F8 genetic analysis strategies when standard approaches fail

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Summary

Haemophilia A is a common X-linked recessive disorder caused by mutations in *F8* leading to deficiency or dysfunction of coagulant factor VIII (FVIII). Despite tremendous improvements in mutation screening methods, in a small group of patients with FVIII deficiency suffering from haemophilia A, no DNA change can be found. In these patients, analysis reveals no causal mutations even after sequencing the whole coding region of *F8* including the flanking splice sites, as well as the promoter and the 3' untranslated region (UTR). After excluding the mutations mimicking the haemophilia A phenotype in interacting partners of the FVIII protein affecting the half life and transport of the protein, mutations or rearrangements in non-coding regions of *F8* have to be considered responsible for the haemophilia A phenotype.

In this review, we present the experiences with molecular diagnosis of such cases and approaches to be applied for mutation negative patients.

Zusammenfassung

Hämophilie A, eine häufige, X-chromosomal rezessiv vererbte Erkrankung, verursacht einen Mangel oder eine Dysfunktion des Blutgerinnungsfaktors VIII (FVIII). Trotz großer Fortschritte in Mutationsscreeningverfahren kann bei einer kleinen Gruppe von Patienten, die unter einem Hämophilie-A-ähnlichen Phänotyp leiden, keine genetische Veränderung im *F8* nachgewiesen werden. Nach Sequenzierung der gesamten kodierenden *F8*-Region einschließlich flankierender Spleißstellen, Promotorbereich und 3'-UTR Region kann in diesen Fällen keine ursächliche Mutation nachgewiesen werden. Nach Ausschluss von Mutationen in Genen der Interaktionspartner des FVIII-Proteins, die Halbwertszeit und Transport beeinflussen, sollte nach der Ursache für den Hämophilie-A-ähnlichen Phänotyp in nicht-kodierenden *F8*-Bereichen untersucht werden.

In dieser Übersicht fassen wir Erfahrungen in der Diagnostik solcher Fälle zusammen und stellen neben molekulargenetischen Mechanismen, die zur Ausprägung eines solchen Phänotyps führen, eine gestaffelte genetische Untersuchung im Rahmen der Diagnostik vor.

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Haemophilia A is an X-linked bleeding disorder (OMIM 306700) with an occurrence of one in 5000 men (1). Typically, affected patients experience prolonged bleeding caused by lack or reduced residual activity of the coagulant factor VIII (FVIII:C). The severity of the disease is defined based on the amount of the residual FVIII:C level.

> Haemophilia A is classified as
> - severe (FVIII:C<1 IU/dl),
> - moderate (1 IU/dl <FVIII:C<5 IU/dl), and
> - mild (5 IU/dl<FVIII:C<40 IU/dl)
> representing about 40%, 10%, and 50% of patients, respectively (2).

While most of the patients are referred to haemophilia centers based on a family history of haemophilia A, about one third of cases are sporadic. Haemophilia A in women is a rare observation (3–5). The female carriers are usually asymptomatic with bleeding events occurring only in about 10% of cases (6). The disease in carriers is mostly manifested when the carrier status is combined with skewed X chromosome inactivation (7, 8).

During the 1980s and early 1990s, mutation analysis of *F8* primarily focused on carrier detection (9, 10), while in the 1990s, genotype-phenotype associations became the driving force of *F8* analysis (11–14). Also, the impact of genetic factors on the risk of inhibitor development became more evident (15–17). To date, a wide range of mutations, scattered through the coding sequence of *F8*, have been reported that lead to either a qualitative or a quantitative defect of the FVIII protein.

The large and complex *F8* gene is located at the distal part of the long arm of the X chromosome (Xq28). It
- spans 186 kb of genomic DNA and

diagnosis	assays and tests for haemophilia A	
standard	FVIII activity assays	• FVIII:C$_{1st}$/FVIII:C$_{Chr}$
	genetic analysis	• inversion PCR (introns 1 and 22) • *F8* sequencing • MLPA
extended	VWF analysis	• VWF:FVIII binding assay • genetic analysis of *VWF* • multimer analysis
	combined FVIII/FV deficiency	• FV activity measurement • genetic analysis of *LMAN1* and *MCFD2*
	analysis of *F8* mRNA splicing	• in silico analysis • RT-PCR
	rearrangements and CNV	• FISH • CGH
	analysis of whole *F8* locus	• long-range PCR • NGS

Tab. 1
Diagnosis of haemophilia A: algorithm of tests

- consists of 26 exons ranging from
  - 69 bp (exon 5) to
  - 3106 bp (exon 14).
- The *F8* coding sequence comprises only about 0.5% of the whole gene and encodes an mRNA of 9.1 kb.
- More than 2000 causal mutations in *F8* are described in the haemophilia A mutation database (HAMSTeRS) (http://hadb.org.uk).

