Severe and moderate hemophilia A: identification of 38 new genetic alterations

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ABSTRACT

Hemophilia A is an X-linked recessive disorder caused by a lack or decrease of factor VIII activity. Its socio-economic impact is high given its high bleeding expression and treatment cost. Our aim was to establish the mutation of each patient to improve family management. A total of 116 unrelated families with severe and moderate hemophilia A were involved. Non-carriers of intron 22 and intron 1 rearrangements were included in *F8* gene screening. Intron 1 and 22 inversion frequencies were 3% and 52.5% respectively. Putative mutations were identified in all the families; 38 were new. The cumulative inhibitor incidence was 22%. Approximately half the families carry non-recurrent mutations, which were unique in around one third. Harmful effects for mutations predicting null alleles are expected. Missense mutation consequences are not easily predictable, despite the help of some bio-informatics tools.

Key words: hemophilia A, *F8* gene, intron 22 inversion, intron 1 inversion, severe hemophilia, moderate hemophilia.

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Introduction

Hemophilia A (HA) is an X-linked recessive disorder. Its incidence is around 1 in 5,000 males who may have several female relatives at risk of being carriers. HA is caused by deficiencies of factor VIII (FVIII), which circulates in the plasma at a very low concentration (0.5 µg/mL), but plays a key role in the amplification phase of the coagulation cascade. FVIII is encoded by the *F8* gene (OMIN #306700). At first, the polymorphism segregation analysis was widely used for carrier diagnosis since it required more basic technology. In 1993, studies from two laboratories described the intron 22 inversion as a recurrent mutation.[1,2] This represented a great step forward in our understanding of the molecular basis in severe HA. In 2002, another less frequent inversion in the intron 1 of the *F8* gene was described.[3] Nowadays, a wide spectrum of heterogeneous mutations has been identified in the *F8* gene (HAMSTeRS database, *http://europium.csc.mrc.ac.uk*). At present, 943 unique mutations have been registered in this database, and their number increases daily.

The search for mutations first began in severe and moderate patients at our center to investigate what causes hemophilia and to improve family management. The detection rate of the genetic defect was 100% in the cohort of the families studied. The characterization and spectrum of these genetic alterations are reported in this paper.

Design and Methods

Subjects

A total of 116 unrelated families were included in this study, 99 of them with severe HA, 16 with moderate HA, and 1 family with HA of an unknown phenotype, involving more than 525 individuals altogether (family studies).

Hemophilia A diagnosis

The following analyses were performed in basal samples: FVIII activity by the one-stage coagulative method (FVIII:C), and antigen of FVIII (FVIII:Ag) by ELISA (Diagnostic Stago). Type 2N von Willebrand disease[4] and a combined FV-FVIII deficiency were ruled out. Assays FVIII:C and antigenic von Willebrand factor (VWF:Ag) were performed in all women in parallel with genetic studies. The Bethesda assay was used for anti-FVIII antibodies titration.[5]

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Genetic studies

The nature of the study was explained to all family members who gave their consent for research. Polymorphism segregation analyses were performed in around half the families. The intron 22 inversion was performed by *southern blot* or by multiplex polymerase chain reaction (PCR) using the enzyme *Takara LA Taq™ with CGI buffer*. The intron 1 inversion was analyzed by the PCR method. Negative patients for either mutations were included in the study to search for mutations by sequencing methodology.[6] The promoter region and the 26 exons and their boundaries from the *F8* gene were amplified with the Ex Takara Taq™ polymerase under the same PCR conditions. These and primer localization can be provided upon request. Sequencing reactions were performed with BigDye Terminator® v1.1 kit (AB Applied Biosystems), and purified products were electrophoresed in the ABI Prism 310 Genetic Analyser (AB).

The nomenclature is based on current recommendations. The nucleotide number is assigned according to the FVIII cDNA sequence from A of the initiator ATG site as +1. The amino acid sequence numbering assigns the first residue of mature FVIII as +1, and hence the initiator methionine is at the -19 position, as used in the HAMSTeRS database.

