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The molecular basis of hemophilia B

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Summary

Hemophilia B (HB) is a genetically determined bleeding disorder characterized by deficiency of the coagulation factor IX (FIX). The severity of bleeding phenotype is associated with FIX plasma level. More than 1200 F9 gene variants have been identified in hemophilia B patients at different locations which only confirms the marked heterogeneity of this bleeding disorder. The HB bleeding phenotype does not always correlate thoroughly with FIX:C plasma level, so identification of the molecular mechanism of HB may be helpful in understanding the heterogeneity of the hemorrhagic phenotype and may contribute to better diagnosis and therapy of the affected patients. This article presents the molecular determinants of hemophilia B and discusses their pathomechanisms in particular FIX domains.

Key words: hemophilia B, factor IX, FIX domain, *F9* gene, causative mutation

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Clotting factor (FIX) is a vitamin K dependent protein, and its deficiency causes hemophilia B (HB), an X-linked recessive bleeding disorder. The first hemophilia B patient, Stephen Christmas, was described in 1952, therefore hemophilia B is also called Christmas disease. In contrast to hemophilia A, hemophilia B is much less common and is estimated to account for 15–20% of all hemophilia cases with an incidence rate of approximately 1 in 30,000 liveborn boys. The bleeding intensity in HB correlates with FIX:C activity in the patient's plasma. Based on the FIX:C level in plasma, hemophilia B has been classified into three types: severe (FIX:C activity < 1% (< 0.01 IU/mL), moderate (1–5% (0.01–0.05 IU/mL) and mild (5–40% (0.05–0.40 IU/ml).

It is known for a fact that the clinical phenotype of hemophilia B does not always perfectly correspond with this classification based on FIX:C level. In addition, it is also unclear why patients with the same HB causative mutation present different bleeding tendencies, ranging from mild to severe. It is therefore, believed that identification of the molecular mechanism of HB may be helpful for understanding the heterogeneity of the bleeding phenotype and will provide significant insight into patient diagnosis and treatment. The large number of different genetic variants identified in HB (>1200) confirms the molecular heterogenicity of this disease.

The FIX gene (*F9*) was cloned in 1982. It spans 34 kb of the long arm of the X chromosome (Xq27.1) and consists of 8 exons and 7 introns. The *F9* gene transcript mRNA (NM_000133) is 2,8 kb in size and encodes a precursor protein containing 461 amino acids (aa), which include a N-terminal signal peptide (1-28 aa), a propeptide (29-46 aa) and the 415-aa mature protein in which the following are distinguished: Gla domain (47-92 aa), two epidermal growth factor-like domains (EGF) (93-171 aa), a linker sequence (172-191 aa), activation peptide (AP, 192-226 aa) and serine protease (SP) domain (227–461 aa). Exon 1 encodes a signal peptide, exon 2 — the propeptide and Gla, exon 3 — part of Gla, exons 4 and 5 — EGF, exon 6 — AP, and exons 7 and 8 — SP.

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FIX belongs to a group of vitamin K-dependent glycoproteins which are mailny synthesized in the liver. Before secretion into the blood, FIX undergoes various post-translational modifications. The signal peptide and propeptide are the regulatory sequences engaged in secretion and carboxylation of the FIX respectively which are removed from the mature protein molecule. The Gla domain is present in vitamin K- dependent coagulation factors and is essential for FIX activation and binding to phospholipid membranes during blood clotting (N-terminal calcium-dependent Gla domain). Additionally, the Gla domain in FIXa contributes to its binding to the C2 domain of FVIIIa and collagen IV. The EGF domains contain two similar domains EGF1 (93-129aa) and EGF2 (130-171aa). EGF1 participates in FIX activation through interaction with FXIa or the TF/FVIIa complex and the FVIIIa co-factor and also induces FIX interaction with FVIIIa and FX. The EGF2 FIXa domain, but not FIX, may be involved in binding to the platelet phospholipid membrane surface as well as to FVIIIa and FX.

