MUTATION IN BRIEF

Prevalence of Small Rearrangements in the Factor VIII Gene F8C Among Patients with Severe Hemophilia A

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Hemophilia A is a common X-linked bleeding disorder caused by various types of mutations in the factor VIII gene F8C. The most common intron 22-inversion is responsible for about 40% of the severe hemophilia A cases while large deletions, point mutations and small (less than 100 bp) deletions or insertions are responsible for the disease in the rest of patients. We report on nine novel (6 deletions, two indels and one partial duplication) and five recurrent small rearrangements identified in 15 German patients with severe hemophilia A, negative for the intron 22-inversion. c.2208-2214delTTATTAC/c.2207-2215insCTCTT and c.4665-4678del/c.4664-4678insAAGGAA identified in the present study are the first small indels described in the factor VIII gene. Our analyses suggest that the prevalence of this type of mutations (predominantly located in exon 14) among patients with severe phenotype and negative for the common intron 22-inversion, is about 30%. The correlation between these molecular defects and formation of factor VIII inhibitors as well as the parental origin of the de novo mutations are evaluated. Finally we show that denaturing HPLC (DHPLC) and classic heteroduplex analysis (HA) are able to detect these sequence alterations on 100% and could be preferred as a screening approach when analysing for mutations in factor VIII in severely affected patients.

KEY WORDS: factor VIII; F8C; mutation analysis; hemophilia A; HEMA; rearrangement; German; DHPLC

INTRODUCTION

Hemophilia A (MIM# 306700) is a clinically heterogeneous X-linked bleeding disorder affecting 1 to 2 per 10 000 males in all ethnic groups. It is caused by a defect or reduced activity of the factor VIII protein – an essential cofactor for the factor IX-mediated activation of factor X in the intrinsic blood coagulation cascade [Fay, 1993]. The severity and frequency of hemorrhages in hemophilia A is inversely related to the amount of residual factor VIII. About 50% of patients have severe hemophilia A with a residual factor VIII activity less than <1% of normal. These patients have frequent spontaneous bleedings into joints, muscles and internal organs. Moderate (factor VIII activity 2-5% of normal) and mild hemophilia A (factor VIII activity 5-30% of normal) occur in about 10% and 30-40% of patients respectively. It has been reported that individuals receiving recombinant factor VIII therapy develop antibodies (s.c. "inhibitors" of factor VIII), leading to autoantibody production against the endogenous protein [reviewed by Scharrer et al., 1999]. The question of correlation between autoimmune response and type of

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molecular defect in this coagulation factor is still open.

Various types of mutations in the factor VIII gene [Gitschier et al. 1984], located on the long arm of chromosome X, are responsible for the bleeding disorder [Kemball-Cook et al. 1998]. The most common sequence alterations leading to a severe disease condition are the partial gene inversion with a breakpoint in intron 22 of the factor VIII gene, responsible for about 40% of the severe hemophilia A cases and different types of large (more than 100 bp) deletions, found in ~5% of the severely affected patients [Lakich et al., 1993; Naylor et al., 1993a].

Point mutations and small (less than 100 bp) deletions or insertions are responsible for the disease in the rest of patients. These alterations are spread throughout the 7 kb coding sequence of the factor VIII gene. The factor VIII mutation database contains to date 629 unique mutations with more than 400 single nucleotide substitutions (http://europium.csc.mrc.ac.uk). Small deletions and insertions in the coding region of the gene, in most cases resulting in frameshifts, have been reported in >150 unrelated patients and more than the half of them are in the large exon 14. These mutations with only few exceptions are leading to a severe clinical phenotype.