The line-up of multiple alignment from the four mammal species was that generated at the UK MRC HGMP Resource Centre. SIFT (Sorting Intolerant From Tolerant, *http://blocks.fhcrc.org/sift/SIFT.html*) and PolyPhen (Polymorphism Phenotyping) were used to evaluate the nature of missense mutations (for more information see *http://genetics.bwh.harvard.edu/pph*).

Table 1. Non missense mutations in severe and moderate hemophilia A.

ID	*FVIII:C (IU/dL)*	*FVIII:Ag (IU/dL)*	*Genetic alteration in F8 gene*	*Exon/Intron*	*Inhibitors*
VSA123	<1	<1	Large deletion#	PR+1	HR
VSA036	<1	na	c.1259dupC (p.D403RfsX2)#	8	N
VSA009	<1	<1	c.1326delC (p.Y423X)#	9	N
VSA022	<1	na	Large deletion#	PR-E8	HR
VSA081	<1	<1.5	c.1753-1G>A#	IVS11	N
VSA172	<1	<1.5	[c.1861C>T(p.Q602X) + c.3780C>G (p. D1241E)]	12	N
VSA058	<1	<1.5	Large deletion#	3-13	HR
VSA008	<1	na	c.2909delG (p.G951VfsX2)#	14	N
VSA133	<1	1.5	c.3292C>T(p.Q1079X)#	14	N
VSA186	<1	<1.5	c.3300dupA (p.E1082RfsX17)	14	N
VSA169	2	3	c.3624delT (p.Q1190KfsX9)#	14	N
VSA128	na (w)	na (w)	c.3637delA (p.I1194FfsX5)	14	N
VSA187‡	1.5	na	[c.3637delA (p.I1194FfsX5) + c.1090G>A(p.D345N)]	14 8	na
VSA132	<1	<1.5	[c.3637dupA (p.I1194NfsX28+ c.3780C>G (p. D1241E]	14	N
VSA184	<1	<1.5	c.3870dupA (p.G1272Rfs29)	14	HR
VSA201	<1	na	[c.3694C>T (p.Q1213X)# + c.3780C>G (p. D1241E)]	14	N
VSA026	<1	na	c.3913C>T (p.Q1286X)#	14	N
VSA108	na	na	c.3949-50delTC (p.P1299SfsX2)#	14	na
VSA041	<1	<1.5	c.4379dupA (p.N1441K fsX2)	14	N
VSA095	<1	<1.8	c.4379dupA (p.N1441K fsX2)	14	N
VSA113	5	na	c.4379dupA (p.N1441K fsX2)	14	na
VSA038	<1	na	[c.3864A>C(p.S1269) + c.4450delA (p.S1465VfsX83) #]	14	N
VSA170	<1	<1	[c.4428_31del2(AG) (p.E1458LfsX89) + c.3780C>G (p. D1241E)]	14	N
VSA147	<1	na	c.4473C>G (p.Y1472X)#	14	N
VSA189	<1	<1.5	c.4542delT (p.P1496QfsX52)#	14	HR
VSA134	<1	<1.5	c.4895delT (p.I1613KfsX10)#	14	N
VSA149	<1	na	c.4841delA (p.K1595RfsX7)#	14	N
VSA024	<1	na	c.5574delT (p.D1840MfsX12)	16	N
VSA004	<1	<1.5	c.5816-14delGTdelinsTA#	IVS17	N
VSA090	na (w)	na (w)	ins180pb#	18	na
VSA030	<1	na	[Large deletion# + c.3780C>G (p. D1241E]	16-20	HR
VSA138	<1	na	c.6243G>A (p.W2062X)#	21	N
VSA166	<1	na	Large deletion#	2-22	HR
VSA066	<1	na	c.6489delT (p.I2145LfsX22)#	23	N
VSA015	<1	<1.5	c.6517_9dupACT (p.T2154dup)#	23	HR
VSA200	<1	<1.5	c.6574+1G>T#	IVS23	N
VSA035	<1	<1.5	c.6682C>T(p.R2209X)	24	N
VSA067	<1	na	c.6682C>T(p.R2209X)	24	N
VSA136	<1	<1.5	c.6723+1G>A#	IVS24	LR (t)
VSA107	<1	<1	Large deletion#	23-26	HR
VSA190	na (w)	na (w)	c.6748C>T(p.Q2231X)#	25	na
VSA192	1.5	<1.9	c.670 + 5G>A	IVS5	N

