The problem of novel FVIII missense mutations for haemophilia A

genetic counseling

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**Summary**
Molecular genetic testing for factor VIII (FVIII) mutations is indicated in haemophilia A since determination of FVIII activity cannot reliably identify female carriers. Given the large number of FVIII mutations, the identification of novel mutations is not uncommon. Since amino acid polymorphisms of FVIII are rare, missense mutations in patients with haemophilia A which are not found in the normal population are considered as causative in general practice when no other mutation can be detected by complete FVIII gene sequencing.

We report a novel rare missense variant (P2311S) in a haemophilia A family that was mistakenly considered as pathogenic leading to amniocentesis, prenatal diagnosis and influenced the peripartal management of the putatively affected child. Subsequently, we identified the novel causative mutation V197G in the family’s index case which could be detected neither in the neonate nor in his mother. **Conclusion:** This case emphasizes the necessity to establish the molecular diagnosis in the family’s index case and to perform expression studies of novel mutations to prove their causative nature.

**Keywords**
Factor VIII, haemophilia A, molecular genetics, recombinant FVIII

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**Das Problem neuer Faktor-VIII-Missense-Mutationen für die genetische Beratung der Hämophilie A**

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**Schlüsselwörter**
Faktor VIII, Hämophilie A, Molekulargenetik, rekombinanter FVIII

**Zusammenfassung**

Genetic counseling and molecular testing for factor VIII (FVIII) mutations is offered to families with haemophilia A. Besides its value to identify carriers with haemophilia A, the molecular defect may also predict the risk of therapy related complications like the development of alloantibodies against infused FVIII (2). The large number of 943 unique mutations in the HAMSTeRS FVIII mutation database (4) illustrates considerable molecular heterogeneity. Therefore, the identification of novel mutations is common. Besides mutations which are considered as clearly pathogenic like deletions, insertions, inversions, nonsense mutations and splice site mutations,

583 missense mutations are listed in the HAMSTeRS database that may affect the expression, secretion, stability and/or the function of FVIII. However, many missense mutations have been identified without proven pathomechanism which could be studied, e.g. by expression and functional assays of recombinant mutant FVIII. Amino acid polymorphisms of FVIII are rare and only nine such polymorphisms are published in the HAMSTeRS database and in a recent publication (3), another three in NCBI dbSNP Build 129 (5). Therefore, FVIII missense mutations which are not found in the normal population are considered as causative in general practice when no other mutation can be detected by complete F8 gene sequencing.

We report on a novel missense mutation in a German family that was mistakenly considered as pathogenic, leading to amniocentesis, prenatal diagnosis and an impact on the peripartal management of the putatively affected child.
Family, material, methods

Family study

Phenotypic and molecular studies were part of our routine diagnostic work-up and were carried out according to our institutional guidelines and the amended version of the declaration of Helsinki (Tokio 2004) after having obtained informed consent from the proposita and the index patient.

The proposita is the first grade cousin of two brothers with moderately severe haemophilia A (Fig. 1). After becoming pregnant she sought molecular carrier testing which revealed a single candidate missense mutation CCT/TCT at codon 2311 in exon 26 (c.6931C>T) predicting the exchange of proline to serine (P2311S). Her affected cousins were at that time not available for molecular testing and their mutation was unknown. Assuming a pathogenic nature, hemizygosity for P2311S in cells from amniocentesis was seen as evidence for the male fetus being affected.

After cesarean section, however, the newborn’s FVIII:C was 126 IU/dl. Repeat molecular testing of the newborn’s leukocytes confirmed the mutation P2311S. Later, one of the index patients in this family agreed on genetic testing for the causative F8 mutation.

Material and methods

FVIII activity (FVIII:C) was determined by conventional one stage functional assays. DNA was extracted from peripheral blood leukocytes and amniotic fluid cells, respectively by standard protocols. Amplification of individual F8 exons by PCR and direct sequencing was performed according to the different protocols in two laboratories (J.S., F.O.). PCR sense and antisense primers were used also for sequencing. A list of primers and PCR conditions can be provided upon request.

