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ORIGINAL ARTICLE

Analysis of mRNA in hemophilia A patients with undetectable mutations reveals normal splicing in the factor VIII gene

O. EL-MAARRI,* U. HERBINIAUX,* J. GRAW,† J. SCHRÖDER,‡, A. TERZIC,†, M. WATZKA,¶ H. H. BRACKMANN,* W. SCHRAMM,§ P. HANFLAND,* R. SCHWAAB,* C. R. MÜLLER‡ and J. OLDENBURG*‡¶

Institute of Experimental Haematology and Transfusion Medicine, Bonn, Germany; †GSF-National Research Center for Environment and Health, Institute of Developmental Genetics, Neuherberg, Germany; §Medical Clinic Department of Transfusion Medicine, Munich, Germany; ‡Institute of Human Genetics, Würzburg, Germany; and ¶Institute of Transfusion Medicine and Immunohaematology, DRK Blood Donor Service Baden-Württemberg Hessen, Frankfurt, Germany

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Summary. *Background*: haemophilia A (HA) is characterized by partial or total deficiency of factor VIII (FVIII) protein activity. It is caused by a broad spectrum of mutations in the FVIII gene. Despite tremendous improvements in mutation screening methods, in about 2% of HA patients no DNA change could be found, even after sequencing the whole coding part of the FVIII gene including the flanking splice sites, as well as the promotor and the 3′ UTR regions. *Objectives, patients and methods*: In the present study we performed a detailed RNA analysis of three groups of patients. The first included control patients with known splicing defects, the second included two patients with already identified nucleotide changes close to splicing sites, that could potentially alter the normal splicing process, and a third group of 11 unrelated patients whose genomic DNA have already been screened for mutations by DHPLC and direct sequencing with no mutation being identified. *Results*: Both candidate splice site mutations were shown to result in either skipping or alternative splicing of at least one exon, therefore these DNA changes must be considered as causal for the patients' HA phenotype. In contrast, no abnormalities on the RNA level were observed in any of 11 unrelated patients without mutations in the FVIII gene. *Conclusions*: These findings exclude mutations that could be located deep in the introns and affecting either normal splicing or lead to mechanisms causing some unknown rearrangements of the FVIII gene. In fact, our results point to the presence of still unknown factor(s) causing HA, which might be either allelic or in the close proximity of the FVIII gene or non-allelic associated with other genetic loci that are involved in the processing of the FVIII protein.

Correspondence: Johannes Oldenburg, Department of Molecular Haemostasis and Immunohaematology, Institute of Transfusion Medicine and Immunohaematology, Sandhofstr. 1, 60528 Frankfurt, Germany.
Tel.: +49 (0) 69 6782177; fax: +49 (0) 69 6782204; e-mail: joldenburg@bsdhessen.de

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Introduction

The total absence of the factor VIII (FVIII) protein in plasma or its decreased activity causes haemophilia A (HA), which is characterized by spontaneous bleeding. A large variety of mutations have been discovered in the FVIII gene of HA patients including the intron 22 and intron 1 inversions hot spots, point mutations (non-sense and missense mutations) that are distributed throughout all exons, and various deletions and insertions [1]. However, despite applying accurate and sensitive mutation detection methods, such as denaturing high performance liquid chromatography (DHPLC) [2] and denaturant gradient gel electrophoresis (DGGE) [3], and even after sequencing all exonic regions, no mutations can be detected in about 2% of the HA cases [4].

Analysis of cDNA (when available) by reverse transcription-polymerase chain reaction (RT-PCR) represents a powerful tool to search for causative splicing mutations or gene rearrangements. This strategy is generally applied to disease-related genes in patients who do not show mutations in the coding regions at the DNA level [5–7]. The same strategy was applied previously as first-line mutation screening method for both the FVIII and FIX genes [8–10]. In fact, it was only the detailed analysis of the FVIII cDNA by Naylor *et al.* [11] that revealed transcripts interrupted in intron 22 in about 50% of severe hemophilia cases. This observation led to the discovery of the major gene rearrangement in the FVIII gene: the intron

22 inversion [12,13]. A similar experimental strategy led to the discovery of the intron 1 inversion, that occurs at a frequency of 5% of HA cases [14,15].

In this study, we investigated lymphocyte mRNA from three selected groups of patients: (i) control patients with known splicing defects, (ii) patients with putative splice site mutations and (iii) patients with no mutation detectable at the DNA level.