Up to now we systematically analyzed factor VIII gene for pathological defects in 50 cases of severe hemophilia A, referred to our institute for molecular diagnosis [Tavassoli et al., 1997; Tavassoli et al., 1998a; Tavassoli et al., 1998b; Tavassoli et al., 1999; Möller-Morlang et al., 1999; unpublished data]. Large deletions and the common intron 22-inversion, a frequent cause for severity of the disease, were previously excluded in these patients using Southern-blot analyses. Here we report on 9 novel and 5 recurrent small rearrangements identified in 15 of these patients. All of these mutations create reading frameshifts and hence are predicted to result in truncated protein variants. Our analyses suggest that a severe phenotype in the hemophilia A bleeding disorder is largely associated with such type of mutation (insertions, deletions and insertion/deletions) in exon 14 of the factor VIII gene. In this study we make use of two methods, denaturing HPLC (DHPLC) and classic heteroduplex analysis (HA), which are able to detect these sequence alterations on 100%. Both methods based on heteroduplex formation between normal (wild type) and mutant DNA sequences are straightforward in use and HA as a low-cost alternative to DHPLC is applicable also in labs and diagnostic centers where exquisite hardware equipment might be an expensive issue. The abundance of insertions and deletions in exon 14 of the gene leading to a severe phenotype could turn such an easy protocol screen into regular diagnostic practice when analyzing for mutations in Factor VIII.

PATIENTS AND METHODS

Fifteen unrelated patients of German origin (14 male and 1 female) affected by severe hemophilia A are included in this study. The clinical severity of the hemophilia A was classified according to standard criteria based on the remaining activity of factor VIII (Antonarakis et al., 1995). In addition, information about development of factor VIII inhibitors was gathered appropriately.

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

DNA extraction and amplification

Genomic DNA was extracted from peripheral blood lymphocytes as described by Miller et al. (1988). PCR was performed on 50 – 100 ng of extracted DNA essentially using the amplification primers and cycling conditions described by Schwaab et al. (1997).

Exon 14 of the factor VIII gene was amplified in nine overlapping formats as presented in Table 1. PCR was 35 cycles of denaturing at 94^{o}C for 30 seconds, annealing at 55^{o}C for 1 minute and extension at 72^{o}C for 1 minute. Reactions were performed in a total volume of 50µl and the reaction mix contained the following: 50 mM Tris.Cl pH 9.5, 20 mM $(NH_4)_2SO_4$, 1 mM DTT, 0.005% NP-40, 1.5 mM $MgCl_2$, 0.5 M betaine, 20 pmol of each primer, 600 µM dNTP, 100 - 200 ng DNA and 1.25 U Taq polymerase produced and purified as previously described (Pluthero, 1993).

Dideoxy fingerprinting and sequencing analysis

To perform dideoxy fingerprinting (ddF) (Sarkar et al., 1992; Blaszyk et al., 1995), PCR products were purified with the PCR product pre-sequencing kit (Amersham Pharmacia Biotech) according to the manufacturer's protocol. Forward and reverse 4µl cycle-sequencing reactions using 15 pmol of the amplification primers, the ddGTP terminator and α-[^{33}P-dCTP] (20 pmol, Hartmann Analytics) were performed with 1.25 U Sequitherm

DNA Polymerase (Epicentre Technologies) in 30 amplification rounds of 15 sec. at 95°C, 1min. at 60°C and 1 min. at 70°C. The resulting products were electrophoresed at room temperature on a 0.5xMDE Serdogel (Serva) nondenaturing gel (350 x 400 x 0.4mm) at 35W constant power for 3h. Upon completion of electrophoresis gels were dried under vacuum prior to autoradiographic exposure on Biomax MR films (Kodak).

All fragments showing aberrant ddF pattern as compared to normal controls were sequenced with the Applied Biosystems (ABI) BigDye Terminator Cycle Sequencing Ready Reaction Kit according to manufacturers instructions and sequencing reactions were analysed on an ABI 310 or ABI 3700 automated sequencer.

