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RESEARCH ARTICLE

Spectrum of Molecular Defects and Mutation Detection Rate in Patients With Severe Hemophilia A

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Hemophilia A is the most frequently occurring X-linked bleeding disorder, affecting one to two out of 10,000 males worldwide. Various types of mutations in the *F8* gene are causative for this condition. It is well known that the most common mutation in severely affected patients is the intron 22 inversion, which accounts for about 45% of cases with *F8* residual activity of less than 1%. Therefore, the aim of the present study was to determine the spectrum and distribution of mutations in the *F8* gene in a large group of patients with severe hemophilia A who previously tested negative for the common intron 22 inversion. Here we report on a mutation analysis of 86 patients collected under the above-mentioned criterion. The pathogenic molecular defect was identified in all patients, and thus our detection rate was virtually 100%. Thirty-four of the identified mutations are described for the first time. The newly detected amino acid substitutions were scored for potential gross or local conformational changes and influence on molecular stability for every single *F8* domain with available structures, using homology modeling. Hum Mutat Res 26(3), 249–254, 2005. **© 2005 Wiley-Liss, Inc.**

KEY WORDS: F8; severe hemophilia A; spectrum of mutations; molecular modeling; F8 inhibitors

DATABASES:
F8–OMIM 306700; GenBank: X01179.1, NM_000132.2, NM_019863.1, P00451 http://europium.csc.mrc.ac.uk/ (HAMSTeRS, The Haemophilia A Mutation, Structure, Test and Resource Site)
http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html (Human Gene Mutation Database at the Institute of Medical Genetics in Cardiff)

INTRODUCTION

Hemophilia A (MIM# 306700) is the most frequently occurring X-linked bleeding disorder, affecting one to two out of 10,000 males worldwide. It is caused by a defect or decreased activity of the coagulation *F8* protein—an essential cofactor for the F9-mediated activation of F10 in the intrinsic blood coagulation cascade. The amount of residual *F8* (FVIII:C) present determines the clinical variability of the disease. About 50% of these patients have severe hemophilia A with an FVIII:C activity less than 1% of normal. These patients experience frequent spontaneous bleeding into joints, muscles, and internal organs. Moderate (FVIII:C 2–5% of normal) and mild (FVIII:C >5% of normal) forms occur in about 10% and 30–40% of patients, respectively.

The *F8* gene is located on the most distal band of chromosome X (Xq28) and spans over 180 kb of genomic DNA. It comprises 26 exons, encoding a polypeptide chain of 2,351 amino acids. This includes a signal peptide of 19 and a mature protein of 2,332 amino acids. The *F8* protein is a large multidomain glycoprotein composed of a heavy chain (domains A1-A2-B) and a light chain (domains A3-C1-C2) [Vehar et al., 1984]. It can be converted in its active form by proteolysis in both the heavy and light chains by various serine proteases, including thrombin and activated F10 (for review see Lenting et al. [1998]).

Various types of mutations in the *F8* gene are responsible for the bleeding disorder in patients with severe hemophilia A [Kemball-Cook et al., 1998]. The most common mutation is the intron 22 inversion (IVS22-inversion) [Lakich et al., 1993], which accounts for 40 to 50% of cases in this group. Point mutations and different small/large deletions/rearrangements are responsible for the disease in the rest of the patients. The aim of this study was to determine the spectrum of these (non-IVS22-inversion) *F8* mutations, their distribution throughout the *F8* protein, and the mutation detection rate on the genomic level in a large group of IVS22-inversion-negative patients with severe hemophilia A.

The Supplementary Material referred to in this article can be accessed at www.interscience.wiley.com/jpages/1059-7794/suppmat.

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MATERIALS AND METHODS

Eighty-six unrelated patients of German origin who previously tested negative for the common IVS22-inversion were included in the study. The molecular analyses of 21 of these cases were previously published [Bogdanova et al., 2001, 2002; Leuer et al., 2001] and the remaining 65 patients were analyzed in the present work. All of the individuals were affected by severe hemophilia A according to the standard criteria [Antonarakis et al., 1995], including one person (PL) [Bogdanova et al., 2001] who had severe hemophilia A and was initially incorrectly reported to us as being moderately affected. Information about the presence of *F8* inhibitors was systematically collected.

The current study complied with the ethical guidelines of the institutions involved. Informed consent was obtained from all analyzed subjects.

