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 28 patients. Five different patterns of rearrangement have been noted, although two of these patterns (pattern 1 [70%] 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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HEMOPHILIA A (inherited factor VIII deficiency) is the result of heterogeneous mutations within the factor VIII gene on the X chromosome.1 The molecular genetic analysis of hemophilia A has been complicated by the large size of the gene, its complex genomic organization, and the mutational heterogeneity exhibited by this disease. After the cloning of the factor VIII gene in 1984,2,3 initial studies identified infrequent mutations that involved either partial or total deletions of the gene4 or nucleotide substitutions affecting endonuclease recognition sites.5,6 Later studies using mutation screening methods on polymerase chain reaction (PCR)-amplified factor VIII sequences improved the yield of mutations significantly.7,8 Using denaturing gradient gel electrophoresis of the factor VIII promoter, 99% of the coding sequence, and 94% of the splice junctions, mutations were reported in 85% of patients with mild and moderately severe phenotypes11 but in only 55% of the severe hemophiliacs tested.12 This observation was later complemented by the recognition that in approximately half of the patients with severe hemophilia A it was impossible to detect a contiguous mRNA transcript across the exon 22-23 boundary.13 The explanation for this defect has recently been reported to involve an inversion of the factor VIII gene between exon 1 and intron 22 and translocation of this region of the gene to a site 5' of the factor VIII locus.14,15 The mechanism responsible for this rearrangement likely involves an intrachromosomal recombination event between homologous sequences upstream of the factor VIII locus and within intron 22.

As noted above, direct mutation testing in hemophilia A has not been a feasible proposition for most molecular diagnostic laboratories involved in carrier detection and prenatal diagnostic studies. In the majority of these cases, a strategy using linked polymorphic markers of the factor VIII gene has been used where possible.16-18 However, linkage analysis has significant limitations in certain circumstances, the most restrictive of which is in families in whom there is an isolated case of hemophilia A and in whom the origin of the factor VIII mutation is uncertain. In these families, the ability to detect the disease causing mutation would be of great benefit.

In this report, we describe the study of a large severe hemophilia A population for the factor VIII inversion mutation. Our results confirm its utility in genetic testing for this patient group and indicate that analysis for this mutation should constitute the first stage in diagnostic protocols for severe hemophilia A families.

MATERIALS AND METHODS

Study population. DNA from 71 severe hemophilia A patients (factor VIII:C levels <0.01 U/mL) was analyzed for the factor VIII inversion mutation. These samples had been referred to the DNA Diagnostic Laboratory at the Kingston General Hospital (Kingston, Ontario, Canada) over the past 7 years for studies of carrier status and prenatal testing. The patient population is representative of the general Ontario population with Caucasian subjects predominant in a diverse ethnic mix.

Molecular genetic studies. DNA was extracted from blood samples by a standard technique.19 In most instances, the patient samples had previously been studied for the pattern of several intragenic factor VIII polymorphisms. Analysis of the biallelic Bcl I,20 Xba I,21 and intron 7' polymorphisms was performed as previously described. The samples were tested for the factor VIII inversion mutation after digestion of 6 μg of DNA with Bcl I according to the manufacturer’s directions (Pharmacia, Lachine, Quebec, Canada). The digested DNA was electrophoresed in a 0.8% agarose gel at 40 V for 22 hours before Southern transfer to Gene Screen Plus nylon membrane (Dupont Canada Inc, Lachine, Quebec, Canada). Prehybridization was performed at 65°C for 2 hours in 0.3 mol/L NaCl, 1% sodium dodecyl sulfate (SDS), and 10% dextran sulfate. The membrane was

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subsequently hybridized with 50 ng of $e^{32}P$ 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 µL 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 0.1 × 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 3) 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 the first family, the analysis of polymorphic haplotypes indicates that the X chromosome on which the hemophilia mutation has occurred originated in the normal maternal grandfather (1.1). 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 direct analysis for this mutation. Previous polymorphism analysis had been uninformative in deriving the likely origin of direct analysis for this mutation.

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**Factor VIII Inversion Mutation**

Fig 1. Autoradiograph of a Southern blot in which genomic DNA samples have been digested with the restriction enzyme Bcl I and analyzed with the probe p482.6. The lanes contain the following samples: lane 1: N, normal female; lane 2: N, normal male; lane 3: 1, pattern 1 inversion mutation; lane 4: 2, pattern 2 inversion mutation; lane 5: 1/N, carrier female for the pattern 1 inversion mutation; lane 6: 2/N, carrier female for the pattern 2 inversion mutation; lane 7: samples have been digested with the restriction enzyme of the factor VIII; lane 8: 4, pattern 4 mutation; lane 9: 5, pattern 5 mutation.

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**Fig 2.** (A) Pedigree of a severe hemophilia A family with an isolated case of hemophilia. All family members have been genotyped for the factor VIII Bcl I, intron 7, and Xba I polymorphisms as well as for the inversion mutation. The inversion mutation is shown to have originated in the germline of the maternal grandfather (I.1) and has been transmitted to only one of his two daughters (II.2). (B) A severe hemophilia A family genotyped for the Bcl I polymorphism and the factor VIII inversion mutation. By coagulation studies (mean of two F.VIII:C and vWF:Ag levels), all three of the second generation females appear to have a high risk of being hemophilia carriers. However, direct analysis for the inversion mutation shows that only individuals II.2 and II.4 have inherited the mutant factor VIII gene from their carrier mother.
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.\(^\text{28}\) 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 hemophiliacs tested.\(^\text{12}\) 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.\(^\text{13}\) 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.\(^\text{14,15}\) 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 \(^\text{16}\) 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.\(^\text{14}\) 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.\(^\text{17}\) 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 hemophilic 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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**REFERENCES**


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

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