The molecular basis underlying haemophilia A is well characterized. In about half of severely affected patients, large genomic inversions are responsible for the disease. These inversions are the result of homologous recombination events between two copies of repeated DNA sequences in intron 1 (18) and intron 22 (19, 20) and their respective inverted homologous copies outside *F8*, and they have been reported in 1% (intron 1 inversion) and 40% (intron 22 inversion) of severe haemophilia A cases, respectively. One in 30 000 male infants are born with an inversion in *F8* and a residual FVIII:C level below 1 IU/dl.

In the remaining cases, various types of point mutations (missense, nonsense and splice site mutations), small and large deletions, insertions, and duplications have been reported scattered throughout the *F8* coding sequence, its flanking intronic regions and the 5´and 3´ UTRs (21–23). However, in a small group of patients suffering from the „haemophilia A-like" phenotype, no detectable mutations in *F8* can be found. To elucidate the molecular mechanism(s) leading to FVIII deficiency in these cases an extended molecular diagnostic procedure is needed.

Diagnosis and mutation detection strategies

Generally, clinical diagnosis of haemophilia A is based on the FVIII:C level. Based on the residual FVIII:C, the severity of the disease is determined. This is critical for the choice of genetic analysis. The evaluation of the disease severity must be performed with caution; apart from preanalytical considerations (e.g. correct storage and amount of citrate anticoagulant, storage time) (24), the discrepancy between the results of one stage (FVIII:C$_{1st}$) and chromogenic (FVIII:C$_{chr}$) assays have to be considered (25, 26). Thus, initial diagnosis of non-severe haemophilia A phenotypes should be based on results from both assays.

Most of the mutations in *F8* (in exons and their flanking intron boundaries) can be identified using routine PCR-based methods with a mutation detection rate of up to 97% (23). The current mutation screening strategy applied for patients with a bleeding tendency and reduced FVIII:C is shown (▶Tab. 1).

1. In the first step intron 22 (19, 20) and intron 1 (18) are screened for inversion mutations. This will identify the causal mutation in approximately 50% of severe haemophilia A patients.
2. In cases where no inversion is detected, the second step is the complete mutation analysis of *F8* (exons and adjacent flanking intronic regions) to detect all mutations located in the exons of *F8* as well as in their adjacent intronic regions.
3. The third step is the search for duplications and deletions using Multiplex Ligation-dependent Probe Amplification (MLPA). Duplications have been reported with an incidence of about 0.07% and can lead to mild-severe haemophilia A depending on their effect on the reading frame (27, 28).

Patients with FVIII deficiency and without detectable causal mutation after these three steps are designated as „mutation negative" (29, 30).

Mutation negative patients

To elucidate the molecular mechanism behind FVIII deficiency in mutation negative patients further investigations beyond the above three steps are needed.

4. The forth step is to exclude misdiagnosis of haemophilia. Absence of mutations in genes that are known to be associated with FVIII deficiency provides further confirmation of the haemophilia A phenotype (▶Tab. 1).
5. In a fifth step, the integrity of the F8 locus has to be analyzed, as these regions might comprise complex rearrangements and/or DNA changes difficult to identify or escaping the current mutation screening protocols.
6. This last step is to clarify whether changes in intronic regions of *F8* possibly affect RNA splicing and transcription of mRNA.

As the last three steps are not routinely applied in genetic laboratories, the flowchart (▶Tab. 1) may simplify the choice for extended analysis to be applied in such cases.

Investigating the effect of known determinants of FVIII:C (step 4)

Causative mutations leading to haemophilia A are located within *F8*. However, mutations in interaction partners of the FVIII protein can lead to FVIII deficiency and mimic a „haemophilia A like"-phenotype. This includes mutations in

- lectin mannose-binding protein (*LMAN1*) (31) and
- multiple coagulation factor deficiency 2 protein (*MCFD2*) (32) as well as
- von Willebrand factor (*VWF)* (33–35).

Mutations in these genes could affect the secretion as well as the half-life of the FVIII protein (31, 36, 37). Genetic analysis of these genes avoids misdiagnosis of haemophilia A in patients suffering from combined FV/FVIII deficiency (38) and von Willebrand disease type 2 Normandy (VWD 2N).

