Biomacromol. J., Vol. 8, No. 1, 70-75, July 2022.

Biomacromolecular Journal
www.bmmj.org

Identification of a 7-Gene Panel by Sanger Sequencing for Inherited Coagulation Factor x Disorders

M. Saeedifar[a,b], G. Karimi[a,*], S.A. Angaji[a,*], F. Zafarghandi Motlagh[b], H. Bagherian[b], S. Rafatian[b,c] and T. Shirzadeh[b]

[a]*Department of Biology, Kharazmi University, karaj, Iran*

[b]*Dr. Zeinali's Medical Genetics Lab, Kawsar Human Genetics Research Center, No. 41 Majlesi St., Vali Asr St, Tehran 1595645513, Iran*

[c]*Department of Biotechnology, Faculty of Biological Science and Technology, University of Isfahan, Isfahan, Iran*

(Received 20 September 2023, Accepted 19 January 2024)

ABSTRACT

Problems with coagulation factor X (FX) inherited in families. Consanguineous marriage is common in Iran. Therefore one may predict a greater prevalence rate there than in the Western populations. FX represent a category of very uncommon bleeding diseases caused by defects in the factor X (F 10) gene. Blood clots can't form without factor X, an essential protein in the coagulation cascade. Factor X deficiencies or abnormalities may cause aberrant bleeding behaviors and provide substantial clinical management problems. Autosomal recessive inheritance pattern characterizes the F10 gene. To examine potential deleterious effects on encoded proteins, in silico approaches were applied. All exons and their boundaries of patient's sample were sequenced using Sanger sequencing. Members of the family were also tested. F10 gene sequencing revealed several novel mutations. In addition, the disease-causing properties of the identified mutations were validated via segregation analysis and in-silico evaluations. Inherited coagulation factor X disorder is a rare bleeding disorder caused by FX protein deficiency or absence. These results would help affected families and those who are carriers for similar mutations. Prevention of morbidity and improvement also aid to conduct genetic counseling, prenatal diagnosis and clinical management of FX deficiency in the Iranian population.

Keywords: Inherited coagulation factor X disorders, F10 gene, genetic heterogeneity, novel mutations, Iran

INTRODUCTION

The vitamin K-dependent plasma protein factor X (FX) is necessary for coagulation, and a lack of FX results in a hemorrhagic phenotype, which is related to the degree of deficiency [1]. FX (human coagulation factor) is a vitamin K-dependent zymogen that plays an important part in the coagulation cascade. Both the intrinsic and extrinsic routes can activate it [2]. The activated FX (FXa), which comprises the His236, Asp282, and Ser379 catalytic triad residues, works with the activated co-factor V (FVa) to cleaves prothrombin to create thrombin cleaves prothrombin to create thrombin.

Congenital FX deficiency is very rare, involving 1 in 1,500,000 persons, according to studies. Due to the possibility of heterozygotes showing both a modest bleeding propensity and clotting patterns consistent with a degree of FX activity between 40 and 60%, the hereditary transmission is autosomal incompletely recessive [3]. However, the prevalence of rare coagulation disorders is 3-5% in normal populations in western countries, but the Frequency of carriers of autosomal recessive inherited coagulation disorders it is 10-20% more in areas where consanguineous marriages are frequent, such as Iran, Saudi Arabia, Kuwait, or even South India [4]. The F10 gene, which extends 27 kb and has eight exons, encodes several functional domains of the FX protein. Presently, 171 mutations in the F10 gene have been associated with FX deficiency, with nearly all of them occurring in the gamma-carboxyglutamic and catalytic domains [2].

Patients with mild FX deficiency could emerge with

*Corresponding authors. E-mail: angaji@khu.ac.ir; g.karimi@khu.ac.ir

Umbilical cording bleeding, hemarthroses, intracranial hemorrhage, mucosal bleeding, muscle bleeding, &hematomas. Spontaneous bleeding episodes are common in these patients. Hematomas, gastrointestinal bleeding, and joint bleeding are possible symptoms of patients with a severe type of FX deficiency [5]. There have also been reports of more severe incidents, such as cerebral hemorrhage, spinal cord bleeding, and umbilical cord bleeding that have been reported in the paper [5]. The diagnosis can be verified using a PT-based assay to detect FX coagulant activity (FX: C).