Factor IX is a serine protease that circulates in the blood as an inactive zymogen. Activation of FIX to FIXa occurs through proteolytic cleavage by FXIa in the intrinsic coagulation pathway, or by the TF/FVIIa complex in the extrinsic pathway, with the release of the activation peptide. A FIXa molecule contains an N-terminal light chain (Gla domain and two EGF domains) and a C-terminal heavy chain (SP domain). It is not only the proteolytic cleavage of FIX by FXIa or the TF/FVIIa complex that is responsible for FIX activation, because the entire activity of FIX itself is too weak to cleave FX. In order to achieve full enzymatic activity, FIXa forms a Ca^{2+} dependent complex with the FVIIIa cofactor on phospholipid-containing membranes, called the tenase complex, which increases FIX activity > 200,000 times.

The interactive Factor IX Gene (*F9*) Variant Database (http://www.factorix.org/) currently registers over 1,200 unique *F9* variants reported in 4713 HB patients. The mutations responsible for HB occur in the coding (~80%) and non-coding regions (including the promoter, introns and 3' untranslated regions) of the *F9* gene.

The coding region mutations are mostly localized in the SP domain (56.9%), and they are quite evenly distributed in particular FIX domain: Gla (12%), EGF1 (9.4%), EGF2 (9.3%). On the other hand, the variants in coding regions are relatively rarely detected in the activation peptide (2.3%) (Fig. 1). The non-coding region mutations are also less frequently detected: signal peptide (3%) and propeptide (3.3%).

Among the mutations identified in *F9*, point mutations predominate (71.9%), while other mutations are less frequent; deletions (17.1%), insertions (1.1%), duplications (3.9%), indels (1.4%), polymorphisms (4.1%) and complex rearrangements (0.4%) (Fig. 2). The HB causative mutations are most often detected in exons 2 and 8 of the *F9* gene. The detection frequency is slightly lower in exons 4, 5, 6 and 7, while it is the lowest in exons 1 and 3. The higher mutations frequency in exons 2 and 8 of *F9* suggest that the encoded FIX molecule domains, propeptide, Gla and SP, are much more important for FIX activity in the clotting pathway than the others.

F9 gene mutations lead to FIX deficiency through affecting its structure, transcription, splicing, translation, post-translational modifications, protein folding, and formation of a functional complex with other clotting factors. More than 70% of the detected *F9* mutations are point mutations. Less frequent are deletions (17.1%), insertions (1.1%), duplications (3.9%) or indels (1.4%) in the coding sequence and most of them cause a frame shift that generates a truncated polypeptide. Most patients with frame shift and inframe mutations localized in coding regions suffer from severe HB. The similar condition occurs when mutations are localized in the flanking exon/intron regions which usually cause aberrant splicing leading to severe HB. About 2% of the unique mutations affect multiple regions of the *F9* gene and correspond to large, multiexon deletions of the *F9* gene that also lead to severe HB. It should be noticed that individuals with large deletions of the *F9* gene are burdened with the highest risk (43%) of developing FIX inhibitor. The bleeding phenotype for HB patients with point mutations varies from severe to mild, and there are several mechanisms responsible for FIX deficiency. In general, point mutations in the promoter region result in HB Leyden, those in the exons cause missense, nonsense or silent mutations, and those in the introns result in abnormal splicing.

Mutations in the promoter region of the *F9* gene often lead to HB Leyden, which was first described in 1970. Men affected with HB Leyden have low FIX:C levels at birth which then rise during puberty and often reach normal levels in adulthood. More than 20 *F9* mutations have been identified as Leyden mutations which are located in the proximal promoter region from c.-50 to c.-18, and cluster at three particular regions:

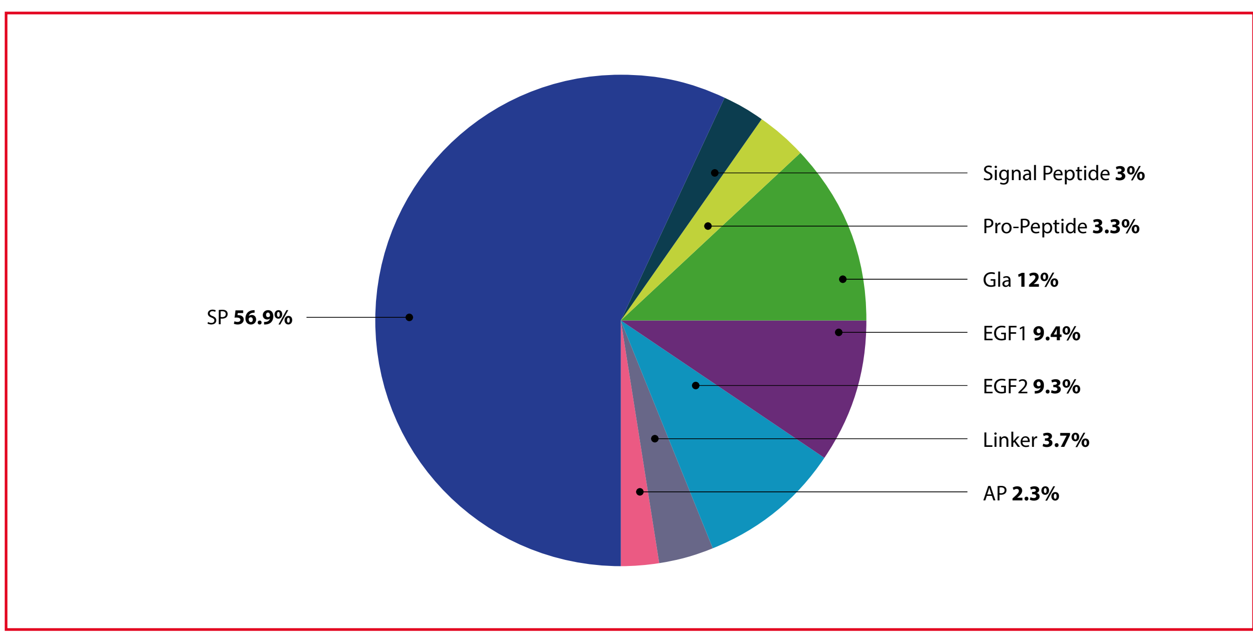

Figure 1. Distribution of the mutations in the particular FIX domains (according to https://dbs.eahad.org/) [13]. EGF1 — epidermal growth factor 1; EGF2 — epidermal growth factor 2; SP — serine protease; AP — activation peptide

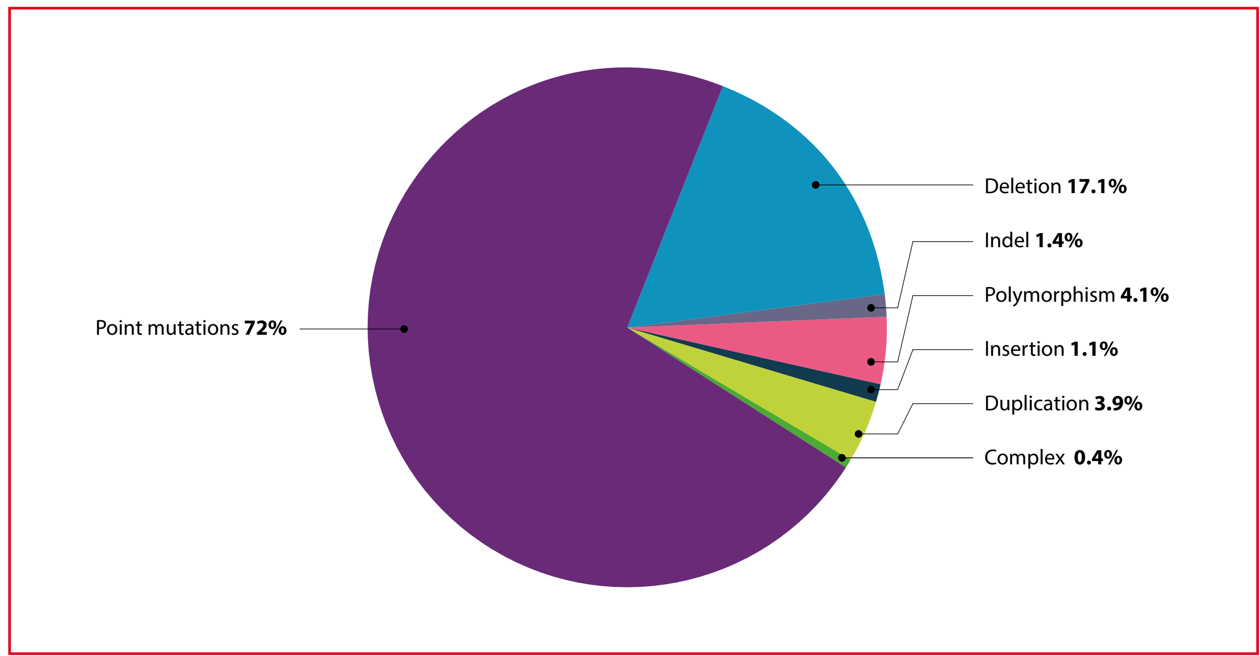

Figure 2. Types of HB causative mutations in the *F9* gene (according to https://dbs.eahad.org/) [13]

