with formerly reported cases (Table 2).

As presented in (Table 2), in-frame single nucleotide substitutions such as c.715G>C (Gly239Arg), c.1090C>T (Arg364Trp), c.1222C>T (His408Tyr), c.1264G>T (Val422Phe) and c.1264G>C (Val422Leu) have been already reported. Novel variations affecting the same amino acid including c.715G>A (Gly239Arg), c.1090C>G (Arg364Gly), c.1222C>A (His408Asn), and c.1265T>C (Val422Ala) are identified in this study. The molecular impact of those variations in corresponding patients is discussed below.

While the reported c.715G>C and newly identified c.715G>A similarly affect the amino acid 239 of FVII protein by replacing glycine with arginine, The c.715G>C was identified in 2 heterozygous cases, one asymptomatic and one presenting a mild phenotype [9]. Here, we report 2 severe FVII-deficient individuals with <1% FVII:C from 2 unrelated pedigrees carrying homozygous c.715G>A. Segregation analysis revealed the parents of both patients as asymptomatic carriers of the mutation.

We identified heterozygous c.1090C>G (Arg364Gly) in a patient presenting moderate clinical symptoms of FVII deficiency. The c.1090C>T (Arg364Trp) has been reported in forms of hetero- and homozygous in several individuals presenting asymptomatic to mild clinical phenotypes [9,28]. Diversity in patient's phenotype could be due to the combinational effect of various SNPs. In addition to the c.1090C>G, a homozygous c.525C>T (His175His) was also detected in our patient.

The c.1222C>A (His408Asn) is another novel variant that is identified in this study. The patient was a homozygous carrier of the mutation and presented severe phenotype with 2% FVII:C. At the same nucleotide position, heterozygous transition of C to T has been once reported in a patient with an unknown clinical phenotype [17].

The transition of T to C at c.1265 that replaces valine with alanine at position 422 of FVII protein is also a novel substitution at this position, while the replacement of valine with phenylalanine and leucine has been reported as a result of G > T and G > C changes at c.1264 (g.18081), respectively [17,29]. Interestingly, all reported cases of c.1264G>T and c.1264G>C are heterozygous carriers of the mutations with an unknown phenotype while the carrier of c.1265T>C (Val422Ala) in our study is a homozygote presenting moderate FVII-deficient phenotype. Identification of homozygotes clarifies the potential role of nucleotide variations in reducing FVII activity and their contributions to a certain phenotype.

Interesting to this study, is the number of homozygous carriers of variations that are either novel, as discussed above, or has solely been reported in forms of heterozygotes. In general, 58 out of 105 studied cases carry 30 different mutations in homozygous (Table 2). In addition to the above-mentioned c.1A>G, c.715G>A, c.1222C>A and c.1265T>C (Val422Ala), the c.509G>A (Arg170His), c.634C>T (Arg212Stop) and c.1285G>A (Ala429Thr) could be listed here.

Heterozygous c.509G>A does not seem to affect the phenotype [30] while the homozygous carrier of this variant in the current study presented moderate FVII-deficient phenotype. The c.634C>T, when occurs in homozygous, causes severe phenotype as detected in probands of 3 unrelated pedigrees in our study. Genetically analyzed parents appeared as asymptomatic heterozygote carriers of the mutation. However, one patient from the fourth pedigree presented a mild phenotype. Genetic analysis identified her as a heterozygous carrier of this mutation. The c.634C>T has only been reported in form of heterozygous in several individuals presenting a diverse range of phenotype from asymptomatic to severe [18,26]. The severe phenotype in heterozygous carriers of c.634C>T seems to be dependent on the accompanying variants [31].

Homozygous c.1285G>A also causes severe phenotype as identified in 2 sisters in the current study. Their parents were asymptomatic as expected [9].

Pathogenic single nucleotide substitutions are not restricted to *F7* coding sequence. In addition to in-frame single nucleotide variations (SNVs), c.316+1G>T, c.572-16C>G (found by WES), and c.682-2A>G that seem to affect transcript splicing were also newly identified in this study (Table 2). Homozygous c.316+1G>T and c.682-2A>G resulted in severe phenotype and <1% FVII:C in affected individuals who were probands of 2 independent pedigrees. While the c.316+1G>T seems to disrupt the consensus 5′ donor site of splicing at intron 4, the A>G transition at c.682-2, the junction of intron 7 and exon 8 of *F7* gene, potentially disrupts splicing at this 3′ splicing acceptor site, similar to the proposed effect of c.572-2A>G [4].

The c.572-16C>G is located at intron 6, and based on different splice predictors such as SPIDEX, AdaBoost, and Random Forest, this nucleotide change might have no impact on splicing. However, at similar splicing region; c.572-1G>A, c.572-2A>G, and c.572-12T>A are previously reported as pathogenic [4,32]. Homozygous c.572-1G>A is clearly shown to be associated with moderate FVII phenotype since splicing acceptor site at the junction of intron 6 and exon 7 is lost through conversion of AG dinucleotide to AA [32]. Similarly, the *in vitro* effect of c.572-12T>A and c.572-2A>G on splicing is shown [4]. Therefore, it is possible that the c.572-16C>G reported in this study affect the splicing. Further functional studies are required for clarification.

In addition to several missenses, nonsense, and splicing site mutations, the c.1346delA (Lys449fs) on exon 9 was also newly identified in a patient with 4% plasma FVII activity who presented severe phenotype. The exon 9 is coding most portion of serine protease domain; the largest domain of F7 protein. In general, almost half of mutations (17 out of 30 mutations reported here) are located on exon 9, suggesting a potential hot spot region in our society, in accordance with worldwide *F7* mutation distribution as listed in EAHAD database.

Throughout this study, we were limited by the number of mild cases who were willing to participate in genetic analysis. Moderate and mild cases are valuable to study the cumulative effect of common variants (polymorphisms) on identified mutations [33]. However, mild patients in Iran often tend to ignore their disease due to cultural pressure, especially when complications are not that serious. Another limitation of this study is the restricted number of patients in whom inhibitor development was identified. Understanding the association between genotypes and the risk of inhibitor development deepens our knowledge on the molecular basis of inhibitor development in FVII deficiency and potentially paves the way toward clinical management of this rare but serious therapeutic challenge [34,35]. Inhibitor development often occurs in patients with severe clinical phenotypes. Of particular interest, is the high number of clinically severe patients (48 patients) in this study who need to be carefully investigated for inhibitor development and relation to certain mutations. In conclusion, our study provides the genetic landscape of FVII deficiency in Iran and reports notable numbers of novel mutations.

AUTHOR CONTRIBUTIONS

S.H.R. designed and performed the experiments. M.F. supervised patient management and follow-up. H.R.Z. performed bioinformatics analysis and wrote the manuscript. M.C.A. performed bioinformatics analysis. T.S.H. designed the study, analyzed the data, and wrote the manuscript. All authors read and approved the final manuscript.

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RELATIONSHIP DISCLOSURE

The authors declare that they have no conflict of financial interest.

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INFORMED PATIENT CONSENT

Written informed consent was received from all patients and their families who participated in this study.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author on reasonable request.

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