

Review

Factor VIII genetic mutations and protein alterations in hemophilia A: A review

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Received 20 April 2011; Accepted 29 April, 2014

Hemophilia A represents a severe most common inherited hemorrhagic disorder caused by heterogeneous mutations, which lead to dysfunctional factor VIII protein. Besides the inversion 22 and intron 1 inversion, the mutations may describe 627 missense and 142 nonsense unique mutations. Changes in the protein sequence induce structural or functional impairment. This study aimed to review mutation in different domains and discuss molecular modeling approach to assess the effects of amino acid substitutions on the topology of FVIII protein domains. A comprehensive literature search was done to analyze the mutations and structural alterations reported in the Hemophilia A gene. Further, our experience in small mutation analyzed with structural alterations was added to the review. Mutation types were used at the hemophilia A mutation, structure, test and resource site (HAMSTeRS). Half of the point mutation in the FVIII gene was found in domain A which includes Glu321Lys, Tyr346Cys, Val357Gly, Thr770Ser, Thr751Ser etc. Exon 14 represents about one half of the coding region and encodes for the FVIII B domain. Several recurrent mutations have been found at 2147, 2150, 2159, 2163 {C1} and 2209, 2300, 2307 (C2) amino-acid positions. Different domains play an important role in the function of FVIII as each contains specific binding active site during the clotting cascade. The review brings forth the functional alterations occurring because of causative mutations in hemophilia A gene.

Key words: Hemophilia a, FVIII gene, mutations, protein modeling.

INTRODUCTION

Hemophilia A is the most common severe X-linked recessive bleeding disorder (Ensenauer et al., 2003) caused by decreased activity of factor VIII due to heterogenous mutations in FVIII coding gene (F8). The word 'Hemophilia' was first used by Hopff in 1828. Hemophilia A is also called "the royal disease" because Queen Victoria of England (1837 to 1901) was a carrier, and from her it spread to the royal families of Spain, Germany and Russia. Hemophilia A is a model disorder in the field of molecular human genetics due to several

factors, including the influence of other proteins on the penetrance and characteristics of the disease (for example, factor V, prothrombin and von Willebrand factor). The sequence of the F8 coding gene was first reported by Gitschier in 1984. A large number of mutations that cause hemophilia A have been identified (Gitschier et al., 1984). Mutations in the F8 gene, located at the telomeric end of the long arm of the X chromosome, include the intron 22 and intron 1 inversion hot spots, point mutations (nonsense and missense

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mutations) that are distributed in all 26 exonic regions and various deletions and insertions (Kemball-Cook and Tuddenham, 1998). Today, more than 960 unique mutations of different types are collected in the worldwide hemophilia database (HAMSTeRs). Several studies in reviews have detailed the mutations in F8 gene. However the functional dearrangement in the protein explaining the causative nature of the mutation needs indepth analysis. In the present article we describe the changes in the protein domains resulting from frequent genetic mutation in Hemophilia A.

CLINICAL FEATURES OF HEMOPHILIA A

Hemophilia A affects 1 in 5,000 males worldwide (Forbes et al., 1997; Bolton-Maggs and Pasi, 2003) cases who suffer from joint and muscle bleeds and easy bruising. The severity of bleeding is closely related to the level of activity of coagulation factor VIII (FVIII: C) in blood. Severity of hemophilia A is defined by FVIII: C level in plasma. Severely affected individuals have < 0.01 iu/dl (< 1% of normal); moderate < 0.01 to 0.05 iu/dl (< 1 to 5% of normal) and mild > 0.05 to < 0.40% iu/dl (< 5 to 40% of normal) (White et al., 2001). The frequency of the mild, moderate and severe forms is 50, 10 and 40%, respectively (Antonarakis et al., 1995). Individuals with factor VIII clotting activity higher than 30% usually do not have bleeding (Kaufman et al., 2006). However, a mild bleeding tendency can occur with low to low-normal factor VIII clotting activity in hemophilia A carrier females (Plug et al., 2006) or in those with mild von Willebrand disease. The major cause of disability from bleeding is chronic joint disease (Luck et al., 2004). In developed countries, haemorrhage into the central nervous system (CNS) is a leading cause of death in cases with hemophilia, accounting for almost 20% of non-infective deaths.