Table 1. Amplification Primers for ddF, DHPLC and HA of Exon 14 of the Factor VIII Gene

PCR Format	Primer Sequence	Length (bp)
Exon 14 AC	AF GGGAGAGAACCTCTAACAGAACGTT CR GGCCTGAAGTGTGTCAT	535
Exon 14 DE	DF CCACATGGGCTATCCTTAT ER GGCTATTCATTAAACCTGAT	468
Exon 14 EF	EF GGACAACTGCAGCAACAGA FR CCAGACTGATGGACTATTCTCA	426
Exon 14 GI	GF GCTCATGGACCTGCTTTGTT IR TATGATACTGAGGCAAAACTACA	714
Exon 14 J	JF GGATAATTTACATGAAATAATA JR GAAATGAGCTGTGTGTTTCTT	267
Exon 14 K	KF TCCAGTACTTCAAGATTTTAGGTC KR GGGTGCTCGGGGTCAAA	319
Exon 14 L	LF CAGACTCCCACTAGAAGAAACAG LR AAGATGAGAAGAGTTGTCTTG	295
Exon 14 MO	MF AAAGGTATCATCATTTCC OR GCTATTGCATGATTGCTT	659
Exon 14 PR	PF TGGAAATCCCAAGAGAAGTCACCAG RR TCAAATGTCACAAGAGCAGAGCA	472

Denaturing HPLC (DHPLC) and heteroduplex analysis (HA)

PCR products containing the identified mutations were mixed with PCR products from normal individuals and subjected to denaturing at 95°C for 5min. After denaturing samples were allowed to cool slowly to room temperature for about 30min to allow formation of heteroduplexes. PCR products from normal controls were processed in parallel for all amplicon mixtures.

DHPLC was performed on WAVE™ DNA Analysis System (Transgenomic) as described by Kuklin et al. (1998). The analysed probes were eluted from the DNASep column (Transgenomic) under appropriate temperature and gradients of buffer A, containing 0.1M triethylammonium acetate (TEAA) and 0.025% acetonitrile and buffer B (0.1M TEAA and 25% acetonitrile). The optimal for mutation detection gradient(s) and temperature(s) of the column were calculated for each fragment using the Wavemaker™ software.

The electrophoretic separation of the heteroduplexes was carried out on a Multiphor II Electrophoresis Unit (Amersham Pharmacia Biotech, Freiburg, Germany) with precise temperature control. About 2 µl of the probes were applied for analysis on 10% ultrathin-layer native polyacrylamide gels (%C = 3.3) in a discontinuous buffer system for 45min on 3W constant power at 7°C and 10°C temperature. Leading buffer was 70 mM tris-sulfate, pH 9.0 and 0.14 M tris-borate, pH 9.0 was trailing buffer. After completion of electrophoresis gels were silver stained and dried for documentation.

RESULTS

In the present study we identified nine novel (six deletions, two indels and one duplication) and five recurrent (three deletions and two insertions) small rearrangements in the Factor VIII gene in 14 male and one female hemophilia A patients (Table 2). One recurrent small deletion was identified in two unrelated patients. The mutations are described according to the nomenclature system recommended by den Dunnen and Antonarakis, (2000).

All male patients had remaining FVIII activity less than 1% and the female patient (NB6) - 22% of normal. Both patients with small indels (NB39 and NB2) and two patients (NB58 and NB56) with single nucleotide deletions developed clinically relevant inhibitors 60-70 exposure to factor VIII concentrates (Table 2). The peak inhibitor levels ranged from 20 BU/ml^{-1} to > 600 BU/ml^{-1} (repeatedly measured Bethesda titers).

Table 2. Small Rearrangements in the Factor VIII Gene, Family History, and Incidence of Inhibitors in 15 Unrelated Patients of German Origin Affected by Severe Hemophilia A

