Direct Detection of a Common Inversion Mutation in the Genetic Diagnosis of Severe Hemophilia A

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Two recent reports suggest that approximately 50% of the cases of severe hemophilia A (factor VIII:C <0.01 U/mL) may be caused by a gross rearrangement of the factor VIII gene. The mutation involves genomic sequence from exon 1 to within intron 22 of the gene in an inversion event. This rearrangement can be detected on a Southern blot using a probe that is complementary to sequence from within intron 22. In this report, we describe the analysis of 71 severe hemophilia A patients for the presence of this mutation. Thirty-two of the patients (45%) showed evidence of the rearrangement, a figure that confirms the initial reports on patterns of rearrangement. Five different patterns of rearrangement have been noted, although two of these patterns (pattern 1 [78%] and pattern 2 [16%]) account for the majority of cases. The other patterns of rearrangement appear to be confined to individual families and may represent the result of additional sequence variation within the region of the genome to which the proximal 22 exons of factor VIII are translocated. Analysis of this patient population for the factor VIII inversion mutation has been extremely useful in a molecular diagnostic sense. In 23 of the cases studied (72%), the affected individual was the only documented hemophiliac in the family and, thus, previous linkage analysis had been limited to the provision of exclusion testing only. In conclusion, it appears that testing for the factor VIII inversion mutation will be positive in approximately 45% of severe hemophiliacs and as such should constitute the initial stage in the genetic testing protocol for these patients’ families.

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Subsequently hybridized with 50 ng of \( ^{32}P \) dCTP-labeled probe p482.6 (catalog no. 57203; American Type Culture Collection, Rockville, MD) that had been digested with EcoRI and mixed with 1 mL of 10 mg/mL sheared salmon sperm DNA and 300 \( \mu \)L of 1 mg/mL sheared human placental DNA. Hybridization was performed at 65°C for 16 hours. After hybridization, the membrane was washed to a stringency of 0.1X standard citrate saline (SSC) and 0.1% SDS at 65°C for 30 minutes. The membrane was exposed to Kodak XAR-5 x-ray film (Eastman Kodak, Rochester, NY) at -70°C with an intensifying screen for 18 hours.

**RESULTS**

Thirty-two of the samples tested (45%) showed evidence of the factor VIII inversion mutation. In 70% of these cases, the pattern of rearrangement (pattern 1) is represented by hybridization bands of 18.5, 17.5, and 15 kb (normal: 19.5, 16.5, and 15 kb, respectively; Fig 1), and in an additional 16% of cases (pattern 2) the altered hybridization band sizes are 18.5, 16.5, and 16 kb (Fig 1). We have also noted three other patterns of rearrangement in this patient population. One of these patterns (pattern 5) has been observed in two families, whereas the other two patterns (patterns 3 and 4) have only been seen in single families. The prevalent type 1 and 2 rearrangements have been documented in association with several different factor VIII polymorphic haplotypes, indicating that the mutation has occurred de novo a number of times. Apart from the fact that all the patients found to have the inversion mutation have severe factor VIII deficiency, we have not identified any other phenotypic association with this form of mutation.

One of the major advantages to being able to detect disease-causing mutations directly is the ability to use this information in carrier detection and prenatal diagnosis. This is especially pertinent in those families in whom isolated cases of hemophilia A exist and in whom polymorphism linkage studies can only be used to rule out transmission of the mutant factor VIII allele. The families shown in Fig 2A and B illustrate the benefit of direct mutation detection in kindreds with an isolated case of hemophilia A.

In one of these families, the analysis of polymorphic haplotypes indicates that the X chromosome on which the hemophilia mutation has occurred originated in the normal maternal grandfather (I.1). Testing of his two daughters for the inversion mutation has indicated that only one of them (II.2) has evidence of the rearrangement. Therefore, the mutation in this family has originated in the germline of the maternal grandfather and the risk of recurrent transmission of the mutation to his daughters relates to the frequency of germline mosaicism for this mutation.**

Even then, all potential carriers can be definitively screened for the mutation.