The findings of the present study show that all mutations close to or within a splice site caused skipping or alternative splicing of at least one exon. In the patient group with no detectable mutation no abnormal splicing or rearrangements of the FVIII cDNA were found. Our results indicate that the cause for HA in these patients may indeed lie in other still-unknown regulatory region(s) of the FVIII gene or in non-allelic genes encoding interacting/modifying protein(s).

Materials and methods

Patients

We divided patients into three categories. As a control group we chose two patients with known intron 1 and intron 22 inversions (patients 21 and 30, respectively, Table 1), as well as patients with either a known splicing error that creates a new cryptic site (patient 31, Table 1) or with previously known or anticipated causative splicing mutations, because a 100% conserved base in the consensus sequence (according to Moore MJ [16]) was affected (patients 10, 14, 17, 45 and 48; Table 1). The second group includes two patients with nucleotide substitutions near or within splice sites with an as yet unknown effect on mRNA splicing (patients 4 and 32; Table 1). The third category (12 patients from 11 families) includes patients that had their FVIII genomic DNA screened for all coding regions by DGGE and/or DHPLC and sequencing with no mutation found [4].

Patient's bloods (2.5 mL) were collected in PAXgene^{TM} blood RNA tubes and total cellular RNA was extracted using the PAXgene Blood RNA kit according to the manufacturer's procedure (PreAnalytiX, Hombrechtikon, Switzerland). We achieved RNA yields of 1.6–9.5 μg. All extracted RNAs were stored at −70 °C until further analysis.

RT-PCR amplifications

For reverse transcription FVIII cDNA was divided into four different regions that cover all the splicing sites, exons 1–8 (region A), exons 8–14 (region B), exons 14–22 (region C) and exons 19–26 (region D) (Fig. 1; for primers see Table 2). We performed reverse transcription using the Omniscript Reverse transcriptase according to the manufacturer's protocol (Qiagen, Hilden, Germany).

We performed the RT-amplifications in two rounds of PCR using a nested approach. For the first PCR we used 5 μL (a total of 50 μL) from the reverse transcription. We divided each of the above four regions into two regions (a total of eight overlapping regions) that were amplified, by a nested PCR approach, using 5 μL (a total of 50 μL) from the first RT-PCR as a template. Primer sequences and the conditions for amplifications are given in Table 2. The PCR products were resolved on 3% NuSieve-agarose (2 : 1).

Table 1 A summary table of the patients included in this study with their DNA mutations (when known) and the results of the detailed RNA analysis. Data about clinical severity, putative mutation and inhibitor incidence are also shown

Patient	Severity	Inhibitors	Mutation	Ref.	RNA analysis
Control mutations					
21	Severe	U	Intron 1 Inv	[15]	No RT-PCR across intron 1
30	Severe	U	Intron 22 Inv	[12]	No RT PCR across intron 22
45, 48	Severe	No	IVS 5–3 C > G	[20]	Ex 6 or 5–6 spliced out/IF
10	Severe	No	IVS 6 + 3 A > G	[18]	Ex 5 or 5–6 spliced out/IF
31	Moderate	No	L504 L G > T	[19]	New 3′ splicing site/IF
14, 17	Severe	U	IVS 22 + 2 T > A	[4]	Ex 22 spliced out/IF
Probable splicing mutations					
4	Mod-Sev	No	IVS 22 + 5 G > T	[4]	Alternative splicing exon 22/IF
32	Severe	No	IVS 3 + 5 G > A	[4]	Ex 2 or 2–3 spliced out/OF
Previously unidentified mutations					
7, 8	Severe/F	No	Not Identified	[4]	Normal splicing
13	Severe/NF	No	Not Identified	[4]	Normal splicing
16	Severe/NF	No	Not Identified	[4]	Normal splicing
25	Severe/NF	No	Not Identified	[4]	Normal splicing
29	Mod-Mild/NF	No	Not Identified	[4]	Normal splicing
43	Severe/F	No	Not Identified	[4]	Normal splicing
44	Mild/NF	No	Not Identified	[4]	Normal splicing
47	Severe/F	U	Not Identified	[4]	Normal splicing
49	Severe/F	U	Not Identified	[4]	Normal splicing
51	Severe/F	No	Not Identified	[4]	Normal splicing
62	Mild/NF	U	Not Identified	[4]	Normal splicing

F: familial; NF: non-familial; IF: in-frame; OF: out-of-frame; U: unknown inhibitor status.

