Analysis of Intron 22 Inversions of the Factor VIII Gene in Severe Hemophilia A: Implications for Genetic Counseling


Intrachromosomal recombinations involving F8A, in intron 22 of the factor VIII gene, and one of two homologous regions 500 kb 5' of the factor VIII gene result in large inversions of DNA at the tip of the X chromosome. The gene is disrupted, causing severe hemophilia A. Two inversions are possible, distal and proximal, depending on which homologous region is involved in the recombination event. A simple Southern blotting technique was used to identify patients and carriers of these inversions. In a group of 85 severe hemophilia A patients, 47% had an inversion, of which 80% were of the distal type. There was no association with restriction fragment length polymorphism (RFLP) haplotypes. The technique has identified a definitive genetic marker in families previously uninformative on RFLP analysis and provided valuable information for genetic counselling. Information may now be provided for carriers without the need to study intervening family members and the diagnosis of severe hemophilia A made in families with only a nonspecific history of bleeding. Analysis of intron 22 inversion should now be the first-line test for carrier diagnosis and genetic counselling for severe hemophilia A and may be particularly useful when there is no affected male family member or when intervening family members are unavailable for testing.

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SEVERE HEMOPHILIA A is caused by a deficiency of coagulation factor VIII (FVIII) of less than 2 international units (IU)/dL. Genetic counseling of these severely affected patients and their relatives is an important part of the clinical management of the disorder. Information regarding the carrier status of female family members and antenatal diagnosis of male fetuses is often requested. This information can be provided in a proportion of patients by analysis of restriction fragment length polymorphisms (RFLPs) and variable number of tandem repeat sequences (VNTRs) associated with the FVIII gene. However, gene tracking techniques can usually only be applied if an affected male and intervening family members are available for analysis, and even then RFLPs are uninformative in approximately 25% of families.

It has been assumed that unrelated patients would carry independent mutations of the FVIII gene. The size of the FVIII gene (186 kb and 26 exons) means that directly identifying the mutation in each family, as can be performed for factor IX deficiency, is a large undertaking. One study that undertook this analysis was unable to find a mutation in the promoter, exons, or exon/intron boundaries in about 50% of severe hemophilia A patients. Recently, this finding has been explained by two groups who have independently described a mutation within the FVIII gene that accounts for about half of the cases of severe hemophilia A.

Intron 22 of the FVIII gene contains a second gene, FVIII-related gene A (F8A), that is transcribed in the opposite direction to FVIII. There are two further copies of F8A approximately 500 kb upstream (telomeric) of the FVIII gene both transcribed in the same direction as FVIII. This allows an intrachromosomal homologous recombination to occur, leading to an inversion of this region and separating FVIII exons 1-22 from exons 23-26. Because there are two upstream copies of the F8A gene, proximal and distal inversions are possible, with both leading to the severe hemophilia A phenotype (Fig 1).

Using a restriction enzyme that restricts DNA outside the F8A gene region and a probe within the region it is possible to show changes in fragment size that correspond to the distal and proximal inversions in severe hemophilia A patients and to directly show inversions in hemophilia A carriers. We have used this test to provide new and important information for families that are affected by hemophilia A even in cases in which there is no affected male family member available for analysis.

PATIENTS AND METHODS

Patients. Eighty-five patients with severe hemophilia A who attended The Royal Free Haemophilia Centre (London, UK) or were referred from other hospitals for gene tracking were studied. The mothers of 25 of these patients with no previous history of hemophilia were analyzed in parallel. In addition, 10 female patients (including one mother-daughter pair) with a family history of hemophilia, but with no index case available, were investigated.