MUTATION PROFILING IN HEMOPHILIA A

Factor VIII gene

The gene of FVIII is located at the long arm of X chromosome (Poustka, et al., 1991; Freije and Schlessinger, 1992). It spans over 186 Kb and comprises of 26 exons, which range from 69 bp (exon 5) to 3.1 Kb (exon 14) in size. The Factor VIII mRNA is nearly 9 Kb in size and it encodes a precursor protein of 2,351 amino acids (Vehar et al., 1984) from which 19 N-terminal proteins of the signal peptide are removed to generate the mature, functional product. Factor VIII is a large multi-domain glycoprotein with domain structure A1-a1-A2-a2-B-a3A3-C1-C2 (Vehar et al., 1984; Lenting et al., 1998). The heavy chain is composed of domains A1-A2-B, while the light is composed of domains A3-C1-C2. Binding of

the 2 chains is non-covalent and requires a metal ion-dependent linkage, with the residues responsible contained within the A1 and A3 domains. This metal ion is likely copper and 1 mol of copper ion (Cu^{++}) (Tagliavacca et al., 1997) has been identified in factor VIII (Bihoreau et al., 1994). The A domains display approximately 30% homology to each other. These domains further display a similar extent of homology to the copper-binding protein ceruloplasmin and to factor V, the cofactor in the prothrombinase complex (Church et al., 1984). The C domains are structurally related to the C domains of factor V. The B domain is unique in that it exhibits no significant homology with any other known protein (Figure 1).

The A domains are bordered by short spacers (a1, a2, and a3) that contain clusters of Aspartic and Glutamic residues, the so called acidic regions. Further processing of protein by thrombin leads to the activation of F8. First cleavage at Arg 1689 (at the B-a3) generates a variably-sized (90 to 210 KDa) heavy chain, consisting of domains A1 and A2 and heterogeneous fragments of the partially proteolysed B domain; during the process, a 40-amino-acid acidic peptide (a3) is released from the C-terminal product to form a 73 KDa light chain that consists of domains A3-C1-C2. Further cleavage by thrombin removes most of the B domain and cleaves the protein between the A1 and A2 domains: cleavage at Arg 372 (between the A1 and A2 domains) and at Arg 740 (between the A2 and B domains) generates 54 KDa A1 and 44 KDa A2 domains. Homologues of F8 in the mouse (Elder et al., 1993) and rat (Watzka et al., 2004) are similar in sequence to the human, but are slightly shorter. The overall identity at the protein level between the mouse and human F8 is 74% and between rat and human is only 61%.

Factor VIII mutations

Factor VIII deficiency is caused by broad spectrum mutations which occur along the entire length of the factor VIII gene. The mutations lead to defect at the level of transcription or translation or to changes of individual amino acids in factor VIII protein. Severe hemophilia is typically caused by inversions, insertions, deletions and nonsense mutations and also by missense mutations. Milder forms of hemophilia are usually caused by missense mutations while single nucleotide deletions or splicing errors may occur.

Missense and nonsense mutations

In mild to moderately severe hemophilia A missense mutations within the exons coding for the three A domains or the two C domains account for most of the mutations detected (Kemball-Cook and Tuddenham,

Table 1. List of point mutations in different domains.

Domain	Missense mutations		Nonsense (stop) mutations	
	Unique mutation	Frequency	Unique mutation	Frequency
A1	174	335	20	45
A2	167	411	24	44
B	9	12	46	69
A3	153	440	27	73
C1	62	217	13	56
C2	62	215	12	49
Total	627	1630	142	336

Source: Kemball-Cook and Tuddenham (1998).

1998; Liu et al., 1998, 2000, 2002). The CpG site is one of the hotspots in hemophilia A, with arginine (CGC, CGG, CGT) being the most frequently affected as the cells DNA repair mechanism does not regard thymidine formed by deamination of methycytosion residues, as abnormal. For example, codon with three different amino acid changes are the arginine codon 531 in exon 11 (Higuchi et al., 1990), codon 1781 in exon 16 (Faridi et al., 2011) and codon 2150 in exon 23 (Liu et al., 2002; Cutler et al., 2002; Habart et al., 2002; Fernandez-Lopez et al., 2005), which has been reported 61 times (Table 1), since arginine is involved in 25% of all missense mutations found so far and play a crucial role in protein function. Ahmed et al. (2005) carried out mutation studies in inversion negative hemophilia A patients by the high performance liquid chromatography (dHPLC) method and found 11 missense mutations, and some other mutations (Ahmed et al., 2005), whereas Jayandharan et al. (2005) detected 101 mutations by using multiplex polymerase chain reactions (PCRs) and the Cap analysis gene expression (CAGE) technique, of which 21 were missense mutations (Jayandharan., 2005). There is a report of missense mutation being pre-dominantly found in the A1 and A2 domains (Bogdanova et al., 2005). We have also found the change of arginine 1781 to histidine in 3 unrelated patients in A3 domain (Faridi et al., 2011). Santacroce et al. (2008) were able to detected 384 and 67 (Feng et al., 2010) different mutations and these were mostly effected in the A domains (Table 2).

