Original Article

# Complete Genotype and Clinical Phenotype of Hemophilia B: A Study on Iranian Patients

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## Abstract

**Background:** Hemophilia B which refers to the deficiency or functional defect of factor IX (FIX) is typically an X-linked bleeding condition that arises from heterogeneous mutations of the FIX gene (F9). The number of hemophilia cases in Iran is considerable and currently, about 1118 Iranian patients are suffering from hemophilia B, although a small number of them underwent genetic investigations. Here we assessed molecular defects and also laboratory and clinical findings of 10 Iranian cases with hemophilia B.

**Materials and Methods:** A total of 10 cases with hemophilia B were enrolled in the study. Patients were clinically examined by a hematologist and their previous medical documents were surveyed carefully. Routine coagulation tests and FIX activity and antigen assays were performed for the studied patients. Genotyping of F9 for identifying genetic mutations was conducted by the Sanger sequencing method following PCR amplification of the promoter region and all the eight exons of the F9 gene.

**Results:** The mean age of patients was 4 years (9 months to 16 years) and consanguinity was reported in 80% of cases. Patients were commonly manifested by hematoma (90%), epistaxis (80%), and hemarthrosis (70%) and the severity of the disorder was severe (70%) or moderate (30%). In nine out of 10 patients a genetic defect in F9 gene we detected including three missense (c.304T>C, c.1007T>A, c.191G>A) and three nonsense mutations (c.892C>T, c.880C>T, c.1113C>A). Based on the FIX variant database (http://www.factorix.org), five mutations have been reported previously, but mutation c.1007T>A (p.Ile336Asn) seems to be a novel mutation.

**Conclusion:** Our results indicated the heterogeneous molecular defects of hemophilia B in Iran, as recorded in the FIX mutation database. Moreover, no specific genotype-phenotype association was observed in studied subjects.

**Keywords:** Hemophilia B, Factor IX deficiency, Genotyping, Mutation, Clinical phenotype

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# Introduction

Coagulation factor IX (FIX) is a vitamin K-dependent serine protease with a critical role in the coagulation cascade. Quantitative deficiency or functional defect of FIX which is known as Hemophilia B is a hemorrhagic disorder with an approximate prevalence of one in 30,000 male infants, much less frequent than hemophilia A (one in 5,000 males) (1). The disorder is typically hereditary with an X-linked recessive manner of inheritance, although about 30% of cases result from sporadic mutations (2). Bleeding from joints and soft tissues is the predominant clinical picture of hemophilia B, as of hemophilia A (3), and in some patients disabling arthropathy may occur as a consequence of recurrent joint bleedings (2). The disorder originates from mutations affecting the FIX gene (*F9*). *F9* gene which spans 33.5 kb maps to the long arm of the X chromosome (Xq27.1) and contains eight exons. The transcribed mRNA with 2.8 kb length encodes 461 amino acids as a precursor polypeptide which then undergoes posttranslational modifications (4). These modifications include γ-carboxylation of the initial Glu residues, glycosylation, hydroxylation, and phosphorylation. Furthermore, the signal peptide and the propeptide sequences are removed which then result in a mature zymogen with 415 residues and 57kDa molecular weight (5). FIX contain several domains including a Gla domain at the N-terminal region, a hydrophobic stack, two epidermal growth factor (EGF)-like domains, an activation peptide, and a C-terminal serine protease domain. In the presence of $Ca^{2+}$, inactive FIX can be cleaved and converted to active FIX (FIXa) through extrinsic or intrinsic pathways by tissue factor/FVIIa or FXIa, respectively (5).

Iran has a high rate of hemophilia patients and based on the 2019 global survey of the World Federation of Hemophilia (WFH), a total of 10030 patients with hemophilia has been reported in Iran, among which 1118 patients are diagnosed with hemophilia B (5271 patients with hemophilia A and 3641 cases with unknown type hemophilia) (6). Although several studies have been performed on different aspects of hemophilia in Iran, the molecular studies are limited and only a few Iranian cases underwent genetic investigations (7). Therefore, the current study aimed to analyze the underlying gene defect of 10 Iranian patients with hemophilia B which was performed by the Sanger sequencing method. We also assessed the laboratory findings and clinical presentations of the studied subjects.

# Methods

**Patients and data collection:** This study was conducted on 10 unrelated patients with previously diagnosed hemophilia B. The parents of all patients were requested to fill a written consent and the study was approved by the Medical Ethics Committee of Iran University of Medical Sciences. Patients were carefully examined by a hematologist and their clinical presentations and previous medical history were thoroughly recorded. Demographic data including age, sex, and consanguinity were extracted from medical documents or by interview.