The clinical phenotype linked to the presence of one mutant allele in homozygous patients is described without interference or compensation by the other allele. The genetic study investigation shows that in a significant number of individuals with inherited bleeding coagulation problems, this technique could consistently and precisely detect harmful variations that assist in genetic counseling, prenatal diagnosis, and, in certain cases, disease progression prediction. This allows patients to be followed up on an individual basis [7]. In the article, the molecular and structural basis of FX deficiency had been investigated in 7 Iranian patients from 7 non-related families with a high rate of consanguineous marriages. Functional validation had been performed by Computational approaches method [17] to assess the impact of the mutations on FX activity. However, it was noted that functional validation could be time-consuming, costly, and technically challenging, depending on the gene and the variant.

MATERIAL AND METHODS

Patients' description seven FX-deficient patients from 7 unrelated families (ranging from 1 to 55-year-old) All of the families were Iranian. The patients had been referred to Medical Genetics Laboratory of Dr. Zeinali, Kawsar Human Genetics Research Center, Tehran Iran, by hematologists. The diagnosis was made based on the patient's symptoms, which included repeated bleeding from the nose, bleeding from the umbilical cord, prothrombin time (PT), and partial thromboplastin time (PTT). Using an enzyme-linked immunosorbent test (ELISA), the amount of FX antigen (FX:Ag) was found to be less than 1%. All the Patients were the progeny of first cousin marriages. Prior to the investigation, informed consent was given by all patients.

DNA Isolation and PCR

Leucocytes taken from the periphery of the blood were utilized to collect genomic DNA from members of the family, which was then extracted using the salting out method [6]. All exonic fragments were amplified by PCR in a 25 µl volume. The reaction mixture included 30 ng of genomic DNA, 7.5-15 pmoles of each primer, 250 mM of each dNTP, 10X buffer (Kawsar Biotech Co., Tehran, Iran, KBC), 2.5 µl of DMSO (Sigma-Aldrich, USA), 0.96 mM, 1 U of Taq polymerase (KBC), and 4 mM of MgCl2 (KBC). Each fragment's PCR conditions assumed a 50 mM salt concentration and a 50 nM annealing oligo concentration. Five minutes of initial denaturation at 94 °C was followed by 30 cycles of amplification consisting of denaturation at 94 °C for 40 s, annealing at the specified temperature for 40 s, and extension at 72 °C for 40 s. The final extension was conducted at 72 degrees Celsius for 5 min.

Sanger Sequencing

The primers for this study were designed using both Oligo7 tool and UCSC genome browser (https://genome.ucsc.edu/cgi-bin/hgPcr) (Table 1), and they are available upon request. PCR amplification was then carried out using the primers listed in Table 1. For analysis, the PCR products underwent Sanger sequencing with the Big Dye Terminator cycle sequencing kit for the examination of eight coding exons of the F10 gene in our patients (Applied Biosystems, Warrington, UK). The sequencing runs were performed on an ABI 3130xl genetic analyzer (PE Applied Biosystems, Foster City, CA, USA)

DNA Analysis

The selected DNA sequences were evaluated using Chromas Lite v2.1.1 (Technelysium, South Brisbane, Qld, Australia) and DS Gene v1.5 (Accerlys, SanDiego, CA, USA) software. The data was analyzed with the annotations of genome version hg38/GRCh38. Variations known to cause inherited coagulation bleeding disorders (IBCDs) were searched in HGMD's professional database, PubMed, and the Online Mendelian Inheritance in Man (OMIM) (http://www.ncbi.nlm.nih.gov/omim). Various bioinformatics tools such as Poly-Phen2, SIFT, SNPs&GO

Table 1. List of Sequencing Primers. Primers were Designed for Amplification of the Factor X Gene (RefSeqNM_000504)

Exon	Forward primers	Reverse primers	Amplicon size (bp)	Annealing temperature (C)
1	5' CCTAAGCCAAGTGATAAGCAGC 3'	5'- GTCTCTGTGACCTCTGATCCG-3'	406	60.4
2	5'AAGGGACATGGCAGTCAGG-3'	5'-TTACCAACAGCCAGGCTATCC-3'	281	61.2
3	5'CAGCTTGAGTCACTTAATTATGG-3'	5'-CTATTATGGAAACACCCTGAGG-3'	257	56.5
4	5'CCATGATGCCGGAAACAGC-3'	5'-GGGCATCTGATCTGAAGGTATACC-3'	208	63
5	5'CAGGCAAGTGGATGTAGCTGG-3'	5'-CTCAGTCCTGTCCTCTTGGTGT-3'	361	62
6	5'ATTGTTCACAGGCGGTCACC-3'	5'-GCTATTACAAGTGTCAGGCTGG-3'	565	61
7	5'GGCTTCTCAGTCAGGCAACAC-3'	5'-GTGTTCCCAACAAATTCCATCAGC-3'	279	64.5
8	5'GCAAGGCTGACAGGCACTTT-3'	5'-AGACACTTCACCTTCCCACC-3'	867	61

(http://snps.biofold.org/snps-and-go), SNAP (https://rostlab.org/services/snap/), mutationtaster (https://www.mutationtaster.org)/, fathmm (http://fathmm.biocompute.org.uk)/, varsome (https://varsome.com/), and franklin (https://franklin.genoox.com/clinical-db/home) were used to define the structural impact of novel missense variants on protein function and their pathogenicity. All variations were redefined according to the norms and recommendations established by the American College of Medical Genetics and Genomics [6].