INVERSION

Inversions cause about half of the cases of severe hemophilia A. The most frequent inversion mutation to affect F8 is Intron 22 and has been reported in 40 to 50% of patients with severe Hemophilia A (Lakich et al., 1993). Recently, an intron 1 inversion was also reported to be a recurrent mutation, occurring in 5% of patients with severe hemophilia A (Bagnall et al., 2002). Studies from the west have reported a prevalence of intron 22 inversion ranging from 30 to 50% in severe hemophilia A

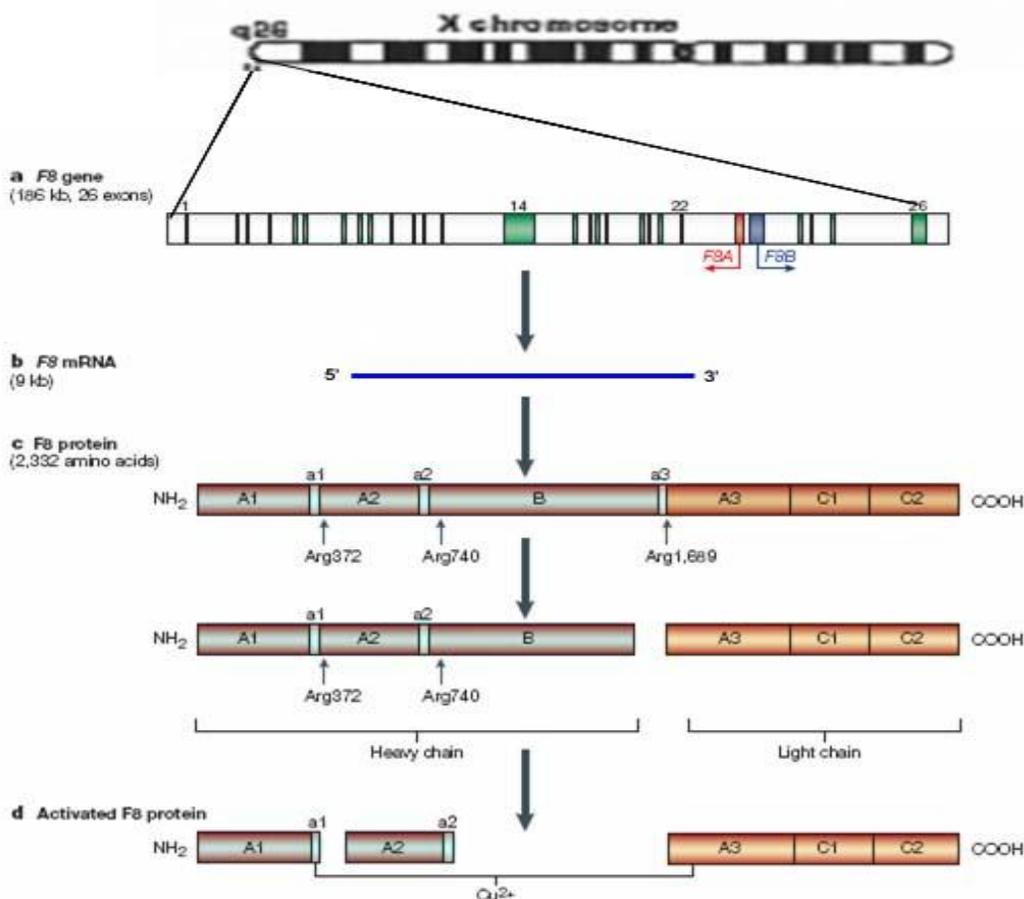
(Enayat et al., 1995; Naylor et al., 1995), which is in accordance with that found in India (Shetty et al., 1998; Jayandharan et al., 2004; Pandey et al., 2002; Ghosh et al., 2004). Shetty et al. (1998) have reported a prevalence of 1.24% of intron 1 inversion positive cases (3 out of a large cohort of 241) in Mumbai population, whereas worldwide incidences record 1 to 5% (Tizzano et al., 1995; Aquila et al., 2003; Rosseti et al., 2004). However, studies by Andrikovics et al. (2003) revealed an absence of intron 1 inversion in 104 unrelated HA cases in Hungary. The intron 22 of the FVIII gene contains a 9.5 kb region, which is present outside of the gene, near the telomere of the X chromosome in two additional copies; these are termed int22h2 and int22h3: int22h1 is the sequence in intron 22 that also include the F8A gene (Figure 3a and b) (Levinson et al., 1990; Naylor et al., 1995). The sequence identity of the three regions is 99.9%. Intrachromosomal homologous recombination between int22h1 and one of its two telomeric copies leads to an inversion of the corresponding parts of the F8 gene. The mechanism leading to the inversion with the break point within intron 1 is similar (Figure 3a and c); however, the consequence is not only the disintegration of the factor VIII gene but also the emergence of a new fusion gene, whose significance remains unknown. Both intron1 and intron22 prevent the formation of full length FVIII mRNA and cause severe Hemophilia A {FVIII activity <1%}.