> To exclude combined FV/FVIII deficiency, determination of FV level is essential.

Combined FV/FVIII deficiency does not cause more bleeding than single deficiency of FV or FVIII, and patients suffer from a mild phenotype (31, 32, 36, 38). Generally, genetic analysis of *LMAN1* and *MCFD2* is applied when FV as well as FVIII levels are reduced. However, in patients with FVIII deficiency where no causal mutation in *F8* is found, irrespective of normal FV activity levels, genetic analysis of *LMAN1* and *MCFD2* is still an appropriate target of investigation. Although no such cases have been described to date, the analysis could lead to the identification of novel mutations in *LMAN1* and/or *MCFD2* that may be specifically involved in the secretion of FVIII and not FV.

The next possible reason for FVIII deficiency is VWD type 2N caused by mutations in *VWF*. Such mutations result in normal VWF activity levels and a normal multimeric pattern, but reduced FVIII:C levels. VWF, a multimeric glycoprotein, is non-covalently bound to FVIII in plasma.

> Reduced binding of VWF to FVIII leads to FVIII deficiency caused by VWD 2N.

The disease is inherited as an autosomal recessive trait and is clinically similar to mild haemophilia A. The FVIII binding assay (37, 39) is the appropriate test for definitive diagnosis of VWD 2N in combination with sequencing the exons comprising the FVIII binding domains of VWF. For VWD 2N the minimum sequencing requirement should cover exons 18 to 20 However, isolated reports of mutations associated with a VWF:FVIII binding defect in exons 17 and 24–27 have been published (39).

Another approach in mutation negative patients, in addition to VWF:FVIII binding assay and genetic analysis of the FVIII binding domain of VWF, is the complete *VWF* mutation analysis. It might lead to the identification of new mutations causing weaker interaction of VWF and FVIII localized in other regions than the typical VWD 2N region of *VWF*.

Furthermore, several intra- and extragenic polymorphisms have been reported to modulate FVIII:C.

- A single polymorphism in exon 14 of *F8* (rs1800291) has been reported to be associated with FVIII:C levels (40).
- Moreover, there is strong support for the association of AB0 blood type and FVIII:C (41, 42).
- Several genetic associations with FVIII:C and VWF:Ag levels have been reported using genome-wide association studies (GWAS) (43, 44).

However, the described SNPs influence FVIII:C levels to a lesser degree which would not result in an haemophilia A like phenotype. Even a combined occurrence of these SNPs is shown not to be associated with FVIII:C deficiency (45, 46).

Integrity of the *F8* locus (step 5)

A wide spectrum of mutation mechanisms has been reported in patients classified as mutation negative patients. This includes both complex genomic rearrangements (47–49) as well as deep intronic mutations (46, 50). In this section, we describe possible gross new rearrangements of *F8* and the ways to detect them.

Copy number variations (CNVs) and gross chromosomal rearrangements

Xq28 is prone, due to its high repetitive content (47, 51, 52), to frequent recombination events (45, 49, 50) (▶ Tab. 1). Fluorescence in situ hybridization (*FISH*) is a widely used molecular cytogenetic technique in the diagnosis of diseases to locate a specific DNA sequence on the chromosomes. This approach can also be applied in haemophilia A diagnostics using a *F8* specific probe. FISH analysis could exclude translocation and gross intra-chromosomal rearrangement of *F8*. However, only cases of haemophilia B in female patients which are due to translocation of *F9* (53–55) have been described to date. Moreover, *FISH* analysis involves laborious cell culturing to generate metaphase chromosomes and can only detect rearrangements that involve >3 Mb of DNA (56).

Comparative genomic hybridization (CGH) (57) is a cost-effective alternative to the traditional cytogenetic methodology. CGH allows genome wide detection of duplication and deletions and helps to understand the role of genomic imbalance to a resolution of as low as 1kb (47, 57–59). It is a fast and robust method of genetic testing and has been applied for identification of deletions and duplications associated with haemophilia A (47, 52, 60). However, balanced chromosomal translocations and inversions with an occurrence of approximately 0.08% (61) are not detectable with the CGH array.

For diagnostic purposes, the described MLPA, due to its simplicity, is a good alternative for detection of CNVs within *F8*. In routine genetic analysis of haemophilia A, MLPA is mostly applied in female patients for detection of deletions and duplications and in male patients for identification of duplications (3, 27, 62).