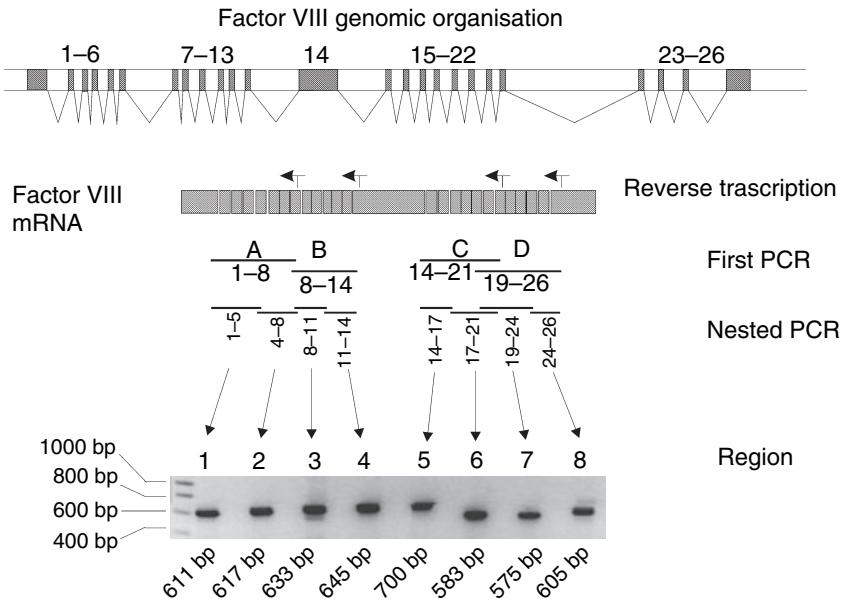

Fig. 1. Schematic diagram of the genomic organization of the factor VIII gene. A representative mRNA is also shown together with the position of the primers used for reverse transcription (horizontal arrows). Positions of the amplified regions in the first and nested PCRs are given below the mRNA with sample PCR products of all eight regions.

Table 2 List of all primers used in this study. *Primers were designed for this study while other primers are as used by Freson *et al.* 1998 [28]. Primers ending by 4 are used for the reverse transcription; primer pair ending with 1 and 4 is used for the first PCR and primer pairs 2-R and F-3 are used for the second nested PCRs

Region/subregion external/nested		Primers 5′–3′	Annealing °C	Product size (bp)
A	Ex1–8/1	CTT CTC CAG TTG AAC ATT TG	52	1254
	Ex1–8/4	TCA GCA GCA ATG TAA TGT AC		
1	Ex1–8/2	GCA AAT AGA GCT CTC CAC CT	58	611
	Ex1–8/R*	TGG CCA GAC TCC CTT CTC TA		
2	Ex1–8/F*	TTG AAT TCA GGC CTC ATT GG	58	617
	Ex1–8/3	GAG CGA ATT TGG ATA AAG GA		
B	Ex8–14/1	AAG TAG ACA GCT GTC CAG AG	52	1275
	Ex8–14/4	CTA GGG TGT CTT GAA TTC TG		
3	Ex8–14/2	AGA AGC GGA AGA CTA TGA TG	58	633
	Ex8–14/R*	GAG AGG GCC AAT GAG TCC TGA		
4	Ex8–14/F*	TGC CTG ACC CGC TAT TAC TC	58	645
	Ex8–14/3	AGA AGC TTC TTG GTT CAA TG		
C	Ex14–19/1	GGG AAA TAA CTC GTA CTA CT	52	1340
	Ex14–19/4	AAC TGA GAG ATG TAG AGG CT		
5	Ex14–19/2	AGT CAG ATC AAG AGG AAA TTG	58	700
	Ex14–19/R*	CCA GCT TTT GGT CTC ATC AA		
6	Ex14–19/F*	CCT GCT CAT GGG AGA CAA GT	58	583
	Ex14–19/3	GAT TGA TCC GGA ATA ATG AAG		
D	Ex19–26/1	TGA GAC AGT GGA AAT GTT AC	52	1285
	Ex19–26/4	TTG CCT AGT TAT ATT GGA AG		
7	Ex19–26/2	AGC ATA AGT GTC AGA CTC CC	58	575
	Ex19–26/R*	TCG AGC TTT TGA AGG AGA CC		
8	Ex19–26/F*	GCC ATT GGG AAT GGA GAG TA	58	605
	Ex19–26/3	AGT TAA TTC AGG AGG CTT CA		

Analysis of the PCR products

When an abnormal-sized product was detected on agarose gel, we sequenced the product using both the reverse and forward primers that were initially used in the nested PCR. When the product was too faint to be directly sequenced we cloned it into pGEM vector (Promega, Heidelberg, Germany) and sequenced the insert using standard M13 forward and reverse primers.