INSERTIONS AND DELETIONS

Insertions and deletions that cause severe hemophilia A are classified into large (more than 50 bp) and small which usually span one or several nucleotides. Various types of repetitive sequences, including SINES and LINES, which are present in the factor VIII gene, may be involved. Insertions and deletions of a single nucleotide cause a shift of the reading frame and thus, usually, a severe form of the disease. During transcription of the damaged gene the RNA-polymerase thus synthesizes some molecules with correct sequence. A minute,

Table 2. Recurrent mutations consist of small deletions or insertions of A nucleotide in the F8 gene.

Exon	Small deletions {50 or less than}			Insertion		
	Size in bp (nucleotides deleted)	Codon	Frequency	Nature of Insertion	Codon	Frequency
14	C3637delA	1191-94	57	1bp A in 9As	1191-94	15
14	C4379delA	1439-41	14	C4379insA	1439-41	29

**Figure 1.** The salient features of the coagulation factor FVIII gene, as well as the production and processing of the domains of protein.

Source: Modified from Graw et al. (2005).

nonetheless immunologically significant amount of normal factor VIII is formed. Hemophilia in such patients is somewhere on the border between the severe and moderate forms. In severe hemophilia A, gross gene alterations (including large deletions or insertions, frameshift and splice junction changes and nonsense and missense mutations) of FVIII account for approximately 50% of mutations detected (Kemball-Cook and Tuddenham, 1998; Bowen, 2002; Goodeve and Peake, 2003; El-Maarri et al., 2005). The most recent update of the HAMSTeRS database (August, 2007) listed more than 200 small deletions (50 or less than) and more than

80 insertions as being causative for Hemophilia A. The recurrent insertions or deletion of a single A has also been reported at shorter stretches of A nucleotides.

THE PRINCIPAL FUNCTIONAL DOMAINS OF THE FVIII PROTEIN

FVIII is a large multi-domain protein containing internal repeats (Vehar et al., 1984). The different domains play an important role in the function of FVIII as each contains specific binding sites for different components of clotting cascade (Lenting, 1998). There are three homologous A-