DNA Extraction and Analysis

A search for mutations in the *F8* gene was performed on genomic DNA extracted from peripheral blood lymphocytes using a salting procedure. The intron 1 breaking inversion was analyzed with breakpoint PCR using the oligonucleotides and conditions published by Bagnall et al. [2002].

PCR amplification of all 26 *F8* exons, including flanking intronic regions, was performed on 50–100 ng of extracted DNA using previously described amplification primers and cycling conditions [Bogdanova et al., 2002; Schwaab et al., 1997]. Amplicons were sequenced in both directions using the ABI PRISM Dye terminator cycle sequencing reaction kit (Applied Biosystems; www.appliedbiosystems.com), and electrophoresis was performed on an ABI-3700 genetic analyzer. The causative nature of the novel mutations was established by the absence of these changes in a set of 100 anonymous DNA samples from healthy male individuals.

In cases in which no PCR product from a given format was obtained, suggesting the presence of a whole exon deletion, a Southern blot analysis of Taq I digested DNA hybridized with an *F8* cDNA probe was performed [Millar et al., 1991].

DNA mutation numbering was based on cDNA sequence NM_000132.2, with nucleotide +1 corresponding to A of the ATG translation initiation codon of the reference sequence.

Conservation Analysis

Amino acid positions that were subjected to changes through mutation were examined for their conservation in murine, porcine, and canine *F8* using the publicly available multiple-sequence alignment lineup on the *F8* mutation database (http://europium.csc.mrc.ac.uk/WebPages/Database/Protein/lineups.html).

Computer-Assisted Molecular Modeling of *F8* Domains

Amino acid sequences of the *F8* domains were excised from the publicly available NCBI submission file (accession number P00451). The physical borders of the respective domains were determined according to Pemberton et al. [1997]. Protein sequence segments with and without the corresponding mutations were submitted for comparative homology modeling [Lund et al., 1997] to the CBS server (www.cbs.dtu.dk/services/CPHmodels). Template files for domain structure prediction were taken from the Protein Data Bank (PDB, www.rcsb.org/pdb/) and included 1KCW (human ceruloplasmin) and 1SDD (bovine factor V) for the A domains, and 1D7P and 1IQD (human *F8*, C2 domain); 1CZV, 1CZS, and 1CZT (human factor V, C2 domain); and 1KEX (human neuorpilin-1, B1 domain) for the C domains. Raw prediction files were downloaded and the models were fitted using the FoldIt utility (freeware, J.C. Jesior, CNRS, Grenoble) and the published 2D crystal structure of the *F8* protein [Stoilova-McPhie et al., 2002] as well as the A domains molecular model [Pemberton et al., 1997]. Structure files were downloaded from the *F8* mutation database (http://europium.csc.mrc.ac.uk/WebPages/Database/Protein/F8Model.htm). The structural data were used to resolve steric clashes, and fitted (FoldIt) molecular model output files were generated on a Macintosh PowerPC workstation with a G3 processor. The fitted files were converted to graphics in a Dreiding (match stick) model mode, and the amino acid selections were entered manually. Graphic outputs with and without the respective mutations were rotated by hand to achieve matching coordinates and were then saved as images.

RESULTS

Using the techniques outlined above, we were able to identify the disease-causing mutation in all of the screened patients. The results are presented according to the type of molecular change observed. Thirty-four novel (seven nonsense, 17 missense, one splice mutation, six small, and three gross deletions) and 22 previously reported mutations in 31 different patients were detected. All mutations (those previously described and those detected in the present work) are summarized in Supplementary Table S1 (available online at www.interscience.wiley.com/jpages/1059-7794/suppmat) and Figure 1.

The description of the mutations on the protein level was based on protein sequence P00451, and the translation initiator Methionine is numbered as +1. Since the codon numbering taken from the literature and reference mutation databases (according to which the 19 amino acids containing signal peptide are numbered in reverse, i.e., the initial Methionine is numbered as –19, and the first Alanine of the mature protein is numbered as +1) differs from the journal-approved nomenclature, the traditional numbering is also shown in the text and tables.