Methods. Blood was taken into EDTA, DNA-separated by standard techniques, and stored at -20°C until analyzed. DNA (10 µg) was digested overnight at 50°C with Bcl I (Boehringer Mannheim, Mannheim, Germany) and electrophoresed on a 0.5% agarose gel (Pharmacia, Uppsala, Sweden) for 48 hours before being Southern blotted overnight using 20× SSC onto a nitrocellulose filter (Hybond N+; Amersham, Amersham, UK) and UV cross-linked. The filters were then prehybridized and hybridized using Church buffer at 65°C overnight with an EcorI/Sac I fragment of p482.6 labeled by random priming (Multiprime DNA labelling kit; Amersham) with 32P dCTP (Amersham, UK) and UV cross-linked. The following day, low and high stringency washes at 25°C and 65°C, respectively, were performed. Bands were visualized by autoradiography.

Gene tracking had previously been performed using intragenic (Bcl I, Bgl I, and Xba I) and extragenic (Bgl II/DX13) RFLPs.

RESULTS

As previously described, normal controls and male subjects without an inversion gave three bands corresponding to 21.5, 16, and 14 kb. Patients with the distal inversion

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Association with the presence of inhibitors. Data on the presence or absence of an inhibitor to FVIII were available on 36 patients, of which 17 (47%) had no inversion, 14 (39%) had a distal inversion, and 5 (14%) had a proximal inversion. Two of the 17 patients with no inversion (12%) had a positive history of an FVIII inhibitor. There was no history of FVIII inhibitors in patients with either distal or proximal inversions.

Carrier identification. It is now possible to directly identify carriers of hemophilia A if an inversion is present. Carriers of the distal inversion have bands of 21.5, 20, 17.5, 16, and 14 kb, whereas carriers of the proximal inversion have bands of 21.5, 20, 16, 15.5, and 14 kb (Fig 2).

Mother-patient pairs. In 25 families in whom there was no previous history of hemophilia the mothers of severely affected patients were studied. Inversions were detected in 12 patients (48%), of which 10 were distal inversions and 2 were proximal. All the mothers of patients with inversions were carriers of the predicted inversion.

There were three mothers who were phenotypically normal on measurements of FVIII and von Willebrand factor antigen (vWF:Ag). The FVIII/vWF:Ag ratios in these mothers were 1.17, 1.04, and 1.14. In each case, no inversion was detected.

CASE REPORTS

The following case reports illustrate the practical benefits for genetic counseling that accrue from the use of intron 22 inversion analysis.

Case 1. A woman presented requesting advice regarding a family history of bleeding. She had one sister who was phenotypically normal and two brothers who had died of hemorrhage, whereas a third brother had recurrent bleeding but was unavailable for testing (Fig 3). She did not know which coagulation factor was deficient in the family but the phenotype was clearly severe in affected male members of the family. Her FVIII level was 73 IU/dL, her vWF:Ag level was 104 IU/dL (ratio, 0.7), and her factor IX level was 80 IU/dL. These results suggested that she was a carrier for

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**Fig 1.** Diagram of a normal FVIII gene region and a distal inversion. A schematic diagram adapted from that published by Lakich et al representing the normal region of Xq28 and a distal inversion. The figures are not drawn to scale. The upper figure shows the FVIII gene with exons 1-22 and 23-26 marked. The shaded area represents intron 22 and contains the F8A gene (A) transcribed in the opposite direction to the FVIII gene. Two further copies of the F8A gene are located 500 kb telomeric to the FVIII gene. In the lower figure, a distal inversion is shown. The intron 22 F8A gene has recombined with the distal extragenic F8A gene separating exons 1-22 from exons 23-26 of the FVIII gene, thus preventing transcription of a complete FVIII mRNA sequence. In the proximal inversion, the intron 22 F8A gene recombines with the extragenic F8A gene closest to the FVIII gene.

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**Fig 2.** Representative autoradiographs showing the normal pattern with bands of 21.5, 16, and 14 kb (lanes 3 and 12); distal inversions with bands of 20, 17.5, and 14 kb (lanes 4, 7, and 9); carriers of the distal inversion with bands of 21.5, 20, 17.5, 16, and 14 kb (lanes 2, 5, and 8); a proximal inversion with bands of 20, 16, and 15.5 kb (lane 14); and a carrier of the proximal inversion with bands of 21.5, 20, 16, 15.5, and 14 kb (lane 13). Lane 15 shows a carrier of the proximal inversion taken from a different gel. Lane 1 shows a Bgl II/ A DNA digest.