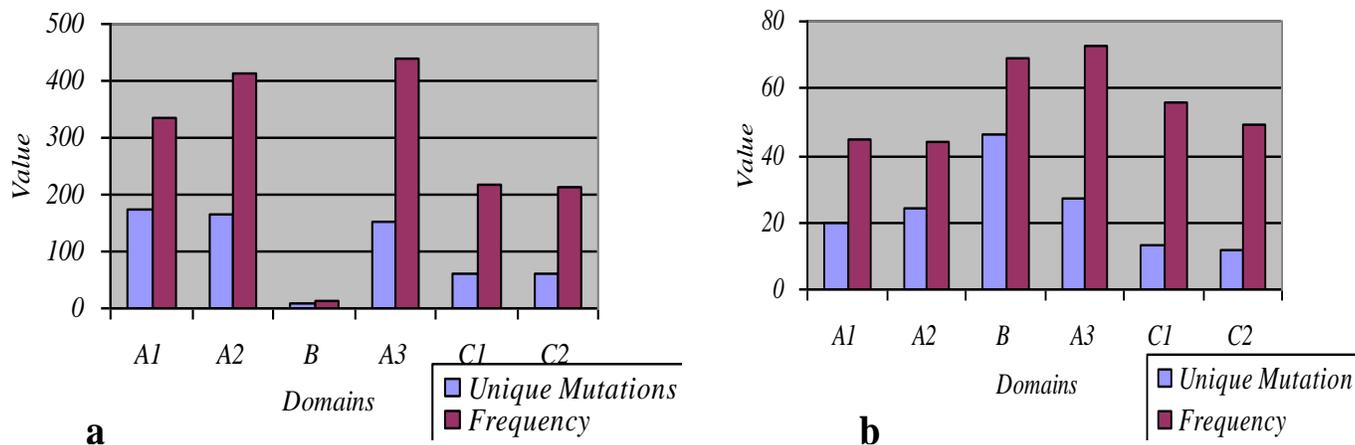


Figure 2. (a) Missense unique mutations and their frequencies (b) Nonsense (Stop) unique mutations and their frequencies.

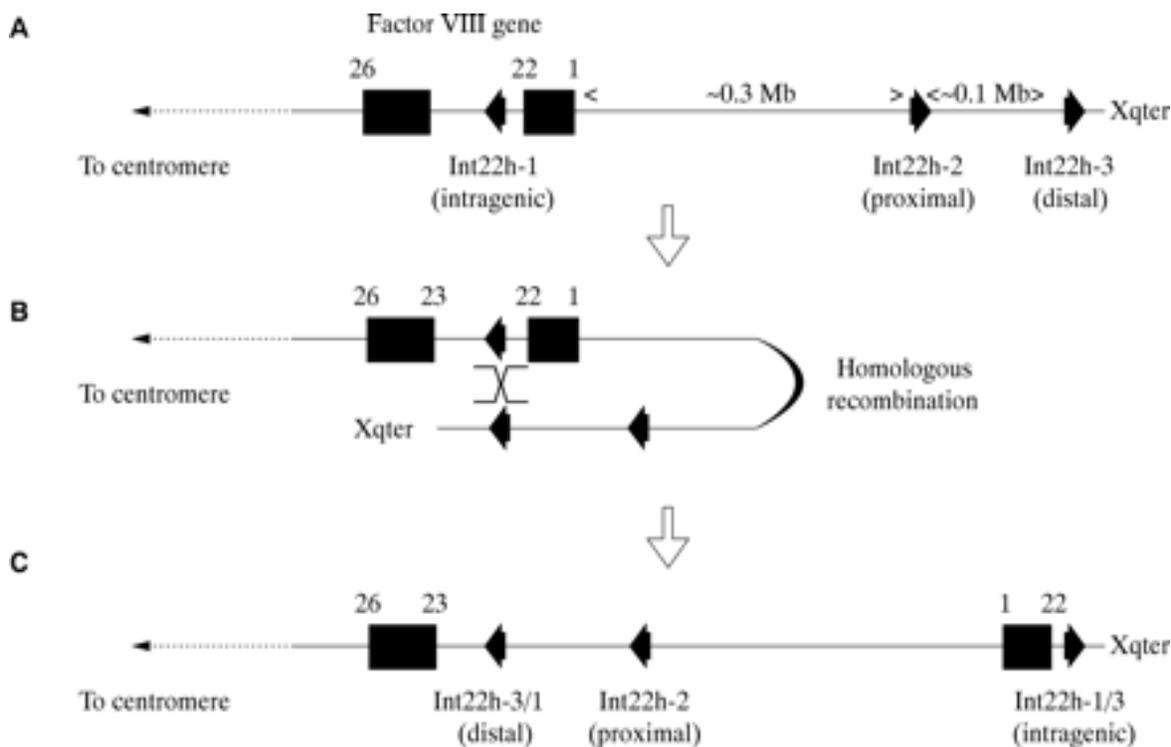


Figure 3. The intron 22 inversion of the factor VIII gene. Source: From molecular pathology Bowen (2002).

type domains (A1, A2 and A3) defined approximately by residue positions 1 to 336, 375 to 719 and 1691 to 2025, respectively. An acidic peptide $\alpha 1$ spans 337 to 374 and separates A1 from A2. A second acidic peptide, $\alpha 2$, (720 to 740) connects A2 with the large, heavily-glycosylated B domain, which encompasses approximately residues 741 to 1648. This leads to a third short acidic peptide, here termed $\alpha 3$, (1649 to 1690), which itself connects with the A3 domain. Finally, there are two homologous C

terminal domains (C1 and C2) each of approximately 155 amino acids. A region of the C2 domain contains a membrane binding site of factor VIII and the site of interaction with von Willebrand factor (Pratt et al., 1999). The domain organization of the FVIII polypeptide can thus be designated as A1. $\alpha 1$.A2. $\alpha 2$.B. $\alpha 3$.A3.C1.C2. The location of seven disulphide bonds within the FVIII molecule has been reported: there are two in each of the A1, A2 and A3 domains and one within the C1 domain

(McMullen et al., 1995).