Missense Mutations

Missense mutations were present in 33 of the patients (Supplementary Table S1). In addition to the three previously published mutations [Bogdanova et al., 2001], 17 novel (Table 1) and 10 recurrent missense mutations were identified in 30 patients tested in the present study. The entire *F8* coding sequence and the exon/intron boundaries were sequenced in these patients to ensure that we would not miss another possible disease-causing mutation. All of the affected amino acids were highly conserved in murine, porcine, and canine *F8*, suggesting an important role for protein function. Any presence of the novel missense mutations was excluded in a control sample of 100 male DNAs.

c.202A>G (p.T68A, T49A) was present in a sporadic case of severe hemophilia A, but was not present in the patient's mother. The remaining missense mutations were detected in index patients with a positive family history, and were found to segregate with the disease phenotype (data not shown).

c.405T>A (p.D135E, D116E), c.676A>T (p.S226C, S207C), c.1332A>T (p.K444N, K425N), and c.5680G>A (p.E1894 K, E1875 K) are conservative mutations. D116G, S207I, and K425R were previously described in severely affected patients [Becker et al., 1996; Higuchi et al., 1991], and E1875G was detected in a moderately affected patient [Tavassoli et al., 1997].

The newly detected amino acid substitutions were scored for potential gross or local conformational changes and influence on molecular stability for every single *F8* domain with available

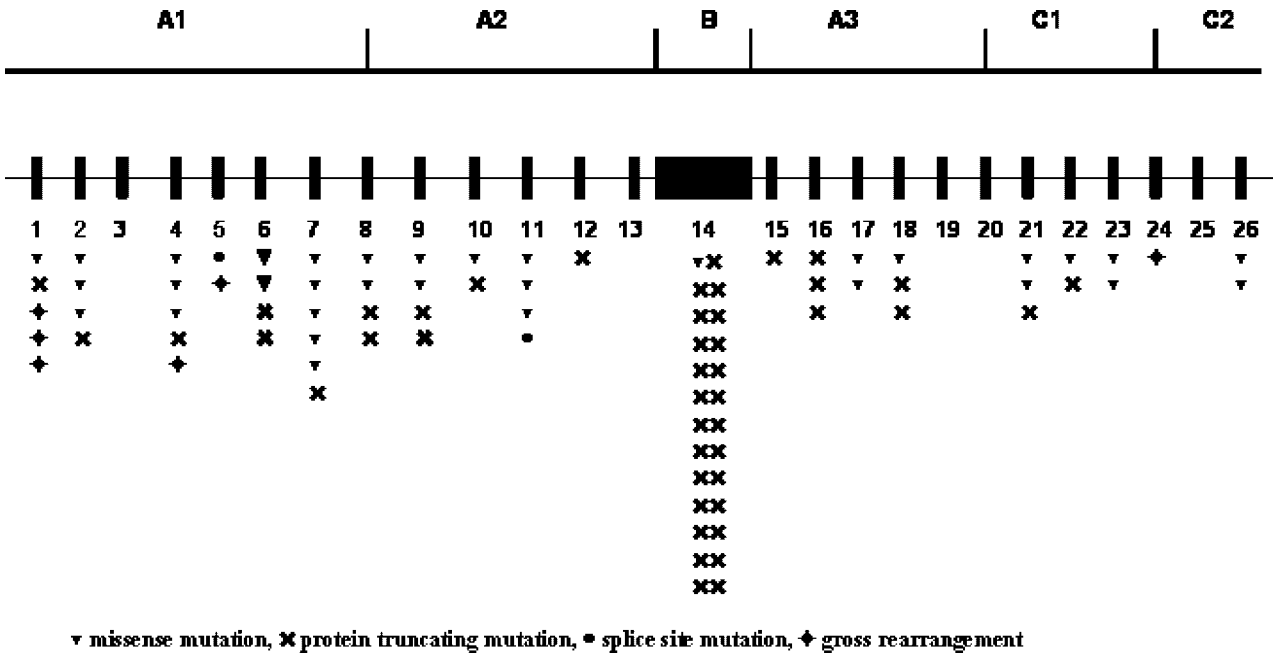

FIGURE 1. Spectrum and distribution of the mutations in the *F8* gene, identified in 86 male patients with severe hemophilia A who were negative for the common intron 22-inversion.