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**Fig 3.** Case 1. Family tree of a woman (arrowed) with a family history of severe bleeding. No other family members were available for testing, but analysis showed that she was a carrier of the distal inversion. (□) Unaffected male family members; (■) affected male family members; (○) female family members. The line through either a circle or square indicates that the individual has died.
hemophilia A but were not definitive. Gene tracking analysis was performed but, because no affected male family members were available, no useful information was generated. Investigation for the intron 22 inversion showed that she was a carrier for the distal inversion.

This finding means that the family was affected by severe hemophilia A and that the consultand is a carrier of the mutation. She had a normal son and two daughters who could now be offered a test to establish whether they were carriers. Similarly, the carrier status of her sister can be established and all female family members can now be analyzed for carriership and be offered antenatal diagnosis by chorion villus sampling, if appropriate.

Case 2. Figure 4 shows a family affected by severe hemophilia A. The aunts (I1 and I3) of an affected male (II10) requested carrier status analysis and potential antenatal diagnosis. Consultand I2 had an FVIII level of 83 IU/dL and a vWF:Ag level of 84 IU/dL. There were no phenotypic data available on I1. Gene tracking using intragenic and extragenic probes was uninformative.

The affected male family member II10 was shown to have a distal intron 22 inversion. The carrier status of the aunts could now be definitively determined. I2 was found to have a normal pattern and was therefore not a carrier, but I1 was found to be a carrier of the distal inversion and can now be offered antenatal diagnosis.

Case 3. Figure 5 shows the family tree of a woman II, who was pregnant for 6 weeks and requesting antenatal diagnosis. She had an FVIII level of 54 IU/dL and a vWF:Ag level of 130 IU/dL, suggesting that she was a carrier. Samples were available from an affected uncle (FVIII level of 0 IU/dL) and her mother. Gene tracking studies were uninformative and a chorion villus sample was therefore not taken. Fortunately, cord blood sampling performed at 18 weeks was uneventful and showed an XX karyotype. A normal female was delivered. Retrospective analysis of stored DNA showed a distal inversion in her uncle and that her mother was a carrier of the distal inversion.

The presenting woman can now be offered confirmation of her carrier status and antenatal diagnosis in future pregnancies. There are also a number of other female family members at risk of carrying the inversion who could be offered counselling and genetic analysis.

Case 4. Samples from two sisters of a severely affected hemophilia A patient were referred for carrier detection and potential antenatal diagnosis (Fig 6). The use of intragenic RFLPs on the family was uninformative, although analysis with Bgl II/DX13 was informative. However, because this is an extragenic probe with a 5% chance of crossover, it is not ideal for use in antenatal diagnosis.

The affected male family member was shown to have a distal inversion and the mother (I1) to be a carrier of the distal inversion.

The two sisters were tested and both were shown to have normal patterns. They can, therefore, now be confidently advised that they are not carriers of hemophilia A.

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**Fig 4.** Case 2. Family tree of a family affected by severe hemophilia A. I1 and I3 requested to know their carrier status, but the family was uninformative on RFLP analysis. I2 was shown to have the distal inversion and analysis of I2 showed that she was a carrier of this inversion, whereas I1 was found to be normal. (◇) Carrier female family member; otherwise, symbols are the same as in Fig 3.

**Fig 5.** Case 3. II, was pregnant for 6 weeks when she made a request to know her carrier status and for antenatal diagnosis. The family were uninformative on RFLP testing. Retrospective analysis now shows that I2 has the distal inversion and that I1 is a carrier of this inversion. II, can now be tested for carrier status and offered antenatal diagnosis in future pregnancies. (◇) Fetus; otherwise, symbols are the same as in Fig 3.