MUTATIONS THAT AFFECT THE A DOMAINS

According to HAMSTeRS database, approximately half of the point mutation in the F8 gene have been reported in domain A (Table 1; Figure 2), which demonstrates the importance of these domains for the activity of the F8 protein. Further regions are highly susceptible to mutations. Common missense mutations in the A domain include Glu321Lys, Tyr346Cys, Val357Gly, Val376Gly, Thr770Ser, Thr751Ser, Glu720Lys; all of the residues that are affected by these mutations lie either adjacent to or within the $\alpha 1$ or $\alpha 2$ acidic region (Stoilova-McPhie et al., 2002; Goodeve et al., 2001; Mumford et al., 2002). Other mutations that have contributed to understanding the structure-function relationship of F8 occur at the thrombin cleavage sites at Arg372 (Arg372Cys) (Pattinson et al., 1990; Vidal et al., 2002) and Arg372His (Jayandharan et al., 2005) and Arg1689 (Arg1689Cys) (Higuchi et al., 1990; Cutler et al., 2002; Arai et al., 1990; Waseem et al., 1990; Hill Deam et al., 2005). Several mutations affect residues that are located at the interface of the A domains (Arg 282Ser, Ala284Glu and Arg531His at the A1-A2 interface, Ser289Leu at the A1-A3 interface, Asn694Ile, Arg698Trp and Arg698Leu at the A2-A3 interface). These mutations facilitate the dissociation of the A2 subunit, which occurs naturally and inactivates the F8 protein (Hakeos et al., 2002). Phe51Ser, Leu 50Val, Leu197Gln, Met1791Ile and Met1772 Ile were disturbed by the core of A1-A3 domain (Feng et al., 2010; Ma et al., 2008). Between the A1 and A2 domains, residues 351 to 365 are thought to include a binding site for coagulation factor X (Ananyeva et al., 2004) and replacement of Leu50 by a smaller Val side chain create a cavity in the core of A1 and disturb the binding site of FX (Ananyeva et al., 2004). Cys310, Met 320Val, His267 and His315 constitute a copper-binding site in A1 domain (Ananyeva et al., 2004), while Cys2000, His1954 and His2005 constitute a copper-binding site in A3 domain (Shen et al., 2008).

Cys310Ser and Cys2000Gly can eliminate the binding sites of copper and destabilize the interaction of heavy-light chain. Cys630 forms a disulfide bond with Cys711 which is located within a FIX-binding region Lys707-Asp712. Substitution by Gly disrupts the disulfide bond that is essential to the tertiary structure of protein (Ananyeva et al., 2004). The FIX interactive site has been localized to residues 558 to 565 and 698 to 712 within the A2 domain and residues 1811 to 1818 within the A3 domain. Residues 1778 to 1823 of the A3 region seem to be located at the surface of the protein and to provide a secondary binding site for F9 (Thompson, 2003). Modeling studies of the mutated FVII gene showed that the mutation is disrupting the salt bridge which was earlier being formed between Arginine 1781 and

Aspartate 1846, while the same could not have been formed due to the larger distance between the Histidine 1781 and Aspartate 1846 residues in the mutated protein. Further, perturbation of the side-chain stacking of Arginine 1781 with Tryptophan 1889 has been observed in the case of mutated Histidine 1781 where no stacking of the side chain has been observed due to a larger distance from Tryptophan 1889. As can be observed from Figure 4a and b, the distance between the Arginine 1781 side-chain nitrogen and Aspartate 1846 oxygen is 3.00 Å, which is quite suitable for salt-bridge formation. On the other hand, the distance between the mutated Histidine 1781 and Aspartate 1846 has been substantially more (4.5 Å), hence the probability of salt-bridge formation between the two is very meagre.

MUTATIONS THAT AFFECT THE B DOMAINS

Exon 14 represents about one half of the coding region and encodes for the F8 domain. Mutations lead to the premature termination of the F8 protein, resulting in hemophilia A (Oldenburg et al., 2004). Some nonsense mutations affect residues that are located at B domain like Trp772stop, Arg795stop, Gln796stop, Ser853stop, Glu861stop, Trp1029stop, Glu1038stop, Gln1122stop, Trp 1535stop (Liu et al., 2002; Aquila et al., 2003), 52 (Habart et al., 2002), 73 (Cutler et al., 2002; Bogdanova et al., 2005; Ahmed et al., 2005). No point mutation has been reported to affect the N-terminal thrombin cleavage site at amino acid 740. Only a few sites in the B domain are of functional importance.