Primary laboratory investigations including prothrombin time (PT) and activated partial thromboplastin time (APTT) were conducted by coagulation analyzer (STart; Stago, Paris, France) using Diagnostica Stago-France kits. FIX activity (FIX: C) and FIX antigen (FIX: Ag) levels were also performed for patients. FIX activity assay was carried out by one-stage PTT-based assay by the STA compact automatic coagulometer (Stago) and using Diagnostica Stago, Franconville, France kit. Furthermore, the ELISA method (ELISA; Diagnostica Stago) was applied for the assessment of FIX antigen level. The severity of disorder was categorized based on the FIX activity level (severe: <1%, moderate: 1-5%, mild: 6-40% FIX activity). For identifying the development of FIX inhibitory antibody, a mixing study was performed.

**Molecular analysis and sequencing:** Genomic DNA was extracted from the whole blood obtained in EDTA anticoagulant, using a DNA extraction kit (TakapooZist, Iran), based on the manufacture's principle. The promoter and all eight exons of the F9 gene were subjected to amplification by polymerase chain reaction (PCR) (Bio-Rad, UK) and specific primers that were designed by Oligo 7 (Molecular Biology Insights Inc, Cascade, CO). The primer sequences are indicated in table 1. Gel electrophoresis with 1% agarose was used to assess the PCR-amplified fragments. Finally, the PCR products were

sequenced by the Sanger sequencing method using ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, California, USA). Analysis of the obtained data was performed through Chromas and CLC sequence viewer software. Detected mutations were searched in 'http://www.factorix.org', a FIX mutation database (8) for more details.

# Results

**Characteristics of patients:** All patients were male and were diagnosed with hemophilia B before the age of 2 years. At the time of this study, the mean age of participants was 4 years, ranging from 9 months to 16 years. Eight patients were from a consanguineous marriage. All patients were treated with on-demand factor IX concentrate and one patient developed an anti-FIX inhibitory antibody. The results of APTT were prolonged in all patients, and the PT results were normal. Seven patients had a severe hemophilia B with FIX: C <1%, while the other three cases were represented with moderate disease (FIX: C 1-5%). The most frequent clinical manifestations among participants were hematoma (90%), epistaxis (80%), and hemarthrosis (70%). Other clinical findings include hematuria (20%), gum bleeding (20%), and delay in wound healing (10%). Demographic data, laboratory findings, and clinical features of studied patients are provided in Table 2.

**Molecular findings:** In nine out of 10 patients we detected a mutation in the *F9* gene. However, no mutation was found in one patient, despite the sequencing of all 8 exons and the gene promoter, which may be due to the presence of mutations in the regions that are not routinely sequenced. The majority of mutations (6 cases) were observed in exon eight, the largest exon, and the other mutations were in exons 2 and 4. No one had a genetic mutation in the promoter region of the *F9* gene.