**Fig 6.** Case 4. Carriership of II, and II, in a family with severe hemophilia A could only be excluded using extragenic probes. II, was shown to have a distal inversion and analysis of his two sisters confirmed that neither were carriers. Symbols are the same as in Figs 3 and 4.
family by gene tracking was uninformative for I. Carrier status in 111 could be excluded with philia A and that I2 and 113 are carriers. Investigation of the data strongly suggest that l2 and 111 are carriers of hemophilia in Fig

Case 5. Figure 7 shows a family tree in which patient II1 had a fatal hemorrhage at the age of 4 days. No coagulation factor levels were performed and there was no other family history of bleeding. FVIII and vWF:Ag levels shown in Fig 7 strongly suggest that the family suffers from hemophilia A and that I2 and I11 are carriers. Investigation of the family by gene tracking was uninformative for Bcl I and Bgl I. Carrier status in III1 could be excluded with Xba I RFLP despite the low FVIII:vWF:Ag ratio.

Analysis for intron 22 inversions show that both I2 and II1 were carriers of the proximal inversion. Analysis of III1 confirmed that she was not a carrier.

A diagnosis of severe hemophilia A could now be confirmed in the family and antenatal diagnosis offered.

DISCUSSION

The demonstration that the genetic defect in half the patients with severe hemophilia A is caused by intrachromosomal inversions involving intron 2267 of the FVIII gene provides the potential for a powerful clinical assay. In a large group of severe hemophilia A patients, we have found that 47% have an inversion, of which the majority (80%) are caused by the distal inversion. These results confirm the incidences reported previously in smaller groups of patients. Lakich et al26 found an incidence of 47% in 19 patients and Naylor et al26 found an incidence of 40% in 23 patients. The screening test described is easy to perform, cheap, and yields relatively quick results. However, the most important feature of this method is that positive results are unequivocal and provide a definitive diagnosis.

We have found no association with RFLP haplotypes or with the presence of FVIII inhibitors, although relatively few patients in this study group had this complication. The carrier status of female relatives of hemophiliacs affected by an inversion can easily be identified and the validity of these results has been confirmed by studying the mother/patient pairs in families with no previous history of hemophilia A. Interestingly, in the three mothers who were phenotypically normal, no inversion was seen. Further study of these patients may be of interest.

Families with severe hemophilia A who were previously uninformative on RFLP and VNTR analysis can now be provided with useful information in about 50% of cases. In the future, using the simple methods described here, if an intron 22 inversion is shown in a single family member, either an affected male or carrier female member, the whole family can confidently be offered advice. Families with severe hemophilia A in whom there is no affected male family member available for testing can also be helped in about 50% of cases, if an inversion is identified. Similarly, a proportion of families, previously uninformative with gene tracking techniques, can now be advised and offered antenatal diagnosis. In some cases in which only a family history suggestive of a severe hemophilia phenotype (either A or B) is available, it is possible to diagnose FVIII deficiency, confirm the severe phenotype, and definitively show whether female members are carriers.

We suggest that in families with severe hemophilia A who seek genetic counselling analysis for an intron 22 inversion should be the first-line investigation because a positive result will provide definitive information, considerably reduce the amount of work required, and limit the number of family members tested, hence decreasing the cost. If no inversion is shown, as will be the case in 50% of families, traditional gene tracking techniques will be required. If a rapid result is required, eg, in pregnancy, all available methods should be used simultaneously. This simple method for establishing the genetic defect in 50% of families may free sufficient resources to allow specific mutations to be sought in the remaining severely affected families.

The information provided by intron 22 inversion analysis will allow many women who previously could not be informed of their carrier status to start a family either confident in the knowledge that they do not carry the affected gene or with the opportunity for antenatal diagnosis. These methods could relieve much of the anxiety experienced by potential carriers of hemophilia A and their partners, allowing them to make informed choices.

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