Exon	Codon(s)	Nucleotide change	Inhibitors	Patient Index Family History	Reference
6	212-213	c.693-696delAAAG	No	NB68 Sporadic	**Present study**
6	233	c.755-766delCA	No	NB43 Sporadic	**Present study**
14	717-719	c.2208-2214delTTATTAC c.2207-2215insCTCTT	Yes	NB39 Positive	**Present study**
14	851	c.2610delT	Yes	NB58 Sporadic	**Present study**
14	963	c.2945-2946insA	No	NB40 Positive	Naylor et al., 1993b
14	1194	c.3638delA	No	NB89, NB91 Positive	Lin et al., 1994
14	1215-1216	c.3702-3705delTACA	No	NB77 Positive	Tavassoli et al., 1998b
14	1271	c.3870delA	No	NB6 Sporadic	**Present study**
14	1488	c.4519delA	No	NB99 Positive.	**Present study**
14	1536-1540	c.4665-4678del c.4664-4678insAAGGAA	Yes	NB2 Positive	**Present study**
14	1575-1577	c.4781-4787del	No	NB57 Sporadic	**Present study**
14	1590	c.4825-4826insA	No	NB134 Positive	Lin et al., 1993
18	1968	c.5961delA	Yes	NB56 Positive	Becker et al., 1996
21	2049-2067	c.6202-6257dupl	No	NB95 Sporadic	**Present study**

Small deletions

We detected three novel single nucleotide deletions occurring in DNA regions of short direct repeats in exon 14 of the factor VIII gene. A deletion of T at cDNA position 2610, codon 581) (c.2610delT) was found in patient NB58 who developed high titre (>10BU) inhibitors before 60 days exposure to factor VIII concentrates. There is no history of hemophilia A in this family although the patients mother was proved to be a carrier.

c.3870delA (codon 1271) was identified in a sporadic female patient (NB2) with residual factor VIII activity 22% of normal who was referred to our institute for genetic testing because of a bleeding episode after a surgery. A novel deletion, c.4519delA (codon 1488) in a run of 5 adenines was detected in patient NB99 with a positive family history.

We also identified three novel deletions of more than one nucleotide, causing frameshift and premature stop-codon in sporadic male patients (NB68, NB43 and NB57). Two of them, c.693-696delAAAG and c.755-766delCA, are located in short stretches of repeated sequences of exon 6 of the factor VIII gene. The third one is a deletion of seven nucleotides (c.4781-4787del) in exon 14 and it was detected in a severely affected sporadic male patient (FVIII:C <1%). The mutation was present in the patient's mother, but not in her two sisters or in the patients grandmother. The haplotype analysis, performed in this family with intragenic polymorphic markers, revealed a grand-paternal origin of the mutant allele (data not shown).

Three recurrent small deletions (c.3638delA, c.3702-3705delTACA and c.5961delA), all occurring in repeated sequences, were detected in four unrelated patients with severe hemophilia A. c.3638delA was found twice in this study. The patient carrying c.5961delA developed persistent high titre (20BU) FVIII inhibitors before 70 days of treating with factor VIII concentrates.

Small insertions and duplications

Two recurrent insertions of adenine nucleotide (c.2945-2946insA and c.4825-4826insA) in poly-A tracts were identified in exon 14 of the factor VIII gene. Both cases have positive family history without information for inhibitors formation.

The variant c.6202-6257dupl, a 56 bp duplication in exon 21 of the factor VIII gene, was identified in a sporadic case (NB95) of severe hemophilia A. The mutation was present in the patients mother but tracing the origin of the duplication was not possible since no DNA from the grandparents was available for analysis.

Small indels

Two small indels (insertion/deletions <100bp), both leading to frameshift and premature stop-codons, were identified in exon 14 of the factor VIII gene.

c.2208-2214del / c.2207-2215insCTCTT was found in a 20 years old severely affected patient (NB39) with a negative family history. This was the only one family member available for DNA analysis. The deleted nucleotides (TTATTAC) are replaced by a completely different sequence of 5 bp (CTCTT), thus predicting to create a premature translation stop at codon 719.

A similar rearrangement, c.4665-4678del / c.4664-4678insAAGGAA, predicted to create translation stop at codon 1593 was identified in patient NB2 and his two daughters. In this case the deleted sequence (TGAAGCAAACAGAC) is replaced again by a simple repetitive element (AAGGAA). Both patients developed high titre FVIII antigens (pick inhibitor levels >600BU/ml^{-1}) which have been detected for a long period of time.