The family shown in Fig 2B illustrates another advantage of direct analysis for this mutation. Previous polymorphism analysis had been uninformative in deriving the likely origin of the inversion mutation. The inversion mutation is shown to have originated in the germline of the maternal grandfather (I.1), indicating that the X chromosome on which the hemophilia mutation occurred originated in the normal maternal grandfather (I.1). Testing of his two daughters for the inversion mutation has indicated that only one of them (II.2) has evidence of the rearrangement. Therefore, the mutation in this family has originated in the germline of the maternal grandfather and the risk of recurrent transmission of the mutation to his daughters relates to the frequency of germline mosaicism for this mutation.**

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of the hemophilic mutation and phenotypic data (the mean of two factor VIII:C and vWF:Ag levels) indicated that all three potential carrier females in the second generation were at high risk of being heterozygous for the mutation. However, despite the coagulation data in this family, the inversion mutation was found in the maternal grandmother and had only been transmitted to two of the three daughters (II.2 and II.4).

**DISCUSSION**

The results reported here confirm that approximately 45% of severe hemophilia A is the result of an inversion mutation affecting exons 1 to 22 of the factor VIII gene. Eighty-five percent of the inversion mutations documented in this study resulted in one of two patterns of genomic rearrangement. We have also identified three additional rare patterns of rearrangement resulting from the inversion mutation. The mutation has been found in association with a number of different factor VIII polymorphic haplotypes, indicating that each mutation likely occurred as an independent event.

The size and genomic complexity of the factor VIII gene along with the demonstrated mutational heterogeneity of hemophilia A has been a major limitation to genetic studies of this disorder. The initial hint of an unusual form of mutation came from studies in which normal candidate regions of the factor VIII gene did not show mutations in approximately 50% of severe hemophilic cases tested. This observation was subsequently followed by the documentation of a possible defect in mRNA processing affecting the exon 22-23 boundary in the same group of patients. This phenomenon has now been elegantly explained by the identification of a common factor VIII gene inversion mutation that translocates exons 1 to 22 of the gene to a site 5' of the factor VIII locus. The mechanism responsible for this mutation likely involves an intrachromosomal recombination event between homologous sequences within intron 22 of the gene and sites approximately 500 kb upstream. The sequence involved in this recombination event within intron 22 is the intronless factor VIII-associated gene A that is transcribed ubiquitously in the opposite orientation to factor VIII. There are normally two upstream copies of gene F8A, both of which are also transcribed, and, in some individuals, the number of homologous upstream repeats may be more than two. This latter phenomenon is likely responsible for the rare variant patterns of rearrangement seen in our population and may be particularly pertinent to the patterns of rearrangement seen in lanes 4 and 5 of Fig 1. In both of these patients, the normal 19.5-kb band, which represents intron 22 of the factor VIII gene, is still seen along with additional aberrant bands. The variant pattern seen in lane 4 of Fig 1 was observed in a familial case of hemophilia A, and thus it is unclear, without further analysis of the factor VIII gene, whether the rearrangement is the underlying cause of the disease or is simply a coincidental normal variation. The fifth pattern (lane 5, Fig 1) was detected in an affected individual who is an isolated case of hemophilia A. DNA marker studies and analysis of the aberrant pattern in other family members showed that the mother of the affected individual inherited it as a de novo rearrangement from her unaffected father, and subsequently transmitted it to her affected son. No other family members, including the grandfather of the affected individual, were shown to carry this new pattern. The simultaneous appearance of the rearrangement with hemophilia A in this family is evidence in support of its causative role in the disease.

The inversion mechanism responsible for this mutation does not usually result in either a loss or gain of DNA; however, in one of our patients, in whom a unique rearrangement pattern was observed (pattern 3), we have previously documented a gross deletion that removes exons 1-22 of the factor VIII gene as well as sequence 5' to the gene. This patient had evidence of a high titer factor VIII inhibitor, one of only two inhibitor patients that we have documented with the inversion mutation.

The practical benefit that results from the ability to detect this mutation is illustrated by the two families shown in Fig 2. In the patient population that we have tested (a random sample of severe hemophiliacs referred to our laboratory over the past 7 years), 23 of the cases showed evidence of only a single hemophiliac in the family. In these families, polymorphism linkage data can be used to definitively rule out transmission of the mutant allele, but, in light of the fact that the point of origin of the mutation is not detectable, it is impossible to positively identify carriers with this strategy. As indicated by our own experiences in a regional molecular diagnostic laboratory offering carrier testing and prenatal diagnosis, referral of families with isolated cases of severe hemophilia A is common. The results obtained in this study indicate that the first step in analysing these families must be to test for the presence of the factor VIII inversion mutation. With this approach, definitive information concerning carrier status or prenatal diagnosis will be available in 45% of these families within 7 to 10 days.

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Direct detection of a common inversion mutation in the genetic diagnosis of severe hemophilia A [see comments]

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