Calculation of 5′- and 3′ splicing scores

Scores for the 5′-and 3′ splicing sites were calculated using web-based software [http://www.fruitfly.org/seq_tools/splice.html] by selecting human as the organism [17]. Scores ranged from 0 to 1 (lowest to highest).

Results

In this study, we performed detailed RNA analysis of all splice sites of the FVIII cDNA in three groups of well-characterized patients (Table 1).

Group 1: control samples

Patients 21 and 30 These two patients have intron 1 and intron 22 inversions, respectively. From patient 21, as expected, we were not able to obtain an RT-PCR product from region A (across intron 1), while from patient 30 no RT-PCR product was obtainable from region D (across intron 22) (data not shown). All other regions in patient 21 (B, C and D) and in patient 30 (A, B and C) gave the expected RT-PCR products.

Patient 10 (IVS-6 + 3-A > G) The consensus 5′ splice site at position + 3 is A or G (a purine). Although the sequence change in this patient does not deviate from the consensus, the RT-PCR product clearly showed two bands of smaller size in comparison to the normal product (Fig. 2a). Sequencing of the products showed the skipping of either exon 6 alone or both exons 5 and 6. Skipping of at least exon 6 associated with the same mutation was also reported by Theophilus *et al.* [18]. Skipping of exon 5 alone or 5 and 6 together produce an in-frame protein lacking 23 aa or 62 aa, respectively.

Patients 14, 17 (IVS-22 + 2-T > A) The T > A exchange at position + 2 is decreasing the 5′ splicing score of intron 22 from 0.98 to an unpredicted splicing site. RT-PCR gave a single smaller band that is missing exon 22, as revealed by sequencing (Fig. 2b); this could explain the severity of the phenotype in these patients. Skipping of exon 22 produce an in-frame protein lacking 52 aa of the C2 domain.

Patient 31 (1569G > T; Leu504Leu, CTG > CTT) This mutation has been reported previously by Diamond *et al.* [1,19]. The G to T transversion is creating a new 3′ splice site that has a splicing score of 0.36 comparable to the original 3′ splice site (splicing score of 0.42). RT-PCR produced a smaller band (Fig. 2c) that lacked the first 36 bases of exon 11. Therefore, the remaining protein sequence remains in frame, resulting in a protein lacking the 12 amino acids encoded by the 5′ end of exon 11. This mutation produces a case of moderate hemophilia.

Patients 45 and 48 (IVS-5–3-C > G) In these two patients, a C > G mutation at the third position is altering a conserved base in the consensus 3′ splice site that should be either T or C (a pyrimidine). The splicing score for this site is altered from 0.99 to 0.38, clearly decreasing the 3′ splicing probability. The RT-PCR across exons 4–8 showed two bands of reduced sizes (Fig. 2d). Sequencing showed the skipping of either exon 6 alone or exons 5–6 together. The same results for the same mutation have been reported previously by Tavassoli *et al.* [20].

Group 2: patients with putative splicing mutations

Patient 4 (IVS-22 + 5-G > T) At position + 5 the 5′ splice site consensus is G. A transversion of G to T reduced splicing score from 0.98 to an unpredicted splice site. The RT-PCR product gave two bands: one of normal size and another one of reduced size (Fig. 2e). Sequencing showed that the normal size band corresponded to the wild-type product, while the smaller one was missing exon 22. In this patient, FVIII activity was about 4% with a moderate–severe phenotype. Because a considerable proportion of the mRNA is correctly spliced, this could explain the moderate hemophilia A phenotype. Although alternative splicing involving intron 22 has been previously reported [8], in this study the presence of two spliced products was not observed in any other controls or studied patients. Therefore, it is likely that this is a causative mutation.