Nucleotides are numbered starting with the A of the initiator codon ATG as +1 and amino acids (aa) numbering accordingly with the first methionine as aa 1. Accordance

Fig. 1
Pedigree of the family studied: Patients II-10, III-4, III-5 and III-8 had moderately severe haemophilia A, patient III-5 was studied to identify the causative mutation in this family subsequent to the unexpected results in the newborn IV-1.

Fig. 2
F8 expression vector for either full length (fl) or B-domain deleted (delB) FVIII expression
with the numbering of the mature FVIII protein and the HAMSTeRS mutation database can be obtained by subtracting 19 aa.

Expression studies were performed using the mammalian expression vector pIREShygroTO harboring either full length or B-domain deleted (delB) F8 cDNA cloned from a liver tumor library (Fig. 2). delB F8 cDNA was obtained by loop out mutagenesis of full length F8 cDNA. Site directed mutagenesis of full length and delB F8 cDNA was carried out by using the Quick Change kit (Stratagene, Kassel, Germany). Mutagenesis primers carrying the respective base exchange at a central position were 40 bp in length.

Transient expression of mutant (m) in comparison to wildtype (wt) FVIII cells was performed in three independent experiments after liposomal transfection (Lipofectamine 2000, Invitrogen) of HEK 293 EBNA cells (Invitrogen, Karlsruhe, Germany) with 4 μg mutant or wt F8 cDNA. The cells were grown for 72h (24h in DMEM (Invitrogen) with 10% (v/v) FBS and 48h in serumfree IMDM (Sigma) + 2% (v/v) Ultraser G (Invitrogen)). FVIII secreted in the medium was concentrated in Centricon tubes to one tenth of the original volume prior to subsequent analysis. FVIII:C was then measured as described above. FVIII:C of mutant FVIII is given in % of wt FVIII.

Results

Unexpectedly the male neonate carrying the mutation P2311S had normal FVIII:C of 126 U/dl and subsequently was no longer considered at an elevated risk of bleeding. After recruitment of an index case with haemophilia from this family (Fig. 1) we could identify the novel mutation V197G (c.590T>G) but not P2311S in this patient who had a FVIII:C of 6 U/dl.

To clarify a causal relationship of the two mutations with the haemophilic phenotype we carried out expression studies and could show that only V197G resulted in a significantly decreased FVIII:C of 7 ± 1% of wild type FVIII. Due to the very low expression level of mutant full length FVIII we could reliably analyze only the recombinant mutant B-domain deleted FVIII which gave a 10-fold higher yield.

In contrast, compared to wt FVIII, P2311S mutant FVIII correlated with non significantly lower functional levels of 87 ± 9 and 87 ± 4% FVIII:C of recombinant full length FVIII and delB FVIII, respectively (Fig. 3).

Discussion

The presence of P2311S in the neonate with a normal FVIII:C suggested that this mutation was only a non-pathogenic variant which was then proven by our expression studies of recombinant mutant full length and delB FVIII which resulted in 87 ± 9% and 87 ± 4% FVIII:C, respectively, compared to recombinant wild type (wt) FVIII. Although the difference was not significant it may well be that this mutation correlates with lowered FVIII:C to some extent, however, not sufficiently to cause a bleeder phenotype. The novel mutation V197G that seems to segregate with haemophilia in the family in contrast, clearly causes a significant reduction of secreted FVIII:C in our expression studies. Compared to recombinant wt FVIII the functional activity was determined as 7 ± 1%, a value which fits well to the FVIII:C level of 6 U/dl in the patient.

Carrier testing without an index case is problematic. If no index cases are available for molecular testing the significance of novel missense mutations in possible carriers can be considered, however, the significance must further be supported e. g.

- studying the conservation status of factor VIII in different species and homologous proteins,
- analyzing the possible molecular consequences of the mutations concerning their spatial position on the structural level, and
- their putative location in known factor VIII functional regions (1).

However, this will nevertheless be insufficient for decision making towards prenatal diagnosis and perinatal management.

Conclusion

Considering the potential consequences, in case of novel missense mutations, the pathogenic nature of the mutation should be proven by recombinant expression of mutant FVIII.

References

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