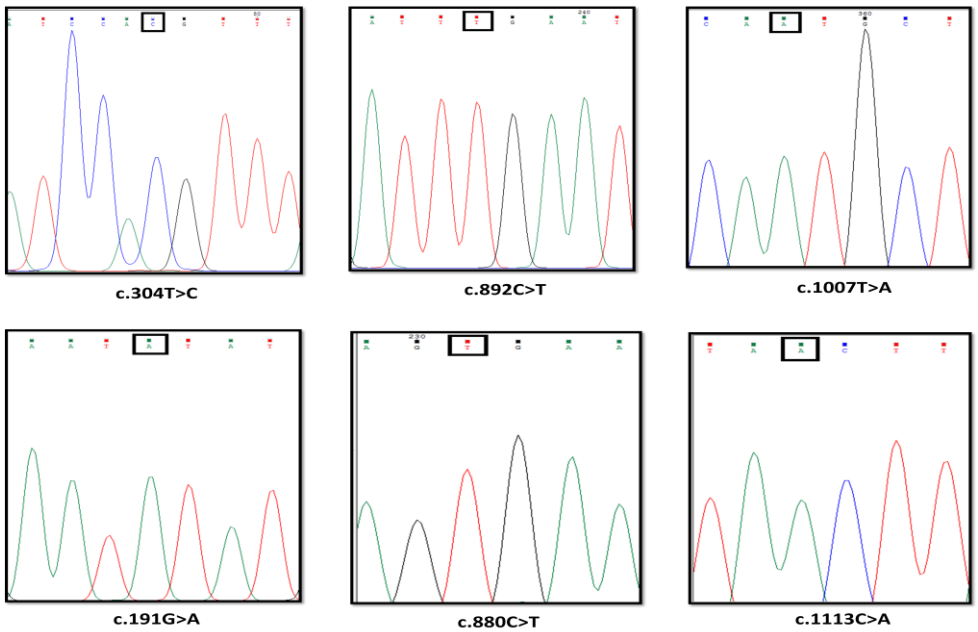


**Figure 1.** The sequence diagram of detected mutations obtained by Chromas software.

**Table 1:** The primer sequences used for PCR amplification.

| Region | Forward primer | Reverse primer | Product size |
|---|---|---|---|
| Promoter | AGGACAAAAGACAAGCTACAGG | TGTTCACGCGCTGCATAACCT | 529 |
| Exon 1 | CCACTGCCCATTCTCTTCACT | AGAGACACACCTCATTACATAC | 743 |
| Exon 2 | GCTCCATGCCCTAAAGAGAAA | TGCTTACCAACATACTGCTTCC | 515 |
| Exon 3 | GTAGTTTTGAAGAAGCACGAG | GCAAGAAGGGTAATGGGGAG | 683 |
| Exon 4 | AATGAGTATCTACAGGGGAGG | GAGGGAAACTTTGAACCATGA | 234 |
| Exon 5 | CCCCAATGTATATTTGACCC | AAGGAAGCAGATTCAAGTAGG | 330 |
| Exon 6 | ATCATAAGCAGCAGAAGTCCC | TAAAATAGCCTCAGTCTCCC | 707 |
| Exon 7 | GCTTCCCTGTCTCTCATTGT | TGACCCTTCTGCCTTTAGCC | 470 |
| Exon 8 (1) | CAGCATGAGTGAACAGAACC | GACATGAATCTCTACCTCCT | 601 |
| Exon 8 (2) | TTCCACTTGTTGACCGAGCC | GATTGAGGAATAGGGTAGAGG | 792 |
| Exon 8 (3) | CCCGATCTTCTTTGCTTCTCC | CTCTGAACACTGCTGAAGACAC | 778 |
| Exon 8 (4) | ACAGCTAGTAGAGACTTTGAGG | AAGGAACTAGCAAGAGTGAGG | 480 |

All detected mutations with their characteristics are shown in table 3. Mutation nomenclature was based on the Human Genome Variation Society (HGVS) guideline. Two types of mutation were detected in our participants including missense and nonsense mutations that were identified in 4 and 5 patients, respectively. Based on the FIX variant database (http://www.factorix.org), five mutations have been reported previously, but mutation c.1007T>A which results in p.Ile336Asn substitution seems to be a novel mutation. Figure-1 illustrates the sequence diagram of all identified mutations obtained by Chromas software.

In two patients with the same mutation (c.304T>C), a polymorphism in intron 1 (c.88+75A>G, 192A>G) was also detected. This polymorphism was previously reported in a Japanese individual and is not a disease-causing variation (9).

## Discussion

Hemophilia B is a bleeding disorder due to coagulation FIX deficiency because of *F9* gene mutations. Underlying mutations are usually classified as type I and type II mutations which are indicative of quantitative deficiency and functional defect, respectively (2). Mutations may occur throughout the F9 gene, including the promoter, 3' and 5' untranslated regions (UTR), and all the eight exons and seven introns, although a vast number of mutations (approximately 37%) involve exon 8 which encodes for a part of the serine protease region (8). Furthermore, specific nucleotides in the *F9* gene are more susceptible to be mutated and are known as hot spot regions.

In contrast to hemophilia A in which rearrangements, particularly intron 22 inversions, are the most frequent gene defect, the majority of mutations causing hemophilia B are point mutations that comprise about 64% of all reported mutations (2, 3). Point mutations include missense mutations which give rise to an amino acid exchange, nonsense mutations leading to premature termination of translation because of an early stop codon, and splicing defects. If point mutations involve the regulation region, it may result in an altered gene expression level (4).

According to the reported mutations in Iranian

**Table 2:** Demographic data, laboratory findings, and clinical manifestations of 10 studied patients with hemophilia B.