Patients 32 (IVS 3 + 5 G > A) This mutation is altering a conserved base in the 5′ splicing site (position + 5 should be G). The splicing score is decreased from 0.99 to 0.42. RT-PCR produced two bands that are smaller than the normal product (Fig. 2f). Sequencing showed that the products are missing either exon 3 alone or exons 2 and 3 together; the complete absence of a normal transcript explains the observed severe phenotype in this patient. Skipping of exon 3 alone produce an in-frame protein missing 41 aa, while skipping of exons 2 and 3 together produce an out-of-frame protein lacking 82 aa.

Group 3: patients with previously undetected mutations

In this group we included study 12 patients that were previously screened for point mutations by either DHPLC or DGGE and by direct sequencing [4], without mutations being detected. Our RT results showed that all eight regions were amplified in all 12 samples. However, the success rate of obtaining a RT-PCR product was lower than in groups 1 and 2, as more samples had to be repeated for RNA extraction, the reverse transcription and the RT-PCR until a product was obtained. In some of these patients (numbers 13, 29 and 51) we observed alternative splicing involving exon 19 (Fig. 2g). The same observation has been reported previously by Maugard *et al.* [8].

To further analyze the reasons of alternative splicing in patient 29, we sequenced exons 18, 19 and 20 together with intron 18 and about 1 kb into the terminal part of intron 19. All sequences were identical to a normal control (data not shown). We further amplified by RT-PCR mRNA from controls samples in the same region (exon 17–21) to see if this alternative splicing also exists in the normal population. The same pattern observed in this patient was also present in about one-quarter of RT-PCRs from normal RNA samples extracted from peripheral lymphocytes (four normal control samples); however, this alternative splicing from the same RNA sample