RESULTS

This study utilized 7 families to identify mutations that cause inherited bleeding coagulation issues in all cases, as detailed in Table 2. The study focused on the impact of gene mutations in the 7 families. all study participants were of Iranian origin. The families had come for disease confirmation, carrier screening, and prenatal diagnosis. Each family received professional counseling.

The identified mutations affected multiple functional domains, including the serine protease catalytic domain and the Gla domain. As depicted in Fig. 1, the majority of mutations were discovered within the catalytic serine protease domain.

Furthermore, sequencing data from the 7-gene panel were verified in another parallel lab using samples collected from four patients who had previously received molecular characterization through Sanger sequencing of a single gene. The sequencing data were fully concordant.

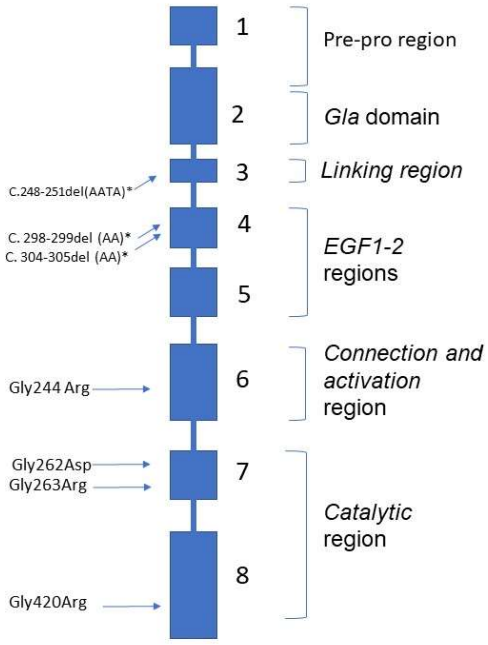

Fig. 1. Schematic representation of factor X gene mutations identified in this study. *Novel mutations [11].

All 7 patients had severe FX deficiency, and there was at least one affected child in each of the families. Among the notable clinical features were a tendency to bleed easily (as

Table 2. Clinical Phenotype, Laboratory Findings and Molecular Diagnosis in a Cohort of 7 Patients with Coagulation Factor X Deficiencies

ACMG Classification (Criteria Used)	Ref.	Functional domain	exon	cDNA mutation with Protein change	Consanguinity marriage	Affected child	CADD PHRED	Mutation Taster	Factor X activity (%)	Place of birth/Residence	Clinvar accession number	Family ID
Likely pathogenic (PP3,PM2,PP2)	[8]	catalytic domain	7	F10 (NM_000504) c.785 G>A (p.G262D)	✓	1	24.9	Disease causing	<1	Khoy, West Azerbaijan Province	N/A	0001215
Likely pathogenic (PP3,PM2,PP2)	[9]	catalytic domain	7	F10 (NM_000504) c.787G>A(p.G263R)	✓	2	27.5	Disease causing	Not measured	Khash, Sistan and Baluchestan Province	N/A	39942657
Likely pathogenic (PP3,PM2,PP2) & Likely pathogenic (PVS1,PM2)	Novel	Linking region & catalytic domain	3&7	F10(NM_000504) c.785 G>A(p.G262D) & (NM_000504)c.248-251del(AATA)	-	1	24.9 & N/A	Prediction Disease causing	<1	Tehran, Tehran Province	SCV003932674	9960857
Likely pathogenic (PP3,PM2,PP2)	[8]	catalytic domain	7	F10 (NM_000504) c.785 G> A (p.G262D)	✓	2	24.9	Disease causing	<1	Quchan County, Razavi Khorasan Province/Sirvan County, Razavi Khorasan Province	N/A	39113088
Likely pathogenic (PVS1,PM2)	Novel	EGF-1	4	F10 (NM_000504) c.298_299del (AA)	✓	2	N/A	Prediction Disease causing	Not measured	Hamadan,Hamadan Province	SCV003932675	39320949
Likely pathogenic (PVS1,PM2)	Novel	EGF-1	4	F10(NM_000504) C.304_305del(AA)	✓	1	N/A	Prediction Disease causing	Not measured	Hamadan,Hamadan Province	SCV003932676	39529308
Likely pathogenic (PP3,PM2,PP2)	[8]	Connection and activation region	6	F10 (NM_000504) c.730 G>A (p.G244R)	✓	1	26.9	Disease causing	<1	Shahrood, Semnan Province/ Tehran, Tehran Province	N/A	39738246