New mutations are marked with #. Nomenclature is based on the cDNA and mature processed Factor VIII. fs: frameshift; N: no; na: not available; (w): mutation detected in an obligatory carrier; HR: high response; LR: low response; t: transient. (‡): patient with double mutation.

Results

Laboratory phenotype data and bleeding history allowed us to classify 99 families with severe HA (FVIII:C <1IU/dL), 16 with moderate HA (1 ≤ FVIII:C ≤ 5 IU/dL), and 1 family with HA of an unknown phenotype since no hemophilic male had yet been born in the family (VSA098), but the FVIII:C/VWF:Ag ratio from the woman was 0.58. The intron 22 inversion was detected in 52 families and the intron 1 inversion in 3 families with severe HA (52.5% and 3.0%). Screening for the mutation within the *F8* gene was carried out in the index case, or in an obligatory carrier from the remaining 61 families. Genetic alterations associated with the HA phenotype were identified in all families. Different mutation types which predict possible null alleles, such as large deletions, small deletions/insertions causing frameshift, premature termination codons, and changes in the splice sites, were identified in 41 families. These alterations, along with another with a duplication of a threonine codon (VSA015), are shown in Table 1, where the 28 new characterized mutations are marked with symbol #. Ten of the patients in Table 1 developed a FVIII antibody in response to replacement therapy. A double mutation was detected in only 1 patient, VSA187 (Tables 1, 2). Missense mutations were found in 19 families (Table 2), 10 are new and 2 were detected twice in unrelated families. Two of the patients developed low-response and transient inhibitors. No missense mutation was located in exon 14.

Overall, 28 of the 125 patients from the families included, in whom the tests of inhibitors were available, developed antibodies after infusion of the exogenous FVIII, this being a cumulated incidence of approximately 22% in severe and moderate HA.

All amino acid residues affected by substitutions in Table 2 were conserved in pig, murine and canine FVIII sequences. Each missense mutation reported in this paper, whether already described or new, was predicted by SIFT as an intolerable amino acid change.

High PSIC values in the PolyPhen program may indicate that substitution is rarely or never observed in the protein family (Table 2).

Discussion

From the cohort studied in this paper, a total of 38 (Table 1 and 2) alterations have been identified that are not in the HAMSTeRS database and recent reports.[7-9]

The intron 22 inversion causes severe HA in approximately half the families studied. The frequency of the inton 1 inversion observed was within the range reported (approximately 5-1.7%).[3,10-12] Even though frequency can be very low, their detection is highly recommended. In our laboratory, both inversions are analyzed in severe and moderate patients.

Six different large deletions have been identified, and all these patients developed high-response inhibitors, in agreement with previous reports.[13] Patients VSA184, VSA189, and VSA015, who carry small insertions or deletions, were also high responders of inhibitors. Patient VSA136, with a mutation in a splicing donor site, developed a transient inhibitor, despite the fact that the inhibitor prevalence in these mutation types is low. However, other genetic or environmental factors may have contributed to inhibitor development. Therefore, routine periodic screening is recommended for all patients. Overall, inhibitor prevalence was approximately 22%, which was in the range of that known in severe HA. We obtained a frequency of 27% in relation to the

Table 2. Missense mutations in severe and moderate hemophilia A.