TABLE 1. Novel Missense Mutations Identified in the Present Study

Exon	*F8* domain	Nucleotide change[a]	Amino acid change[b]	Amino acid change[c]	Patient index	Conformational change
1	A1	c.86T>G	p.V29G	V10G	NBK01	Hydrogen bonding
2	A1	c.202A>G	p.T68A	T49A	NB42	Hydrogen bonding
2	A1	c.202A>C	p.T68P	T49P	NB29	Helix-breaking
2	A1	c.230T>C	p.L77P	L58P	NB166	Helix-breaking
4	A1	c.405T>A	p.D135E	D116E	NB38	Ion bonding
6	A1	c.676A>T	p.S226C	S207C	NB97	Loop conformation
7	A1	c.899A>T	p.H300L	H281L	NBK02	Loop conformation
8	A2	c.1241A>G	p.Y414C	Y395C	NB227	Loop conformation
9	A2	c.1332A>T	p.K444N	K425N	NB100	Ion bonding
9	A2	c.1337G>C	p.R446P	R427P	NB264	Helix-breaking
11	A2	c.1696C>T	p.L566F	L547F	NB148	Loop conformation
11	A2	c.1641C>G	p.C547W	C528W	NB225	Disulfide bridge
14	B	c.4979C>T	p.P1660L	P1641L	NB48	Cleavage site
17	A3	c.5680G>A	p.E1894K	E1875K	NB122	Ion bonding
18	A3	c.5934T>G	p.S1978R	S1959R	NBK03	Ion bonding
21	C1	c.6213A>T	p.R2071S	R2052S	NB126	Ion bonding
26	C2	c.6986C>T	p.P2329L	P2310L	NBK04	Helix-breaking

[a]DNA mutation numbering is based on cDNA sequence (GenBank no. NM_000132.2), with nucleotide +1 corresponding to A of the ATG translation initiation codon.
[b]Sequence changes at protein level following the Nomenclature for Description of Genetic Variations approved by the Human Genome Variation Society (www.hgvs.org/mutnomen/).
[c]Sequence changes at protein level, representative of the published literature and the reference mutation databases. The 19 amino acids containing signal peptide are numbered reverse, thus the initial Methionine is numbered as –19. The first Alanine of the mature protein is numbered as +1.

structures, using homology modeling. From our data we can conclude that all of the missense mutations listed in Table 1 change the *F8* domain topology and/or influence the molecular stability of the corresponding chain segments or protein regions. Using the structural information obtained, we can classify these alterations as A) gross conformational changes, and B) local conformational changes. All newly discovered missense mutations are grouped in Table 1 with the indicated type of conformational change. Class A includes the following mutations: 1) destroying disulfide bridges, 2) destroying or creating ionic bonds, 3) conformational mutations involving proline, and 4) mutations changing hydrogen-bonding patterns. Examples of class A mutations are presented in Supplementary Figure S1. Figure 2 presents another drastic example of the effect of the mutation c.5680G>A (p.E1894 K, E1875 K) on the conformation of the A3 domain. The charge reversal prevents the ionic bond between E1875 and R1941 in the mature protein, which results in conformational relaxation of the whole A3 domain.

Since there is no available model structure for the protein region contained in the B domain, no homology modeling was possible for this portion of the molecule. Judging from the position of the affected amino acid and the general effects of proline mutations,

c.4979C>T (p.P1660L, P1641L) most probably influences the proteolytic cleavage efficiency at residue R1648.

Recurrent missense mutations were identified in 13 patients (Supplementary Table S1). In the HAMSTeRS database (http://europium.csc.mfc.ac.uk), there is as yet no entry for four of these mutations (c.491G>A (p.G164D, G145D), c.785C>T (p.P262L, P243L), c.1475A>G (p.Y492C, Y473C), and c.6356A>G (p.Q2119R, Q2100R)) for severe hemophilia. They were found in six moderately affected patients up to date. Obviously, these molecular defects may have a variable clinical expression similar to that of c.901C>T (p.R301C, R282C), c.902G>T (p.301L, R282L), and c.6535G>A (p.R2182 H, R2163 H), which have been described in many patients with a severe or mild/moderate phenotype, and were found in five of our severely affected patients.

Nonsense Mutations

Seventeen nonsense mutations were detected in the present study in addition to the three previously described mutations (Supplementary Table S1). Seven of these are novel (listed in Table 2). Four of the changes are located in exon 14, thus eliminating the light chain of the *F8* protein. c.5301C>A (p.Y1767X, Y1748X) and c.5415T>A (p.Y1805X, Y1786X) lead to the same effect, since the premature stop leaves an A3 peptide consisting of only about 50 and 90 amino acid residues, respectively.

Seven recurrent nonsense mutations were detected in 10 patients. These are systematically represented in Supplementary Table S1. As expected, all of these mutations were found in severely affected patients.