Rearrangement involving F8 introns

The two recurrent mutation hotspots in intron 1 (18) and intron 22 (20) of *F8* are prevalent examples of complex rearrangements in *F8*. Recently, a third inversion due to homologous recombination of inverted repeats within intron 1 of *F8* was described in a single severe haemophilia A case (52). The three inversions occur due to the presence of homologous sequences in an in-

Tab. 2 Deep intronic mutations causing mild haemophilia A in mutation-negative patients

mutation in *F8*	intron position	experimental consequence: inclusion of	effect	reference	patients (n)
c.143+1567A>G	1	191 bp	pre mature stop codon	(73)	2
c.1537+325A>G	10	226 bp		(71)	1
c.2113+1152delA	13	112 bp		(50)	1
c.5587−93C>T	16	56 bp		(46, 50)	1
c.5998+530C>T	18	105 bp		(46)	1
c.5998+941C>T		126 bp		(46)	1
c.5999−227G>A		35 bp / 55 bp	in-frame protein with extra 30 aa	(50)	3

aa: amino acids

verted orientation and/or are due to the high homology of the segmental duplications in Xq28. The high frequency of micro/macro homologies and repetitive sequences predispose to pathological genomic rearrangements. Such complex rearrangements evade standard PCR screening strategies. These above describe inversions that disrupt on the introns of *F8* may not be the only ones and thus a universal strategy to detect novel breakpoints must be applied in patients with as yet no detectable mutations in the *F8*.

A convenient method to search for the intronic breakpoints in the *F8* locus is the amplification of whole genomic region of *F8* using a long range PCR (LR-PCR) approach (28, 52) (▶Tab. 1). The complete *F8* locus (approx. 186 kb) can be amplified in 28 overlapping LR-PCR fragments (52). This approach can identify possible breakpoints, abnormalities and rearrangements in patients with a severe haemophilia A phenotype (28, 52). In patients where a breakpoint in *F8* locus based on the absence of a given LR-PCR fragment is detected, the junctions of the breakpoint can be characterized using an inverse PCR approach.

Inverse PCR is the principle of the diagnostic PCR applied in most genetic laboratories for the detection of intron 22 inversion (63). Recently, a new generation of inverse PCR-based diagnostic tests for the identification of intron 22 and intron 1 inversions was developed by Rossetti *et al.* (64). Using an inverse PCR approach, the unknown sequences flanking the known sequences can be amplified and the junctions of the breakpoints can be characterized. Inverse PCR is principally a multistep protocol whereby the genomic DNA is first digested using an appropriate restriction enzyme, followed by a self-ligation step of the digested products. Next, PCR amplification is performed using primers located in the vicinity of the unknown sequences. Sequencing the inverse PCR fragments could lead to identification of the junctions of the breakpoints and subsequently the involved sequences and their genomic locations.

Disease associated intronic mutations leading to haemophilia A (step 6)

Intronic mutations might activate a cryptic donor or acceptor splice site and splicing between this novel splice site and a pre-existing splice site results in the inclusion of a cryptic exon. The evidence of deeper intronic sequence variations affecting the splicing process is now becoming more obvious (65–67). The importance of screening for splicing errors has also been underlined in haemophilia A (50, 68, 69).

The most direct approach to identify DNA changes that might affect RNA splicing is qualitative reverse transcription-PCR (RT-PCR) (▶Tab. 1). The effect of several intronic variants in *F8* located close to or within the splice site consensus sequences has been analyzed by several groups (69, 70). These groups have shown that not only changes close to or within the consensus splice sites but also single nucleotide substitutions in deep intronic regions of *F8* may lead to alternative splicing. Such aberrant mRNAs mostly lead to haemophilia A due to the generation of premature stop codons (▶Tab. 2) (46, 50, 71). Such transcripts would lead to a production of a truncated protein or coupled surveillance degradation of the mRNA (72).

The evidence of the pathogenic impact of such deep intronic mutations in haemophilia A was first reported in 1999 when Bagnall et al. described a mutation in intron 1 of *F8* (c.143+1567A>G) that activates a cryptic exon and generates a premature stop codon in *F8* mRNA (73). This mutation was found in two patients suffering from severe and moderate haemophilia A phenotype, with FVIII:C of 1 IU/dl and 2 IU/dl, respectively. Other deep intronic mutations also lead to aberrant mRNA splicing. However, RT-PCR analysis for this mutation shows the availability of the wild type *F8* transcript along with the aberrant transcript, which leads to a milder haemophilia A phenotype (▶Tab. 2).