MUTATIONS THAT AFFECT THE C DOMAINS

Defect in vWF interaction

The role of the FVIII-vWF interaction in stabilizing FVIII in plasma, extending its half-life and reducing clearance was well described. Missense mutations which disrupt this interaction lead to Hemophilia A. These mutations have been identified primarily within the C1 and C2 domains and may alter the core structure of these domains or disrupt surface site interactions (Liu et al., 2000). Clusters of residues have been identified as hemophilia yielding sites that may disrupt vWF interaction and are: Gln2100, Tyr2105, Ser2119, Arg2150 and Thr2154 within the C1 domain and Pro2300, Arg2304 and Arg2307 within the C2 domain. Additional mutations have been described near the C1 cluster at Glu2087, Arg2090, Ile2098, Asn2129 and Pro2153 (Jacquemin et al., 2000).

One important vWF-binding site is between amino acids Glu1, 649 and Arg1, 689, with Tyr1, 680 also being an important site. The abnormal amino acid sequence lies in the C2 domain (residues 2173 to 2332) and affects binding to von Willebrand factor (with residues 2248 to

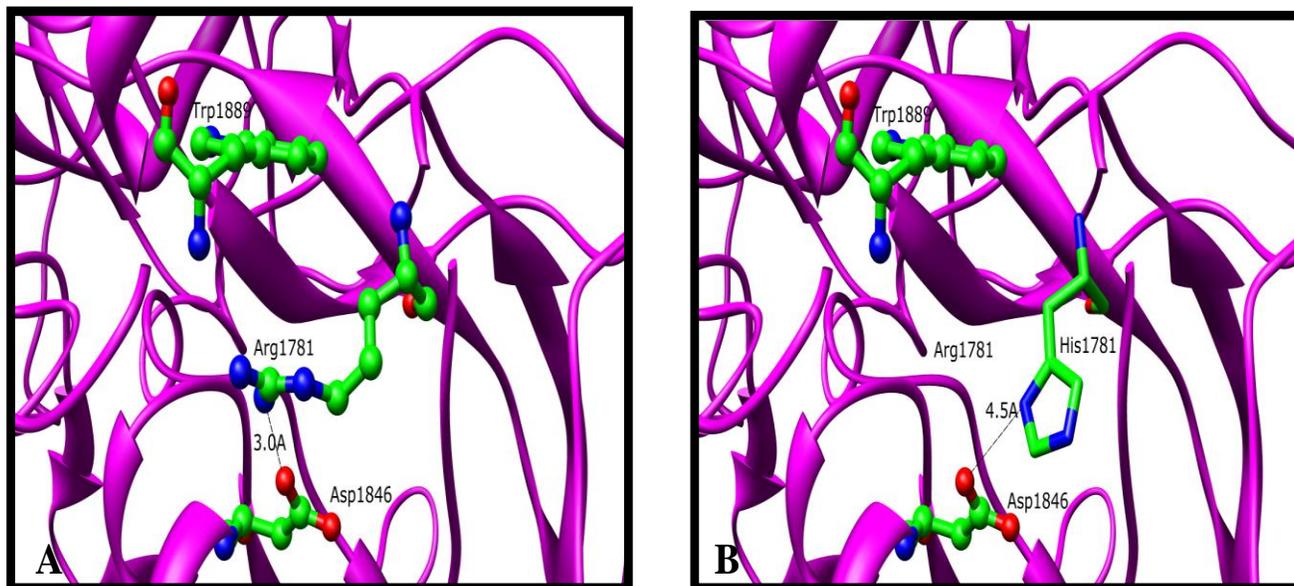


Figure 4. A Normal protein model of FVIII of A3 domain showing the distance (3.00 Å) between the native Arginine 1781 and Aspartate 1846 which is suitable for salt-bridge formation; B. Mutated protein model of FVIII of A3 domain showing increased distance (4.5 Å) between the mutated Histidine 1781 and native Aspartate 1846 not suitable for salt-bridge formation.

2312) (Viot et al., 1998). The C-terminal 2303 to 2332 sequence of the C2 domain has been suggested to form an interactive site for both vWF and Phospholipids (Saenko et al., 1994; Hua Bao-jai et al., 2010; Fay and Jenkins, 2005; Foster et al., 1990).