c.-34/c.-35, c.-49 and c.-19. Mutations at nucleotides c.-34 and c.-35 account for more than half of HB Leyden cases. Depending on the mutations, some HB patients initially present a severe phenotype which then evolves to a mild one, or represent only a milder phenotype. Initially it was thought that the increase of FIX:C levels with age was related to androgen receptors. The mechanism is not fully understood so far.

More than 200 patients with point mutations in the introns have been reported in the *F9* gene database which accounts for approx. 6% of the total of the patients (Table 1). Most of the intron point mutations were found near the splice sites (within 25 bp). However the deep intron mutations were rarely identified. Point mutations located in the introns usually lead to abnormal splicing and consequently affect the expression of the functional

Table 1. Distribution of point mutations in particular regions of *F9* gene (modified from [8])

Region F9	Mutation type	% of total patients in the *F9* mutation database
Promoter	HB Leyden	2
Exons	Missense	65
	Nonsense	13
	Synonymous (silent)	1
Introns	Splice	6
3`UTR		0,6

protein. According to the *F9* database, most HB patients with intron mutations demonstrate severe to moderate phenotypes which may indicate that this mutation could be involved in interference with FIX alternative splicing.

Point mutations in 3`UTR were reported in 22 HB cases. The most frequently identified mutation was c.2545A>G with severe or moderate phenotype, since this mutation leads to activation of the cryptic splice site, which presumably destabilizes the mRNA or alters the splicing of the previous intron.

Point mutations in the *F9* coding regions account for almost 80% of the mutations responsible for HB. These mutations include 336 residues of the 461 residues in the FIX precursor and may cause silent, nonsense, and missense mutations.

Synonymous (silent) mutations alter the nucleotide but not the encoded amino acid. They may appear clinically neutral, although some may affect protein production due to aberrant splicing, mRNA instability, or abnormal translation. According to *F9* database, 16 unique silent mutations have been identified so far, but their mechanisms are not yet fully understood.

Nonsense mutations account for approximately 13% of point mutations in the coding region and are usually responsible for severe HB. Individuals with nonsense mutations are at a higher risk of developing a FIX inhibitor. A few patients have been identified with moderate or mild HB, which suggests that spontaneous ribosome readthrough may occur with some nonsense mutations. It is worth noting that the plasma from HB patients with p.Arg294* and p.Arg298* mutations revealed traces of full-length FIX. This was confirmed by in vitro studies. It is speculated that ribosome readthrough in nonsense mutations that allow at least the lowest level of protein production, has impact on both the severity of the disease as well as the likelihood of FIX inhibitor development.

Missense mutations in the signal peptide and propeptide are relatively rare. The signal peptide and propeptide are regulatory sequences that are cleaved off in mature FIX. These mutations cause FIX deficiency by interfering with FIX's co-translational translocation to endoplasmic reticulum (e.g. p.Ile17Asn, p.Leu20Ser, p.Leu23Pro, p.Leu24Pro) or signal peptide cleavage (e.g. p.Ala26Asp and p.Cys28Arg)/Tyr/Trp. There are two significant elements in the structure of the propeptide: the GGCX recognition site and the propeptidase recognition site. Mutations of the propeptide sequence result in FIX deficiency through decreased expression, abnormal carboxylation and impaired protein secretion (e.g. p.Arg43Gln, p.Arg43Trp, p.Arg46Ser), or may lead to the formation of an uncleaved propeptide in the mature FIX protein (e.g. p.Arg43Gln, p.Arg43Trp, p.Arg46Ser), which disrupts the structure of the Gla domain.