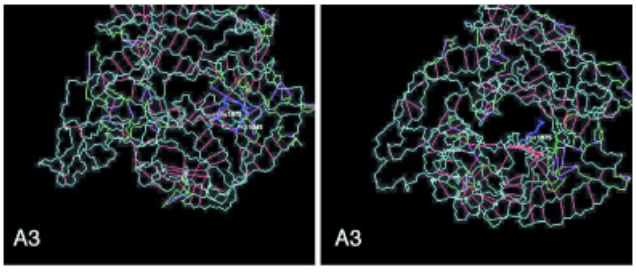

FIGURE 2. **Dreiding model image of *F8* A3 domain (left panel) with p.E1894 K (E1875 K) mutation (right panel). Images are oriented according to the disulfide bond C1832-C1858 lineup. Protein chains are colored in light blue, and selected amino acids are dark blue. Disulfide bonds are presented in yellow, hydrogen bonds in red, and ionic bonds in magenta. Residues participating in ionic bonds are highlighted in green (where not selected in dark blue). Amino acids are numbered according to the NCBI data submission P00451.**

Small and Gross Rearrangements

Twenty-five of the patients were affected by small rearrangements in the *F8* gene. Six of these molecular defects are described for the first time (Supplementary Table S1).

In three patients we were unable to amplify exons 4, 24, and 5 to part of exon 14 (nucleotide position c.2660-?) of the *F8* gene, which suggests that there are large deletions involving the respective exons. Subsequently performed Southern blot analyses confirmed missing bands corresponding to these exons, suggesting that they are deleted in these patients (for details see Supplementary Table S1). The exon 4 deletion is in frame, whereas the exon 24 deletion is out of frame, causing a frame shift and premature stop. Finally, the recently published intron 1 breaking inversion was detected in three of our patients.

Splice Mutations

Only two splicing errors were identified in our patient group (Supplementary Table S1). c.1753–1G>A (IVS11–1G>A), identified in Patient NB118, is a novel change that is predicted to delete the acceptor splice site of intron 10. The other splice mutation, c.671–2A>G (IVS5–2A>G), was observed in one severely affected patient [Naylor et al., 1991].

Spectrum and Distribution of the Mutations in the F8 Gene in Severely Affected Patients

A full spectrum of mutations, including missense, nonsense, small, and gross deletions/rearrangements and splice mutations, was detected in our group of patients. To prevent ascertainment bias, we analyzed the spectrum and distribution of the mutations in all 86 severely affected individuals who were negative for the IVS22-inversion, including the previously described 21 cases [Bogdanova et al., 2001, 2002; Leuer et al., 2001]. Supplementary Table S2 describes the frequency of all mutations identified in the present study and in our three previous works. The distribution of the different types of changes is schematically presented in Figure 1.

The missense mutations accounted for 33 (38.3%) of all 86 cases. They were distributed throughout the whole sequence of the *F8* gene, but predominantly in the A1 and A2 domains. The protein truncating point mutations were present in 45 patients (52.3%). More than half (about 60%) of these types of molecular defects were located in exon 14 of the *F8* gene.

Gross rearrangements were detected in six severely affected cases (about 7%). Three of these patients (about 3.5% of the cases

TABLE 2. **Novel Nonsense Mutations Identified in the Present Study**

Exon	*F8* domain	Nucleotide change[a]	Amino acid change[b]	Amino acid change[c]	Patient index
4	A1	c.560T>A	p.L187X	L168X	NB111
14	B	c.4006C>T	p.Q1336X	Q1317X	NB88
14	B	c.2912T>G	p.L971X	L952X	NB85
14	B	c.3967C>T	p.Q1323X	Q1304X	NB23
14	B	c.3844A>T	p.K1282X	K1263X	NB9
15	A3	c.5301C>A	p.Y1767X	Y1748X	NB170
16	A3	c.5415T>A	p.Y1805X	Y1786X	NB83

[a]DNA mutation numbering is based on cDNA sequence (GenBank no. NM_000132.2), with nucleotide +1 corresponding to A of the ATG translation initiation codon.
[b]Sequence changes at protein level following the Nomenclature for Description of Genetic Variations approved by the Human Genome Variation Society (www.hgvs.org/mutnomen/).
[c]Sequence changes at protein level, representative of the published literature and the reference mutation databases. The 19 amino acids containing signal peptide are numbered reverse, thus the initial Methionine is numbered as –19. The first Alanine of the mature protein is numbered as +1.

in our group) carried the intron 1 breaking inversion. The large deletions identified accounted for 3.5% of the cases. Most probably, these would have been missed if we had analyzed a female carrier instead of a male index patient.