Defects in phospholipid binding

The FVIII C1 and C2 domains are important interactive surfaces for the PL membrane. But C2 domain of amino acid (2173 to 2332) is important for interactions with phospholipid, but also for binding to vWF. An analysis of 57 reported mutations corresponding to substitutions at 43 separate residues within the C1 and C2 domains have been analysed (Liu et al., 2000). A synthetic peptide encompassing residues 2303 to 2332 of the C2 domain of FVIII inhibits both phospholipid (Foster et al., 1990) and vWf binding (Saenko et al., 1994).

Deletion of Alanine 2201 in the FVIII C2 domain results in hemophilia A by impairing both FVIII binding to vWF and phospholipids (d' Oiron et al., 2004). Several recurrent mutations at amino-acid positions 2147, 2150, 2159, 2163 (C1) and 2209, 2300, 2307 (C2) have been found (Table 3). Recently, the disulfide bonds between Cys2021 and Cys2164 within the C1 domain and between Cys2174 and Cys2326 within the C2 domain have been reported (Kaufman and Pipe, 1998; Nogami et al., 1999). The C2 domain (residues 2303 to 2332) have been shown to be involved in factor VIII interaction with phospholipids. Gly2325Cys mutation affects the conformation of the phospholipids binding sites in the C2

domain (Akkarapatumwong et al., 2000; Foster et al., 1990).

CONCLUSION

Worldwide mutation analysis of Hemophilia-A patients has revealed the underlying causative mutation in a large variety of cases. Approximately 40 to 50% of the severe cases are caused by inversion of intron 22 of the F8 gene and sequences outside the gene. For smaller deletions, insertions or point mutations, the degree of severity depends on the functional defect caused in the affected domains. Mutational analysis has contributed to a better understanding of the structure-function relationship of FVIII protein and shown how alterations in a single gene can be modulated by changes in its interaction partners. The involvement of each residue in the FVIII function is not fully understood, except for the key residues located within the activation cleavage or the FVIII interaction sites.

Mutational analysis is also clinically valuable in order to assess risk of inhibitor development. A serious complication is in the treatment of hemophilia A and genetic factors, in particular, the types of mutations that seem to play an important role in its formation.

Conflict of Interest

The authors declare that they have no conflict of interests.

Table 3. Recurrent mutations in the coagulation factor VIII (F8) gene.

Exon	Nucleotide change	Amino Acid	Domain	Amino-acid change	Inhibitors	Frequency (no. of cases)
4	GTG>ATG	162	A1	Val>Met	No	26
8	CGA>TGA	336	A1	ARG>Stop	Yes	15
10	GGA>AGA	479	A2	Gly>Arg	No	19
11	CGG>TGG	527	A2	Arg>Try	No	23
11	CGC>TGC	531	A2	Arg>Cys	Yes	27
11	CGC>CAC	531	A2	Arg>Cys	No	18
12	CGA>GGA	583	A2	Arg>Gly	No	21
12	CGC>TGC	593	A2	Arg>Cys	Yes	49
14	TAT>TTT	1680	B	Tyr>Phe	Yes	24
14	CGC>TGC	1689	a3	Arg>Cys	No	24
16	CGT>CAT	1781	A3	Arg>His	No	19
18	CGA>TGA	1941	A3	Arg>Stop	Yes	17
18	CGA>TGA	1966	A3	Arg>Stop	Yes	18
18	CGA>CAA	1966	A3	Arg>Gln	No	38
19	CGG>CAA	1966	A3	Arg>Try	Yes	37
19	GTG>GCG	2016	A3	Val>Ala	No	47
22	CGA>TGA	2116	A3	Arg>Stop	Yes	17
23	CGA>TGA	2147	C1	Arg>Stop	Yes	25
23	CGT>CAT	2150	C1	Arg>His	Yes	61
23	CGC>TGC	2159	C1	Arg>Cys	No	31
23	CGC>CAC	2163	C1	Arg>His	Yes	19
24	CGA>CAA	2209	C2	Arg>Gln	Yes	25
24	CGA>TGA	2209	C2	Arg>Stop	Yes	21
25	TGG>TGT	2229	C2	Tyr> Cys	Yes	17
26	CCG>CTG	2300	C2	Pro>Leu	Yes	19
26	CGA>TGA	2307	C2	Arg>Stop	Yes	16
26	CGA>CAA	2307	C2	Arg>Gln	Yes	19

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