All mutations identified in this study using ddF as a screening approach were successfully detected by DHPLC under gradient and temperature conditions as predicted by the Wavemaker^{TM} software. In most of the cases all mutations in a certain format were shown at a single DNA-Sep temperature (Table 3). The heteroduplex analysis performed at 10^{o}C did not detect the insertions and the deletions of adenine. These types of mutations were detectable by HA only at 7^{o}C (Table 3).

Table 3. Comparison of the DHPLC and HA for Detection of Small Rearrangements in the Factor VIII Gene

Exon / Format	Mutation	DHPLC @ ^{o}C	HA @10^{o}C	HA @7^{o}C
6	c.693-696delAAAG	59	+	+
	c.755-766delCA		+	+
14 AC	c.2208-2214delTTATTAC c.2207-2215insCTCTT	57	+	+
14 DE	c.2610delT	57	+	+
14 EF	c.2945-2946insA	55	+	+
14 J	c.3638delA	55	-	+
	c.3702-3705deTACA		-	+
14 K	c.3870delA	57	-	+
14 MO	c.4519delA	58	-	+
	c.4665-4678del c.4664-4678insAAGGAA		+	+
	c.4781-4787del		+	+
	c.4825-4826insA		-	+
18	c.5961delA	57	-	+
21	c.6202-6257dupl	56	+	+

DISCUSSION

In the present study we identified small rearrangements in about 30% (15 out of 50) of the patients with severe hemophilia A, not carrying a large deletion or the common intron 22-inversion. Ten patients (20%) were shown to carry small deletions, two (4%) – small insertions, two (4%) were carrying insertion/deletions and one (2%) – a partial duplication. Similar proportion of small deletions (19.7%) and insertions (2,8%) have been reported by

Becker et al., 1996 in 71 severely affected patients negative for the common intron 22 inversion. Even higher proportion of these types of defects in the factor VIII gene have been estimated by others [Lin et al., 1993; Ljiung and Sjörin, 1999]. Of altogether 629 unique mutations included in the hemophilia A mutation database (http://europium.csc.mrc.ac.uk) to date, 102 (16%) are small insertions and deletions (below 200 bp). Generally, it is clear that despite the slight differences in distribution found in various studies, the prevalence of small rearrangements (deletions, insertions, partial duplications) in the factor VIII gene among patients with severe bleeding disorder lacking the intron 22 inversion is relatively high. Due to the nature of the sequence alteration, these mutations are expected to be easily detected by different screening approaches with a high rate of sensitivity, which is probably not the case for point mutations. In our study all mutations were successfully detected using all tested screening approaches (ddF, DHPLC and HA), in most cases under universal conditions. A small, but not real exception is the detection of mutations in clustered adenine tracts, changing the number of As, which can be accomplished by HA only at 7^{o}C. It is known that such adenine repeats can project peculiar DNA tertiary structures, which could be the explanation for the lower temperature conditions necessary to visualise the sequence differences.

Since ddF is expensive and laborious and DHPLC requires a special equipment, probably not available in every diagnostic unit, the simple heteroduplex analysis could be recommended as a screening step in mutation detection strategies applied to intron 22 inversion-negative patients with severe hemophilia A.

Five of our hemophilia A patients were sporadic cases and in three of those patient's mothers were available for genetic testing. In all these cases the appropriate mutation was detected also by probands mothers and in one family (mutation c.4781-4787del) a grand-paternal origin of the mutation was proved. Previous investigations on the origin of mutations in the factor VIII gene established a higher ratio of mutation frequencies for males than for females [Oldenburg et al., 1993; Becker et al. 1996] but gave conflicting results on the sex-specific mutation rates. Becker et al., 1996 found that inversions and point mutations show 4-15-fold-higher mutation rate in male germ cells, whereas small deletions show >5-fold-higher mutation rate in female germ cells. Ljung and Stjörn, 1999 found 6-fold higher mutation frequency in males, without sex-specific mutation rate. Although the number of investigated sporadic cases in this study is too small to draw conclusions about this issue, our results confirm the higher mutation frequencies in males but do not support the evidence for sex-specific mutation rate.