| Patient number | Age | Age at diagnosis | Consanguinity | PT (s) | PTT (s) | FIX: C (%) | FIX: Ag (IU/dL) | Disease severity | Manifestations leading to a diagnosis | Clinical manifestations |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2.5 Y | 20 M | Yes (first relation) | 10 | 52 | <1 | 2 | Severe | Epistaxis | Epistaxis, gums bleeding, hematoma |
| 2 | 4 Y | 1 Y | No | 10 | >60 | <1 | 4 | Severe | Hematoma | Hematuria, hematoma, hemarthrosis |
| 3* | 16 Y | 6 M | Yes (first relation) | 9 | >60 | <1 | 3 | Severe | Epistaxis | Epistaxis, hematoma, hemarthrosis |
| 4 | 18 M | 14 M | Yes (first relation) | 8 | >60 | 4 | 8 | Moderate | Hemarthrosis | Hematoma, hemarthrosis |
| 5 | 5 Y | 2 Y | Yes (second relation) | 9 | 49 | <1 | 6 | Severe | Delay in wound healing | Epistaxis, hematoma, hemarthrosis, delay in wound healing |
| 6 | 19 M | 1 Y | Yes (first relation) | 10 | >60 | <1 | 1 | Severe | Hematoma | Epistaxis, hematoma |
| 7 | 5 Y | 2 Y | No | 10 | 52 | <1 | 3 | Severe | Epistaxis | Epistaxis, hemarthrosis, gum bleeding |
| 8 | 9 M | 6 M | Yes (second relation) | 11 | >60 | <1 | 5 | Severe | Hemarthrosis | Epistaxis, hematoma, hemarthrosis |
| 9 | 11 M | 8 M | Yes (first relation) | 9 | >60 | 5 | 2 | Moderate | Epistaxis | Epistaxis, hematoma, hemarthrosis |
| 10 | 2.5 Y | 1 Y | Yes (second relation) | 11 | 48 | 2 | 5 | Moderate | Hamaturi, hematoma | Hamaturi, epistaxis, hematoma |

* Patient number 3 developed anti FIX antibody.

Y: year, M: month, s: second

patients, missense mutations are the most frequently detected mutations in Hemophilia B which comprises more than 50% of all reported mutations in most of the studies. The second common mutation in Iranian hemophilia B patients is nonsense mutations (7). However, the results of the molecular defects of hemophilia B in Iran are limited. The largest study was conducted by Karimipoor et al on 76 unrelated patients with hemophilia B. Mutations were found in 52 out of 76 studied cases and include 33 missense mutations, 11 nonsense mutations, 4 mutations affecting the promoter region, 2 frameshifts, and 2 splicing defects (10). Here we found genetic defects in 9 out of 10 patients by Sanger sequencing which is indicative of the acceptable efficiency of this method. All the detected mutations were point mutations and with missense or nonsense effects, which are inconsistent with the previous reports. As could be expected, most of the mutations involved exon 8 and affects the serine protease domain of the protein. It has been indicated that there is a high rate of heterogeneity in the genetic defects of hemophilia B and therefore there is not a recurring mutation that can be used for the detection of carriers in different ethnicities (7). Furthermore, a genotype-phenotype heterogeneity is also reported in hemophilia B, and so

**Table 3:** Detected mutations in studied patients with hemophilia B.

| Patient number | Nucleotide number | Nucleotide exchange | Amino acid exchange | Mutation type | Mutation effect | Location | Domain |
|---|---|---|---|---|---|---|---|
| 1, 2 | 10418 | c.304T>C | p.Cys102Arg | Point mutation | Missense | Exon 4 | EGF1 |
| 3, 4 | 30875 | c.892C>T | p.Arg298Stop | Point mutation | Nonsense | Exon 8 | Serine Protease |
| 5 | 30990 | c.1007T>A * | p.Ile336Asn | Point mutation | Missense | Exon 8 | Serine Protease |
| 6 | 6428G | c.191G>A | p.Cys64Tyr | Point mutation | Missense | Exon 2 | Gla |
| 7 | 30863 | c.880C>T | p.Arg294Stop | Point mutation | Nonsense | Exon 8 | Serine Protease |
| 8, 9 | 31096 | c.1113C>A | p.Tyr371Stop | Point mutation | Nonsense | Exon 8 | Serine Protease |

In the number 10 patient, no mutation was detected.

* Based on the FIX mutation databases, it seems to be a novel mutation.

different phenotypes and disease severities may be induced by the same mutation (11). This finding can also be elucidated from our study, as two mutations including c.892C>T and c.1113C>A were associated with severe and moderated disease severities. Furthermore, only one of the two patients affected by the c.892C>T (p.Arg298Stop) nonsense mutation developed an anti-FIX inhibitor (patient number 3 with a severe phenotype). The risk of inhibitor development in hemophilia B is much less frequent compared with hemophilia A. Inhibitors are reported in about 2% of patients with hemophilia B, mainly in severely affected patients, and are usually associated with large deletions and nonsense mutations (12).

## Conclusion

Our findings were suggestive of the heterogeneous molecular defects of hemophilia B in Iran, as recorded in the FIX mutation database. Moreover, no specific genotype-phenotype association was observed in studied subjects.

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## Conflicts of Interest

The authors declare no conflict of interest at all.

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