Molecular genetic background of haemophilia A patients with discrepancy between one-stage and two-stage factor VIII assays

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Human blood coagulation factor VIII (FVIII) is an essential protein for the blood coagulation. Its physiological relevance is evident from the severe bleeding disorder haemophilia A associated with FVIII deficiency or dysfunction. The accurate measurement of FVIII coagulant activity (FVIII:C) in plasma is important for

- diagnosis of haemophilia,
- defining the severity of the disease and
- follow up the effect of FVIII replacement therapy.

FVIII activity often correlates with the clinical expression of the disease. It can be measured by the

- one-stage (FVIII:C\textsubscript{1st}) or
- the classical two-stage (FVIII:C\textsubscript{2st}) clotting assay or
- the chromogenic method (FVIII:C\textsubscript{chr}).

The most commonly used method is the FVIII:C\textsubscript{1st} assay, which is based on the activated partial thromboplastin time (aPTT) (1). The FVIII:C\textsubscript{2st} assays consist of two reactions forming initially activated factor X (FX), which is either measured in the second stage by a clotting test (FVIII:C\textsubscript{2st} (2)) or by using a chromogenic substrate (FVIII:C\textsubscript{chr}) (3).

Results from all FVIII:C assays are usually equivalent for patients with severe, moderate or mild haemophilia. Several recent reports have observed discrepancies concerning the haemophilia A phenotype: In approximately one-third of patients with mild haemophilia A discrepancy between results from the two assays has been reported (4–9). A small subset of the mutations are characterised in patients with discrepant results.

The aim of our study was to characterise the mutation profile in patients with discrepancy.

Patients, material, methods

Haemophilia patients

284 patients previously diagnosed in Haemophilia Center Bonn as mild and moderate haemophiliacs were studied. Haemophilia was diagnosed on the basis of reduced FVIII:C (< 30 IU/dl) measured by one-stage and chromogenic methods, normal value of VWF antigen and VWF: ristocetin cofactor activity (VWF:RCo) was determined as previously described (10).

DNA isolation, F8 sequence

High molecular weight genomic DNA was isolated from whole blood by a salting out procedure (11) DNA concentrations were standardized to 100 ng/μL.

The entire coding region of the F8 gene was amplified in all patients and relatives and direct sequenced using primer and experimental conditions described earlier (12). DNA sequencing was performed on both strands, using the BigDye Terminator Cycle Sequencing V1.1 Ready Reaction kit and an automated ABI-3130 DNA sequencer (Applied Biosystems). Sequence Analysis software package (Applied Biosystems) was applied for final sequence reading and mutation documentation.

Results, discussion

284 patients with mild or moderate HA have been analyzed. In 101 patients
(35.5%) discrepant results for FVIII:C were found; 28 different mutations have been detected. Eleven of them showed higher FVIII:C<sub>1st</sub> results then the FVIII:C<sub>Chr</sub> giving rise to a ratio larger then 0.6. (Table 1). Nine mutations have been described for the first time. All mutations (except one, already described in the literature) are clustered in A1, A2, and A3 domains, in the regions at the interface between subunits (A1-A2, A1-A3, A2-A3). Additionally, most of the mutations are presented at the surface of the FVIII protein (Fig. 1). This render the FVIII less stable so that the activated FVIII is inactivated more quickly than normal. Seventeen F8 gene alterations displayed higher FVIII:C<sub>Chr</sub> assay compared to the FVIII:C<sub>1st</sub>. In these, mainly mild hemophilia A cases, the effect of the underlying mutation was only apparent in the FVIII:C<sub>1st</sub> clotting assay. The FVIII:C<sub>Chr</sub> assay yields normal activities. In contrast to mutation by which the FVIII:C<sub>1st</sub> is higher than FVIII:C<sub>Chr</sub>, here the mutations are scattered all over the whole gene. Some of them share the common feature of clustering in the vicinity of thrombin cleavage sites. These mutations produce impaired or delayed thrombin activation and their effect is masked by the usage of unphysiologically high concentrations of thrombin in the two stage assay. This suggests that abnormal FVIII molecules are changed in the regions which are important for function as assessed by FVIII:C<sub>Chr</sub> assay but that those regions react differently during assessment with FVIII:C<sub>1st</sub> method. The most common identified mutations (in 60% of patients) were presented by Tyr295Ala

### Table 1: Mutation spectrum in haemophilia A patients with discrepancy

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<thead>
<tr>
<th>FVIII : C domain</th>
<th>FVIII : Ag (U/dl)</th>
<th>FVIII : Ag (IU/dl)</th>
<th>FVIII : C&lt;sub&gt;1-stage&lt;/sub&gt; chromogenic</th>
<th>FVIII : C&lt;sub&gt;1-stage&lt;/sub&gt; chromogenic</th>
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<tr>
<td>FVIII : Ag</td>
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<td>FVIII : C&lt;sub&gt;1-stage&lt;/sub&gt;</td>
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<td>FVIII : C&lt;sub&gt;Chr&lt;/sub&gt;</td>
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**Mutation number of**  
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Considering our results (109/284) we assume that assay discrepancy is quite frequent finding in patients with mild/moderate haemophilia A. Still it is not known which assay better reflects the haemophilia severity with regard to clinical manifestation. Based on the existence of the discrepancy phenomena, results in measurements of FVIII activity can be misleading and fail to diagnose the haemophilia phenotype by certain patients. The strong association between the phenotype and group of mutations described in this study suggests that there is genetic basis to the discrepancy between FVIII:C₁st and FVIII:CChr assay.

**Conclusion**

Certain point mutations may escape detection if the residual FVIII:C is tested only by either the FVIII:C₁st or the FVIII:CChr assay. This can lead to

- misdiagnosis in some cases of mild haemophilia or
- problems of assigning the correct degree of severity of haemophilia.

**References**