About one half of the small deletions and insertions reported to date are located in the large exon 14 (B domain) and the rest are spread over the whole coding region of the factor VIII gene [http://europium.csc.mrc.ac.uk]. 11 of the 15 mutations identified in our study are disposed in exon 14 of the gene. Although this finding could be biased by the relatively small number of patients, it supports the high frequency of this type molecular defects in the B domain of the factor VIII gene. Some of the mutations in this exon identified in our study have already been described in several other patients, giving evidence for mutation hot-spots, like the A-stretch at position c.3629-3638 (codons 1191-1194) – mutations in 25 unrelated patients described [Becker et al., 1996; Lin et al., 1993; Economou 1992; Pieneman et al., 1995; Becher et al., 1996; Freson et al., 1998; Vidal et al., 2001; Goodeve et al., 2000; Akkarapatumwong et al., 2000, present study]; c.4372-4379, codons 1439-1441 (13 mutations described) [http://europium.csc.mrc.ac.uk]; c.4820-4825, codons 1588-1590 (4 mutations described) [Lin et al., 1993; Freson et al., 1998; Goodeve et al., 2000; present study). Furthermore c.3702-3705delTACA, codon 1215-1216 identified in one of our patients has been described in two other patients [Tavassoli et al., 1998b; Ivaskevicius et al., 2001], showing that this repetitive sequence could be also prone to mutations.

Another possible mutation hot-spot could be the 7A-stretch in exon 18, codons 1967-1968, since deletions or insertion of A at this position have been described in couple of unrelated patients [Becker et al., 1996; Pieneman et al, 1995; Liu et al., 1998; present study]. From crystal structures and studies in solution, it is generally known for A-tracts in DNA that they develop distinctive B' structure, essentially straight and more rigid than the generic B-form DNA (McConnell and Beveridge, 2001; MacDonald et al., 2000). Such structures induce bending in the molecule, the angle of which depends on the surrounding purine/pyrimidine bases and they can cause increased gel retardation due to enhanced conformation and preferential counterion binding (Stellwagen et al., 2001). That is why mutations (insertions/deletions of A) in A-stretches require more attention and specific conditions to be visualised in a heteroduplex analysis set-up.

In addition to the previously described deletions at codons 210-211 and 224 in exon 6 [Lin et al., 1993; Goodeve et al., 2000], two further deletions – c.693-696delAAAG (codons 212-213) and c.755-766delCA (codon 233) were identified in our patient group. Obviously mutations in this exon are not exclusively rare as previously

suggested (Becker et al., 1996). Moreover, the sequence at cDNA positions c.688-698 (codons 211-214) is repetitive (GAAACAAAGAAA) and could be also prone to small deletions or insertions.

The mutations c.2208-2214delTTATTAC / c.2207-2215insCTCTT and c.4665-4678del / c.4664-4678insAAGGAA (both in exon 14) identified in the present study are the first small (<100 bp) complex rearrangements described in the factor VIII gene. Such sequence alterations have been previously described for other genes coding for comparatively large protein molecules, exhibiting channel (SLC26A3 or CLD gene, involved in congenital chloride diarrhea) or receptor functions (LDL receptor gene involved in atherosclerosis) [Heath et al., 2001; Hoglund et al., 2001]. In the case of factor VIII, several gross complex rearrangements have been already reported for the gene [http://europium.csc.mrc.ac.uk ; Kazazian et al., 1988]. A very detailed description of a 316 bp deletion abolishing the acceptor splice site of exon 16 and insertion of 6 bp at the same position have been published by Tavassoli et al (1999). The authors postulate that an intragenic recombination, responsible for the deletion and accompanied by a slipped mispairing during replication (resulting in the 6 bp insertion) is the mutational mechanism in this case. It is very possible that similar mechanism(s) are responsible for the observed small rearrangements in exon 14, although the analysis for repeated elements, potentially mediating recombination events is negative for this particular region (http://ftp.genome.washington.edu:80/cgi-bin/RepeatMasker). On the other hand, there are many short homopolymeric tracts of As and Ts in exon 14, as well as highly recombinogenic palindromic sequences in 200-400 bp distance of one another, which are molecular features very likely to promote slipped mispairing and intragenic recombinations. The last could be true for the observed duplication of 56 bp in exon 21, there is only one similar duplication of 13 bp found in exon 14 of the gene [Laprise et al., 1998].