Assuming a 45% prevalence of the common IVS22-inversion among patients with severe hemophilia A, the extrapolated distribution for the remaining types of mutations would be about 21% missense mutations, 29% protein truncating mutations, and 3.6% gross rearrangements (Supplementary Table S2).

Development of *F8* Inhibitors

None of our patients with missense/nonsense/splicing mutations or gross deletions/rearrangements developed inhibitors during their treatment with *F8*. All four inhibitor-positive patients (the previously published individuals NB2, NB56, and NB58 [Bogdanova et al., 2002], and NB253 (c.3385delC, exon 14)) carried small deletions in exon 14 of the *F8* gene. Thus the inhibitor-positive cases represented 4.6% of our patient cohort.

DISCUSSION

It is well known that the common IVS22-inversion of the *F8* gene accounts for 40–50% of severe hemophilia A cases, and usually such patients are tested for this molecular defect prior to extended mutation screening. Therefore, the purpose of this study was to determine the mutation spectrum and detection rate in a large sample of patients with severe hemophilia A who previously tested negative for the intron 22 inversion. To our knowledge this is the first report of such a homogeneous patient cohort, collected during routine daily practice. Based on our results, the expected mutation detection rate in severely hemophilia A-affected male patients approaches 100% when tests are performed for common inversions and other gross rearrangements, followed by sequencing analysis of negative probands. The sensitivity of such an approach appears to be higher than that obtained by previous methods. Some of the recently published studies concerning mutation detection in the *F8* gene used prescreening techniques such as heteroduplex analysis, SSCP, and conformation-sensitive gel electrophoresis (CSGE), and showed that particular mutations are missed by these techniques [Liu et al., 2002; Habart et al., 2003a; Cutler et al., 2002]. Our results support the opinion that analysis of patients with severe hemophilia A should include DNA sequencing even when pre-screening indicates a negative result [Liu et al., 2002; Habart et al., 2003a; Citron et al., 2002]. Moreover, most of these studies involved patients with heterogeneous phenotypes, and thus the reduced mutation detection rate could be explained to some extent by considering that the sensitivity of the analysis on the genomic level may be lower in mildly affected cases. Another possible reason for the reduced sensitivity is the testing of female carriers, since gross deletions of whole exon(s) would be masked in females by the normal allele. Finally, most recent works did not include the recently published intron 1 breaking inversion. The prevalence of this molecular defect is estimated to be 1.5% in Iranian patients, 1.8% in patients of British origin, and 4% in patients from the Czech Republic [Cumming, 2004; Rastegar Lari et al., 2004; Habart et al., 2003b]; thus, its frequency in some untested populations could be even higher.

It is generally considered that 20 to 30% of patients with severe hemophilia A develop inhibitors during their treatment with *F8*. Several studies have shown that genetic factors and different therapeutic strategies can influence a patient's susceptibility to this major complication. Patients with severe molecular defects, such as the IVS22-inversion, have a higher inhibitor prevalence than patients with milder mutations [Oldenburg et al., 2002]. Moreover, it has been suggested that prophylactic treatment with high doses of *F8* could be a risk factor for inhibitor development [Kreuz et al., 2003]. In our patients the occurrence of inhibitor-positive cases was 4.6%, which is lower than normally observed levels. This could be due to the skewed study population, which we obtained by subtracting those patients who carried the IVS22-inversion from the larger population. In addition, the vast majority of our patients have been treated on demand for bleeding episodes (normally once in 6 months), which could be another reason for the lower proportion of inhibitor-positive subjects in this group.

Nearly half of the molecular defects observed in our study are located in the A1 and A2 domains. The vast majority of the protein truncating point mutations are localized in exon 14 of the *F8* gene, encoding the B domain of the protein. Since exons 1–13 contain about 25% of the coding sequence of the *F8* gene, one should probably start an analysis of patients with severe hemophilia A by screening for point mutations in this part of the gene, followed by (or in parallel with) a search for protein truncating mutations in exon 14. Although such an approach may not represent an advantage in all cases, the distribution of defects in severe hemophiliacs differs considerably in comparison with mild cases (unpublished results), which one should take into account when trying to reduce the number of analyses performed.