ID	*FVIII:C (IU/dL)*	*FVIII:Ag (IU/dL)*	*Mutation in F8 gene (mature FVIII)*	*Exon*	*Polyphen (PSIC score)*	*Inhibitors*
VSA098	35 (w)	na (w)	c.248C>G(p.P64R)#	2	probably damaging (2.3)	na
VSA025	4.4	14	c.545A>T(p.D163V)#	4	probably damaging (2.3)	LR (t)
VSA181	<1	na	[c.902G>A(p.R282H) + c.3780C>G (p. D1241E)]	7	possibly damaging (1.6)	N
VSA164	1	9.8	c.1043G>A(p.C329T)	8	probably damaging (2.7)	N
VSA011	4	12	c.1244C>T (p.A396V)#	8	benign (1.2)	N
VSA121	4.2	4	c.1244C>T(p.A396V)#	8	benign (1.2)	N
VSA037	na	na	c.1619C>G(p.P521R)#	11	probably damaging (2.3)	N
VSA122	50 (w)	na (w)	c.1966T>C(p.W637R)#	13	probably damaging (3.5)	N
VSA001	<1	na	c.2048A>G(p.Y664C)	13	probably damaging (2.4)	N
VSA187‡	1.5	na	[c.3637delA p.I1194FfsX5) + c.1090G>A(p.D345N)#]	8	benign (1.4)	na
VSA126	4.5	7	c.5286T>A (p.F1743L)#	15	possibly damaging (1.8)	N
VSA153	3.6	2.2	[c.5286T>A(p.F1743L)#+ c.3780C>G (p. D1241E)]	15	possibly damaging (1.8)	N
VSA185	2.1	<1.8	c.5399G>A(p.R1781H)	16	possibly damaging (1.6)	N
VSA196	4.5	3	c.5399G>A[p.R1781H]	16	possibly damaging (1.6)	N
VSA106	1.8	na	c.5542 G>A(p.E1829K)#	16	benign (1.3)	N
VSA013	<1	na	c.5594A>G(p.D1846G) #	17	possibly damaging (1.9)	N
VSA010	7	<1.5	c.6506G>A(p.R2150H)	23	possibly damaging (1.6)	LR
VSA021	4.5	< 1	c.6545G>A(p.R2163H)	23	possibly damaging (1.6)	N
VSA168	3	< 1.8	c.6623A>C(p.Q2189P)#	24	possibly damaging (1.9)	N

All changes are conserved in pig, murine and canine F8 sequences. Each mutation was predicted by SIFT as an intolerable amino acid change (see also Table 1).

intron 22 inversion (*data not shown*), and of approximately 30% for the intron 1 inversion. The mutations shown in Table 1 are considered to be the cause of hemophilia. The majority of these mutation types predict premature termination codons and therefore nonsense-mediated mRNA decay (NMD).[14] The least predictable effects are those caused by a missense mutation. With reference to patient VSA015, the addition of a new polar residue (T) in the folded domain C1 could affect the interaction between the A3 and C1 domains, and might be critical either for binding activities or the function of FVIII.[15] In specific cases, like the female subject with no hemophiliacs in her family and a novel missense mutation in the exon 2 (VSA098), the possibility of offering clear genetic counseling is complicated. Although the substitution P64R has a poor prediction, the real magnitude of its effect is still unknown. Bioinformatic tools can help to predict the possible adverse effect of a new mutation. However, their results must be interpreted with caution. The increase in requests for direct genetic analyses by physicians suggests that it is now clinical practice in developed countries to at least offer more accurate family genetic counseling and when necessary select the most appropriate treatment.

Authorship and Disclosures

PC was responsible for designing the study, performing genetic analyses, analyzing the data and writing the manuscript. NC and CE helped to perform the search for mutations. MB performed polymorphism segregation analyses and intron 22 detection by southern blot.

ARC, SH and MAD collected the clinical and analytical data from the patients. VC was responsible for coagulant and antigenic analyses. JAA and all authors supervised and revised the final version of the paper.

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