seen by epistaxis, hemarthroses, gingival bleeding, gastrointestinal bleeding, central nervous system hemorrhage, ecchymoses, hematuria, and a bled umbilical cord). refer to Table 2 for additional clinical and hematological data on the study's participants.

Novel Mutations

A compound heterozygous mutation was found that had significantly decreased FX activity. A heterozygous G>A transition in exon 7 that was discovered using DNA sequencing indicated a Gly262Asp alteration in the catalytic domain. Another heterozygous c.248-251del (AATA) mutation was discovered in exon 3 within a small deletion del (AATA) in the linking region. (Family ID: 9960857)

A heterozygous C. 298-299del (AA) mutation was discovered in exon 4 within a small deletion del (AA) in the EGF-1 region (family ID: 39320949).

A heterozygous C. 304-305del (AA) mutation was discovered in exon 4 within a small deletion del (AA) in the EGF-1 region (family ID: 39529308).

Previously Reported Mutations

The previously reported mutations, namely Gly262Asp [8], and Gly263Arg [9] Gly244 Arg [8] were present in exon 4,6,7,8 respectably`, and affected the catalytic region, EGF-1 domain and Connection and activation region.

DISCUSSION

Given the critical importance of early diagnosis in patients with coagulation disorders, the high rate of consanguineous marriages in Iran, the high cost of laboratory tests, and the difficulty in obtaining these tests in medical centers, this study was conducted to investigate the prevalence of bleeding symptoms in individuals who are heterozygous for recessively inherited coagulation disorders [4].

This is particularly significant as diagnosing bleeding disorders, even those that are extremely uncommon, requires an in-depth assessment of hemorrhagic signs. However, because reporting and interpreting bleeding symptoms are intrinsic, evaluating bleeding symptoms is an obstacle.

Significant symptoms may go undetected because they are deemed healthy and minimal, whereas trivial symptoms may receive excessive attention. This problem is highlighted by the high frequency of bleeding symptoms reported by the general population [10].

This study shows the molecular anomalies in seven unrelated Iranian FX deficient families.

The effects of all the variants found in the patients were categorized according to the guidelines provided by the American College of Medical Genetics and Genomics 2015 (ACMG). (Table 2)

A compound heterozygosity (Gly262Asp, c.248-251del) was brought on FX deficiency in one patient (Family ID: 9960857). The c.248-251del is the novel deletion frameshift mutation, which involves a number of base pairs that is not a multiple of three and hence breaks a DNA sequence's triplet reading frame. Gly262Asp, mutations identified here have been reported previously in patients of Italian, Iranian respectively [12].

The c.298_299del (AA) is the novel deletion frameshift mutation in patient (family ID: 39320949) which involves base pairs that are not multiples of three, breaking the triplet reading frame of a DNA sequence [12].

The C.304_305del (AA) is the novel deletion frameshift mutation in patient (family ID: 39529308) which involves base pairs that are not multiples of three, breaking the triplet reading frame of a DNA sequence [12].

CONCLUSION

In conclusion, seven missense mutations in unrelated FX deficient pedigrees were identified, accounting for type I FX deficiency, which may help explain the correlation between genotype and clinical presentation in this disorder.

The process of validating novel mutations encompasses several stages, including the utilization of Bioinformatics Analysis [14], the implementation of Phenotype-Oriented Target Identification [15], the application of a Networking of Science approach [15], and the execution of Sanger Sequencing [16].

Our findings show that this strategy might be a precise, repeatable, and trustworthy method for the quick genetic identification of Inherited bleeding coagulation disorders. They can facilitate the development of new techniques or tools for gene editing, gene therapy, or genetic testing, that can potentially correct or prevent disease-causing mutations.

ACKNOWLEDGMENTS

We are so grateful to patients and their respected families who kindly consented to join the research. We thank Dr. Zeinali's Medical Genetics Lab staff for technical collaborations, as well. The authors also thank Kharazmi University for their facilities and cooperation.