Missense mutations are usually of particular interest because they pinpoint functionally important amino acids. The richest spectrum of conformations that alter mutations, as identified in the present study, is undoubtedly observed in the A1 and A2 domains, which also harbor most of the ligand interaction sites for *F8*. The rest of the newly detected missense mutations, which are dispersed in the remainder of the molecule (A3 to C2 domains) appear to be responsible for stability issues and reflect the mostly structural function of these protein regions.

The severity of hemophilia due to particular missense mutations appears to vary among different patients. In the international hemophilia A database HAMSTeRS, there are reports of 34 amino acid changes leading to severe or mild/moderate bleeding disease. Examples of such variably expressed mutations are R282C, R282L, and R2163 H, which we detected in six of our severely affected patients. G145D, P243L, Y473C, and Q2100R obviously represent further missense changes that cause variable phenotypes. One possible reason for this phenomenon is the presence of an additional molecular change on some of the mutated or healthy alleles that modifies the pathological expression. The presence of a second mutation in the coding region of *F8* was excluded by the sequencing analysis in our patients. Intronic variations, possibly affecting the splicing efficiency, that are detectable at the RNA level were not covered by our analysis because no RNA from the diagnosed individuals was available. On the other hand, a recent search for splicing defects or rearrangements at the RNA level, in patients with hemophilia A who screened negative for mutations at the genomic level, revealed no further defect affecting the *F8* gene [El-Maarri et al., 2005]. Although molecular defects located deep in the introns of the *F8* gene appear to be rare, it would be worthwhile to test RNA from patients carrying missense mutations that are known to cause variable phenotypes to assess whether a second variation could be the reason for the clinical variability. Apart from the underlying mechanism, which remains to be determined, this study supports previous findings that particular missense mutations are characterized by a variable phenotypic expression that should be taken into account for clinical predictions. Finally, our results further emphasize the importance of searching for factors that modify the hemophilia A phenotype.

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REFERENCES

Antonarakis SE, Kazazian HH, Tuddenham GD. 1995. Molecular etiology of factor VIII deficiency in hemophilia A. Hum Mutat 5: 1–22.

Bagnall RD, Waseem N, Green PM, Giannelli F. 2002. Recurrent inversion breaking intron 1 of the factor VIII gene is a frequent cause of severe hemophilia. Blood 99:168A–174A.

Becker J, Schwaab R, Moller-Taube A, Schwaab U, Schmidt W, Brackmann HH, Grimm T, Olek K, Oldenburg J. 1996. Characterization of the factor VIII defect in 147 patients with sporadic hemophilia A: family studies indicate a mutation type-dependent sex ratio of mutation frequencies. Am J Hum Genet 58:657–670.

Bogdanova N, Lemcke B, Markoff A, Pollmann H, Dworniczak B, Eigel A, Horst J. 2001. Seven novel and four recurrent point mutations in the factor VIII (*F8*) gene. Hum Mutat 18:546.

Bogdanova N, Markoff A, Pollmann H, Nowak-Gottl U, Eisert R, Dworniczak B, Eigel A, Horst J. 2002. Prevalence of small rearrangements in the factor VIII gene *F8*C among patients with severe hemophilia A. Hum Mutat 20:236–237.

Citron M, Godmilow L, Ganguly T, Ganguly A. 2002. High throughput mutation screening of the factor VIII gene (*F8*C) in hemophilia A: 37 novel mutations and genotype-phenotype correlation. Hum Mutat 20:267–274.

Cumming AM. 2004. UK Haemophilia Centre Doctors' Organization Haemophilia Genetics Laboratory Network. The factor VIII gene intron 1 inversion mutation: prevalence in severe hemophilia A patients in the UK. J Thromb Haemost 2:205–206.

Cutler JA, Mitchell MJ, Smith MP, Savidge GF. 2002. The identification and classification of 41 novel mutations in the factor VIII gene (*F8*C). Hum Mutat 19:274–278.

El-Maarri O, Herbiniaux U, Graw J, Schroder J, Terzic A, Watzka M, Brackmann HH, Schramm W, Hanfland P, Schwaab R, Muller CR, Oldenburg J. 2005. Analysis of mRNA in hemophilia A patients with undetectable mutations reveals normal splicing in the factor VIII gene. J Thromb Haemost 3:332–339.

Habart D, Kalabova D, Novotny M, Vorlova Z. 2003a. Thirty-four novel mutations detected in factor VIII gene by multiplex CSGE: modeling of 13 novel amino acid substitutions. J Thromb Haemost 1:773–781.