Sequencing the whole *F8* locus is a possible approach for further investigations in patients where no alternative splicing of mRNA is observed. For this purpose, a PCR fragment can be obtained by LR-PCR approach and sequenced using a next generation sequencing (NGS) platform (▶Tab. 1). The advantage of NGS is that the whole *F8* locus is screened for variations in one single step. This approach might identify intronic variants which may fail to show an effect on RNA splicing due to several limitations of the currently used RT-PCR approach (46) (e. g. the SNP might overlap with the primers used for the reverse transcription or the splicing effect would evade the nested RT-PCR).

However, to identify patient specific variants, all intronic variations need to be filtered using the SNP database (dbSNP) and the 1000 genomes project (▶www.ncbi.nlm.nih.gov/SNP/ ▶www.1000genomes.org/). Furthermore, a panel of healthy individuals has to be screened to exclude rare polymorphisms related to ethnicity of the patient cohort. All variants that pass these exclusion criteria and are recurrent in this group of patients may be considered as potential intronic mutations. However, the causality of such mutations

needs to be proven by functional assays (e.g. luciferase assay).

While the RT-PCR approach is the initial method to screen for abnormal splicing, the NGS approach has the advantage of screening all introns in one step thus localizing all potential changes to be investigated in detail using specific RT-PCR approaches.

Patients without apparent causative mutation

And yet, in some patients, a causal mutation explaining the haemophilia A phenotype cannot be detected. In this group, the reason for FVIII deficiency remains elusive. Several possibilities must be investigated to identify the molecular mechanisms behind the disease in these cases.

� The first one is tissue mosaicism.

A point mutation in *F8* could be present only in the liver among other tissues but not in blood. Although mutations causing haemophilia A usually appear to have arisen in germ cells, a de novo mutation may also occur during early embryogenesis and thus may represent either germline and/or somatic mosaicism (74–78). However, this is only true in sporadic cases, but not for patients with a family history of haemophilia A.

� The second possibility is rapid degradation of FVIII protein or its rapid uptake in blood.

Previous studies have shown that LDL receptor-related protein (LRP-1) and other members of the LDL receptor family affect the clearance of FVIII (79). Moreover, C-type lectin domain family 4 member M (CLEC4M), a calcium-dependent mannose-specific receptor, was recently shown to influence clearance of VWF (80). CLEC4M is expressed in the main site of FVIII production, the liver sinusoidal endothelial cells, and due to the important role of VWF in protection of FVIII from degradation, such mutations or polymorphisms may indirectly contribute to variability in FVIII:C. While it is possible that mutations at other loci play a role in intra-

cellular trafficking and/or secretion of FVIII molecule, it remains unclear whether such mutations lead to FVIII deficiency.

� Another possibility is a putative regulatory region, the so-called locus control region (LCR), located within or upstream of *F8*.

Recent studies on several loci, i.e. β-globin, have offered new insights in support of long distance interaction between enhancers and promoters from 6 to 25 kb upstream of the gene (81). Such LCRs are defined functionally due to their ability to influence the expression of linked genes. The fact that the defect leading to haemophilia A like phenotype in most cases indicates a X-chromosomal inheritance pattern makes the role of such LCRs in regulation of *F8* expression more convincing.

In order to elucidate the exact molecular mechanism behind haemophilia A phenotype in the remaining patients, more genetic and cellular approaches need to be applied. For example,

- a genome wide association study may be carried out if several affected members from the same family are available,
- a cellular approach by establishing a patient specific cellular system.

The improvements in modeling the diseases by induced pluripotent stem cells could facilitate a cellular system to understand and investigate the molecular basis of haemophilia.

Conclusion

Despite remarkable improvements in mutation detection technologies, in a small group of patients with reduced FVIII:C, the cause of haemophilia cannot be found in the exons of *F8*.

� In such cases, it is necessary to look beyond the coding sequences, exon/intron boundary, and promoter region.

Here, a systematic screening protocol is needed to identify the molecular cause of the disease.

In this review, we summarize our experience in analyzing such cases and we

describe a six steps protocol to identify causal mutations. We present several techniques for successful identification and confirmation of novel mutations. These techniques may be applied not only in haemophilia A patients, but also in the genetic analysis of other monogenic diseases, where no pathogenic mutation is found in the coding sequences.

Conflict of interest

The authors declare that they have no conflict of interest.

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