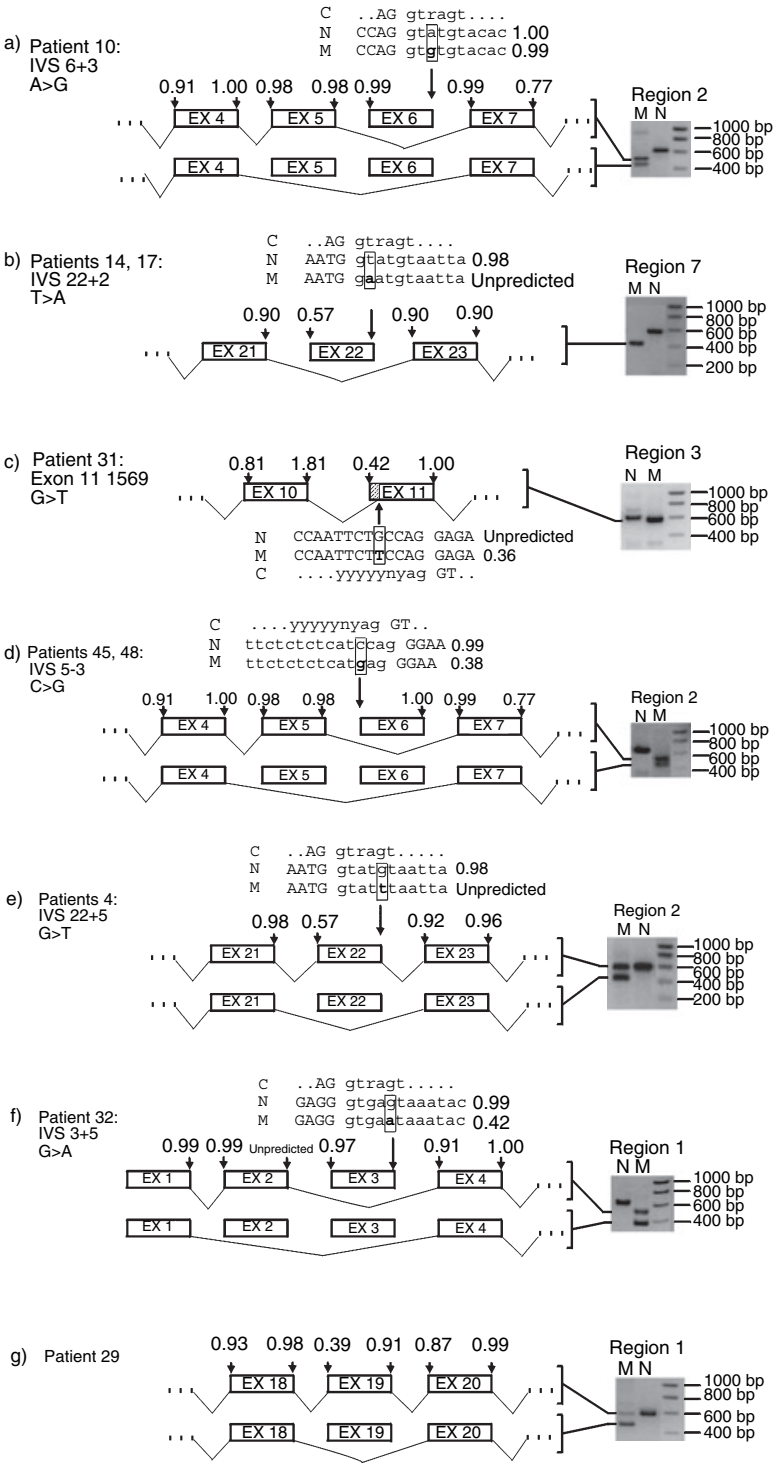

Fig. 2. Schematic diagram of the exons involved in the splicing error and the position of the mutation is shown. (a, b, c, d) Samples with known splicing errors; (e, f, g) samples with putative splicing errors. The splicing score is indicated above each splice site (N: normal or wild-type; M: mutant; C: consensus sequence splice site, according to Moore MJ [16] n: any base, y: t or c; r: a or g).

was not always reproducible from different RT-PCR when the same RNA was used as a template; this may indicate the low abundance of such variation (data not shown). Thus we cannot assume that this is a causative mutation leading to HA. This alternative splicing of exon 19 in ectopic RNA could be explained by the low scores of the 3′ splicing site of intron 18 (0.39) in comparison to intron 19 (0.87) (Fig. 2g). The mRNA product lacking exon 19 produces an in-frame protein lacking 39 aa from the A3 domain. However, although some alternative splicings were also observed in liver tissues from rats [21],

the existence and the extent of this alternative splicing in exon 19 in the human liver tissues is unknown.

We run the RT-PCR products on 3% NuSieve–agarose gels (2 : 1), which allows a size difference of about 30–50 base pairs (bp) to be distinguished. The presence of small insertions or deletions below the agarose detection limit was excluded by DHPLC analysis (data not shown). This left us with the conclusion that in these patients the FVIII cDNA has both normal sequence and normal splicing patterns. Furthermore, the RT-PCR products from patients 13, 16, 29, 47, 49, 51 and 62 were directly sequenced. All sequenced products showed a normally spliced cDNA.

Discussion

HA is caused by a variety of mutations mechanisms in the FVIII gene. One group of mutations occurs within or in the vicinity of splice sites. It is not always clear whether these base exchanges are causing exon(s) skipping or alternative splicing. Web-based software tools are available to calculate the splicing probabilities of 5′ and 3′ splices before and after nucleotide substitution at individual positions. However, to test these changes experimentally we have analyzed some potential splice site mutations by RT-PCR. We were able to show that all point mutations lying in or at the vicinity of a splice consensus sites caused real splicing errors in all patients studied (Fig. 2, Table 1). This highlights the usefulness of the mRNA analysis for routine diagnosis of such ambiguous cases, including the presence of potential cryptic splice sites, and clearly demonstrates the causality of the mutations, which is also important for the genetic counselling of those families.