It is generally accepted for the evolution of protein families that small indels occur in nearly every loop of the protein tertiary structure (Pascarella and Argos, 1992). Taking this idea one step further, it should be possible to map sites of protein-protein interactions using indel modeling. Such task has already been accomplished in the case of the human complement component C3 (Ogata et al., 1998). Part of the engineered indels severely damaged the C3 functional activity, but did not affect the stability or structure of the protein and another part did drastically change the molecule's conformation. In the case of factor VIII, indels in exon 14, which is removed by proteolytic cleavage and should be important for the proper folding of the mature protein, could have a pronounced effect on the structure and stability as well as on the activity of the molecule.

The formation of inhibitors to factor VIII in patients is the most serious complication of the hemophilia A therapy. Up to 35-40% of subjects with severe hemophilia A develop a factor VIII inhibitor at some stage in their treatment [reviewed by Scharrer et al., 1999 and Oldenburg et al., 2000]. Collection of more data on possible factors influencing the inhibitor formation is therefore very important on order to allow correct assessment of inhibitor risk. Recent studies including factor VIII mutation data in patients with severe hemophilia A gave the evidence that mutations like large deletions, inversions or nonsense mutations, leading to a major loss of coding information and lack of circulating factor VIII antigen are associated with a higher risk (about 35%) to develop inhibitors than missense mutations or small deletions (4.3 and 7.4% respectively) [Schwaab et al., 1995a]. Moreover, Goodeve et al. (2000) did not find inhibitors in 11 severely affected patients with small insertions or deletions resulting in alteration of the reading frame. Frameshift deletions in runs of adenine are considered to have a low inhibitor risk, probably due to a restoration of the reading frame by slippage errors of the polymerases [Young et al., 1997]. Although the present study was not designed for estimation of possible correlations between factor VIII genotype and inhibitor development, it brings some knowledge about this issue. Both our patients with small complex rearrangements developed inhibitors during their treatment with rFVIII, thus giving the evidence that this type of mutations are related to a higher inhibitor prevalence. Furthermore, two (20%) of our 10 patients with small deletions - c.2610delT (codon 851, exon 14, B domain) and c.5961delA (codon 1968, exon 18, A3 domain) - were inhibitor-positive. cDNA positions c.5955-5961 for instance contains a stretch of 7 adenine and deletions or insertions at this place are expected to be partially corrected by the slippage of the polymerase enzymes [Schwaab et al., 1995a; Antonarakis 1995]. Indeed, no inhibitor-patient with a frameshift mutation at the A-runs in exon 14 have been reported to date, including the present study. However, this repair mechanism seems to be not so effective in the 7A-stretch of exon 18, since 2 of 5 patients with deletion/insertion of A at this position reported to date developed inhibitors [Becker et al., 1996; Pieneman et al., 1995; Liu et al., 1998, present study]. Most probably not only the type of mutations but also their localization in the factor VIII gene influences the predisposition to inhibitor formation.

Some sequence alterations rendering severe phenotype, detected in this and other genetic analyses on hemophilia A should be invaluable natural probes for important functional domains of the factor VIII protein. The correlation genotype-phenotype in this disease has another therapy relevant dimension as well, that is projecting identified molecular defects on inhibitor formation. This creates opportunity to gain insight into molecular mechanisms of the protein folding and stabilization as well as functional properties. The last, but not least are important issues towards designing appropriate individualized treatment for this most frequent inherited bleeding disorder.

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