The Gla FIX domain is involved in the FIXa binding to the phospholipids membrane, to TF in the TF/FVIIa complex, and to the C2 domain of FVIIIa therefore missense mutations in this domain affect the enzymatic activity of FIXa. They represent approximately 12% of the detected *F9* mutations and of the 12 glutamate residues, point mutations in 9 residues have been reported in HB patients, mostly with severe HB. These mutations lead to destabilization of the FIX structure by disrupting the Ca^{2+} ions binding to polypeptide chain. Point mutations in the Gla domain disrupt the structural integrity of this region and affect the function of FIXa by impairing its interaction with the phospholipids membrane, TF, FVIIIa and collagen IV.

Frequency of missense mutations in the EGF1 and EGF2 domains is slightly higher than in Gla domain (18.7 vs. 12%). Missense mutations of the cysteines (Cys) break the disulfide bonds and cause a severe bleeding phenotype. These cysteine mutations in HB are associated with reduced levels

of FIX antigen, suggestive of the destabilization of FIX. Apart from disulfide bonds, Ca^{2+} ion binding to the EGF1 domain (residues Asp93, Gln96 and Asp110) is vital for stabilizing its conformation and assembly of the Xase complex on the phospholipid surface. Missense mutations in these domains may disrupt the stability of EGF1 domain, which serves to correctly position the SP domain for optimal interaction with FVIIIa. Thus, HB causative mutations in EGF domains, especially EGF2 (residues Ile136, Asn138 and Arg140), may interfere with the clotting process due to defective FIXa to FVIIIa binding. In HB patients, missense mutations in residues Ile136 and Val153 of the EGF2 domain, may disrupt the interaction of FIXa with the activated platelets surface and defective assembly of the Xase complex. In patients with p.Gly94Arg/ /Val mutations, the interaction of FIX with the TF/ /FVIIa complex is disrupted.

The *F9* genetic variant database describes more than 300 HB patients with mutations at the cleavage site of activation peptide, namely at Arg191 or Arg226 residues. Mutations in the other residues of the activation peptide are rarely reported and these are usually considered polymorphisms. Removal of the activation peptide from FIX during activation requires cleavage at both the Arg191 and Arg226 site therefore the missense mutations disrupt these cleavages. Most patients with missense mutations at Arg191 site exhibit moderate to mild bleeding tendency, while those with mutations at Arg226 site present the severe phenotype. Individuals with mutations at Arg191 and Arg226 sites have various FIX antigen levels. Mutations at Arg226 residues are associated with normal or increased antigen levels, while Arg191 mutations are characterized by normally or moderately reduced antigen levels. This indicates the detrimental effects of Arg191 mutations on the protein folding or secretion manifested by various bleeding phenotype in patients with Arg191Cys>Leu>Pro>His mutations.

Among HB patients with missense mutations almost 57% have the mutations located in the SP FIX domain, which only shows how important the domain is. Missense mutations in the cysteine residues involved in disulfide bond formation in the SP domain and protein stabilization usually cause severe HB. Mutations in residues Arg294, Arg298, and Asn310 residues of the Ca^{2+} ion binding loop indicate markedly lower levels of FIX antigen, which suggests that the calcium loop affects the stability of the SP domain. Another mechanism responsible for FIX deficiency is the impaired interaction with FVIIIa due to missense mutations in the involved region of the SP domain (e.g. 378-helix), because missense mutations at 8/9 residues in this domain result in HB through reducing FIXa's affinity to FVIIIa (Lys339 residues, Asn392, Lys362). Moreover, mutations in the catalytic triad of His267, Asp315 and Ser411 of the SP domain impair the active site formation or substrate recognition.

Hemophilia B is one of the most intensively studied genetic disorders. Thanks to wide availability of genetic testing, more than 1,000 unique mutations have already been detected in HB patients, although the molecular mechanisms of the FIX deficiency caused by these mutations is not yet fully understood. Differences in the bleeding tendency of HB patients in the presence of the same causative mutation indicate that identification of the *F9* mutation is not the only one predictor of bleeding tendency. Investigation of the complexity of the underlying pathogenic mechanisms of FIX deficiency may provide insight into new strategies for better therapy of HB patients. Insight into nonsense mutations for example may result in development of drugs inducing ribosomal readthrough which allows for secretion of full-length FIX, or development of medications that modifies aberrant splicing.

Conflict of interest: none declared

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