However, in another minority group of patients (about 2%) no mutations can be detected in coding region of the FVIII gene [3]. We tested the possibility that some mutations may lie in introns and could create new cryptic sites adding new exons or affecting the normal splicing. The FVIII introns are relatively large (up to 32 Kb), which makes it impossible to include them in routine diagnostic sequencing. Therefore, to bypass this problem, we performed detailed FVIII cDNA analysis by RT-PCR. We could not find any abnormal mRNA splicing that could explain the FVIII deficiency or the hemophilic condition. We can think of three possibilities to explain this phenomenon. First the present mutation screening methods (including direct sequencing of PCR products) may not be sensitive enough to detect certain types of sequence changes. Despite considerable advances in sequencing chemistry the sensitivity of any sequence reaction is still dependent on the sequence context to some extent. A small detection failure rate may still be attributable to these technical limitations or to the analysis of the sequencing data. The second possibility (applied particularly to non-familial cases that do not necessarily show typical X-linked inheritance) is that mutation(s) in other proteins may affect proper folding, secretion, modification and/or trafficking of the FVIII protein or modulate the activity of the mature molecule in blood. Examples of such proteins are the ERGIC-53 (also known as LMAN1) and the recently identified MCFD2 protein, which are involved in transporting both FVIII and FV from the endoplasmic reticulum to the Golgi compartment and whose defects cause combined FVIII/FV deficiency [22,23]. Because FV activity in our patients is normal (data not shown) involvement of these two particular proteins could be excluded. However, other as yet unknown proteins could be involved in FVIII protein processing. For example, the secretion efficiency of the FVIII molecule depends on the 'correct' processing of the protein in the ER-Golgi apparatus; this includes (but not only) phosphorylation, sulfotation, glycosylation and proteolytic cleavage in the B domain at aa 1313 and 1648 by a still unknown protein. Recently, the importance of these post-translational modifications is being recognized through its involvement in many human diseases. For example, mutations in several genes involved in glycosylation can cause a group of congenital muscular dystrophies [24,25]. Because the B domain of the FVIII is a target for 19 N-glycosylation and three O-glycosylation sites and taking into consideration the proposed role of this domain in the intracellular trafficking of the FVIII molecule, the involvement of glycosylation defects in the hemophilic phenotype is not unlikely.

The third possibility to explain the hemophilia phenotype is a diminished expression/secretion level of the FVIII mRNA/mature protein that would lead to lower amount of active FVIII protein in circulation. Although we were able to obtain RT-PCR products from patients' lymphocytes, two points should be mentioned here: the first is that the success rate of obtaining a RT-PCR product was lower in group 3 patients compared to patients with known point mutations or to normal controls. This observation may point to a lower abundance of the FVIII transcript in these patients. Further studies, including a quantitative RT-PCR, are needed to clarify the reason for this at present empirical experience. The reason for such a lower mRNA expression could be defects in some regulatory elements such as enhancers or other epigenetics factors; however, in factor VIII little is known about such factors. The second point is that the ectopic mRNA from blood circulating cells (mainly lymphocytes) may not reflect the correct expression level in the main factor VIII-producing cells, which are mainly in the liver and the kidneys. In the liver, sinusoidal endothelial cells and Kupffer cells show relatively higher expression levels [26].

Most of the patients with no mutations detected that were included in this study share two important characteristics: a severe clinical phenotype (nine of 12) and no inhibitor development. The probability of inhibitor development was shown to depend on the type of mutation with a higher incidence associated with the absence of large parts of the FVIII protein due to non-sense mutations, gross deletions or intron 1 and intron 22 inversions [27]. Thus, the absence of inhibitor formation in these patients is in line with the hypothesis that the defect in these patients may be due to either inefficient secretion or expression of the FVIII protein.

Another issue of interest that might point to an allelic or non-allelic mechanism is the pattern of inheritance of the

hemophilia phenotype in the group 3 patients. In six of the 11 families, including five with severe hemophilia A, more than one hemophiliac was known (Table 2), thus favoring an X-linked inheritance. This assumption is strengthened by the fact that all 11 hemophiliacs were males. In five of the 11 families, two with non-severe and three with severe hemophilia A no history of hemophilia has been reported, leaving these families as candidates for a non-X-linked mode of inheritance. Consanguinity, which might point to an autosomal recessive condition, cannot be deduced from any of the family trees.

In conclusion, mRNA analysis has become a routine tool for verifying the causality of mutations with potential effect on mRNA splicing. Most interestingly, a highly selective cohort of patients showed no mutation within the FVIII gene. These rare and well-characterized patients should be gathered throughout genetic centres and subjected to further studies that might reveal novel allelic and non-allelic mechanisms leading to hemophilia A.

Author contributions

O.E-M. planned and supervised the experimental work, conducted part of the experimental work alone and made the major contribution to the writing of the manuscript. U.H. conducted most of the RNA analysis. J.G., H.-H. B., W.S. and R.S. were part of a national consortium, by which a major proportion of German hemophilia A patients were phenotyped and genotyped, thus identifying the very selected patient cohort addressed in the present study. J.S. and C.R.M. run a genetic laboratory and in part coordinated the communication between the clinics and the genetic laboratories. M.W. significantly supported the RNA analysis. A.T. contributed to the sequencing of the RT-PCR products. P.H. provided the infrastructure for the group and contributed to the writing of the manuscript. J.O. initiated, supervised and coordinated the project on the RNA analysis as well as the communication between all partners. He cowrote the manuscript.

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