REFERENCES

[1] Tarantino, M.D., 2021. Occurrence and management of severe bleeding episodes in patients with hereditary factor X deficiency. *Haemophilia*, *27*(4), pp.531-543.

[2] Liang, Q., Chen, Q., Ding, Q., Wu, F., Wang, X., Xi, X. and Wang, H., 2013. Six novel missense mutations causing factor X deficiency and application of thrombin generation test. *Thrombosis research*, *131*(6), pp.554-559.

[3] Girolami, A., Cosi, E., Sambado, L., Girolami, B. and Randi, M.L., 2015, April. Complex history of the discovery and characterization of congenital factor X deficiency. In *Seminars in thrombosis and hemostasis* (pp. 359-365). Thieme Medical Publishers.

[4] Mahmoodi, M., Peyvandi, F., Afrasiabi, A., Ghaffarpasand, F. and Karimi, M., 2011. Bleeding symptoms in heterozygous carriers of inherited coagulation disorders in southern Iran. *Blood coagulation & fibrinolysis*, *22*(5), pp.396-401.

[5] Batsuli, G. and Kouides, P., 2021. Rare coagulation factor deficiencies (factors VII, X, V, and II). *Hematology/Oncology Clinics*, *35*(6), pp.1181-1196.

[6] Riggs, E.R., Andersen, E.F., Cherry, A.M., Kantarci, S., Kearney, H., Patel, A., Raca, G., Ritter, D.I., South, S.T., Thorland, E.C. and Pineda-Alvarez, D., 2020. Technical standards for the interpretation and reporting of constitutional copy-number variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics (ACMG) and the Clinical Genome Resource (ClinGen).

[7] Bastida, J.M., Del Rey, M., Lozano, M.L., Sarasquete, M.E., Benito, R., Fontecha, M.E., Fisac, R., García-Frade, L.J., Aguilar, C., Martínez, M.P. and Pardal, E., 2016. Design and application of a 23-gene panel by next-generation sequencing for inherited coagulation bleeding disorders. *Haemophilia*, *22*(4), pp.590-597.

[8] Peyvandi, F., Duga, S., Akhavan, S. and Mannucci, P.M., 2002. Rare coagulation deficiencies. *Haemophilia*, *8*(3), pp.308-321.

[9] Jayandharan, G., Viswabandya, A., Baidya, S., Nair, S.C., Shaji, R.V., George, B., Chandy, M. and Srivastava, A., 2005. Six novel mutations including triple heterozygosity for Phe31Ser, 514delT and 516T→ G factor X gene mutations are responsible for congenital factor X deficiency in patients of Nepali and Indian origin. *Journal of Thrombosis and Haemostasis*, *3*(7), pp.1482-1487.

[10] James, P., Salomon, O., Mikovic, D. and Peyvandi, F., 2014. Rare bleeding disorders–bleeding assessment tools, laboratory aspects and phenotype and therapy of FXI deficiency. *Haemophilia*, *20*, pp.71-75.

[11] Camire, R.M., 2021. Blood coagulation factor X: Molecular biology, inherited disease, and engineered therapeutics. *Journal of thrombosis and thrombolysis*, *52*(2), pp.383-390.

[12] Hu, J. and Ng, P.C., 2012. Predicting the effects of frameshifting indels. *Genome biology*, *13*, pp.1-11.

[13] Seaby, E.G. and Ennis, S., 2020. Challenges in the diagnosis and discovery of rare genetic disorders using contemporary sequencing technologies. *Briefings in Functional Genomics*, *19*(4), pp.243-258.

[14] Liu, C.K., Chen, Y.H., Tang, C.Y., Chang, S.C., Lin, Y.J., Tsai, M.F., Chen, Y.T. and Yao, A., 2008. Functional analysis of novel SNPs and mutations in human and mouse genomes. *BMC bioinformatics*, *9*, pp.1-7.

[15] Lyon, G.J. and Wang, K., 2012. Identifying disease mutations in genomic medicine settings: current challenges and how to accelerate progress. *Genome medicine*, *4*(7), pp.1-16.

[16] Qian, X., Wang, J., Wang, M., Igelman, A.D., Jones, K.D., Li, Y., Wang, K., Goetz, K.E., Birch, D.G., Yang, P. and Pennesi, M.E., 2021. Identification of deep-intronic splice mutations in a large cohort of patients with inherited retinal diseases. *Frontiers in genetics*, *12*, p.647400.

[17] Ritchie, G.R. and Flicek, P., 2014. Computational approaches to interpreting genomic sequence variation. *Genome medicine*, *6*, pp.1-11.