Habart D, Kalabova D, Hrachovinova I, Vorlova Z. 2003b. Significant prevalence of the intron 1 factor VIII gene inversion among patients with severe hemophilia A in the Czech Republic. J Thromb Haemost 1:1323–1324.

Higuchi M, Antonarakis SE, Kasch L, Oldenburg J, Petersen EE, Olek K, Inaba H, Kazazian HH. 1991. Molecular characterization of mild-to-moderate hemophilia A: detection of the mutation in 25 of 29 patients by denaturing gradient gel electrophoresis. Proc Natl Acad Sci USA 88:8307–8311.

Kemball-Cook G, Tudedenham EGD, Wacey AI. 1998. The factor VIII structure and mutation resource site: HAMSTeRS version 4. Nucleic Acids Res 26:216–219.

Kreuz W, Ettingshausen CE, Auerswald G, Saguer IM, Becker S, Funk M, Heller C, Klarmann D, Klingebiel T, GTH PUP Study Group. 2003. Epidemiology of inhibitors and current treatment strategies. Haematologica 88:EREP04.

Lakich D, Kazazien HH, Antonarakis SE, Gischier J. 1993. Inversions disrupting the factor VIII gene as a common cause of severe hemophilia A. Nat Genet 5:236–241.

Lenting PJ, van Mourik JA, Mertens K. 1998. The life cycle of coagulation factor VIII in view of its structure and function. Blood 92:3983–3996.

Leuer M, Oldenburg J, Lavergne JM, Ludwig M, Fregin A, Eigel A, Ljung R, Goodeve A, Peake I, Olek K. 2001. Somatic mosaicism in hemophilia A: a fairly common event. Am J Hum Genet 69: 75–87.

Liu ML, Nakaya S, Thompson AR. 2002. Non-inversion factor VIII mutations in 80 hemophilia A families including 24 with alloimmune responses. Thromb Haemost 87:273–276.

Lund O, Frimand K, Gorodkin J, Bohr H, Bohr J, Hansen J, Brunak S. 1997. Protein distance constraints predicted by neural networks and probability density functions. Protein Eng 10: 1241–1248.

Millar DS, Zoll B, Martinowitz U, Kakkar VV, Cooper DN. 1991. The molecular genetics of haemophilia A: screening for point mutations in the factor VIII gene using the restriction enzyme TaqI. Hum Genet 87:607–612.

Naylor JA, Green PM, Montandon JA, Rizza CR, Giannelli F. 1991. Detection of three novel mutations in two haemophilia A patients by rapid screening of whole essential region of factor VIII gene. Lancet 337:635–639.

Oldenburg J, El-Maarri O, Schwaab R. 2002. Inhibitor development in correlation to factor VIII genotypes. Haemophilia 8(Suppl 2):23–29.

Pemberton S, Lindley P, Zaitsev V, Card G, Tuddenham EG, Kemball-Cook GA. 1997. Molecular model for the triplicated A domains of human factor VIII based on the crystal structure of human ceruloplasmin. Blood 89:2413–2421.

Rastegar Lari G, Enayat MS, Arjang Z, Lavergne JM, Ala F. 2004. Identification of intron 1 and 22 inversion mutations in the factor VIII gene of 124 Iranian families with severe haemophilia A. Haemophilia 10:410–411.

Schwaab R, Oldenburg J, Lalloz MR, Schwaab U, Pemberton S, Hanfland P, Brackmann HH, Tuddenham EG, Michaelides K. 1997. Factor VIII gene mutations found by a comparative study of SSCP, DGGE and CMC and their analysis on a molecular model of factor VIII protein. Hum Genet 101: 323–332.

Stoilova-McPhie S, Villoutreix BO, Mertens K, Kemball-Cook G, Holzenburg A. 2002. 3-Dimensional structure of membrane-bound coagulation factor VIII: modeling of the factor VIII heterodimer within a 3-dimensional density map derived by electron crystallography. Blood 99:1215–1223.

Tavassoli K, Eigel A, Pollmann H, Horst J. 1997. Mutational analysis of ectopic factor VIII transcripts from hemophilia A patients: identification of cryptic splice site, exon skipping and novel point mutations. Hum Genet 100:508–511.

Vehar GA, Keyt B, Eaton D, Rodriguet H, O'Brien DP, Rotblat F, Oppermann H, Keck R, Wood WI, Harkins RN, Tuddenham EGD, Lawn RM, Capon DJ. 1984. Structure of human factor VIII. Nature 312:337–342.