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Research Article

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Rapid Publications

Carrier Detection of Hemophilia B by Using a Restriction Site Polymorphism Associated with the Coagulation Factor IX Gene

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Abstract. The cloned complementary DNA for coagulation Factor IX (FIX) detects a frequent restriction fragment length polymorphism (RFLP) in human genomic DNAs digested with the restriction endonuclease Taq I. This genetic marker was used, in parallel with coagulation and immunological assays, to follow the segregation of an abnormal FIX gene in a large Hemophilia B family. Among the six potential female carriers, functional assays showed that four had a high probability, and two a low probability of being carriers. Analysis at the DNA level with the cDNA probe was informative in five of the six cases, and in all these five the diagnosis of carrier state was definitively confirmed. This demonstrates the feasibility of using linkage analysis at the DNA level for the genetic screening of Hemophilia B. This method has the advantages over conventional assays of giving a diagnosis of certainty, and of being applicable to early prenatal diagnosis using biopsies of trophoblast villi. At present, the single known polymorphism associated with the FIX gene restricts the application of linkage analysis to informative cases (40%), but findings of additional RFLPs in this region should improve this figure.

Introduction

Hemophilia B is a severe X-linked hemorrhagic disorder due to a deficiency of coagulation Factor IX (1). Immunological assays for Factor IX (FIX)[1] can distinguish between two main types of Hemophilia B patients (2): the B^- patients who lack detectable FIX protein and the rarer B^+ patients where the gene encodes an abnormal FIX protein. Genetic screening of Hemophilia B (carrier detection and prenatal diagnosis) represents an important issue (3, 4). Carrier detection is presently based on the study of the transmission of the trait in the family, on assays for the procoagulant activity (FIX:C), and on the level of antigenic protein in plasma (FIX:Ag). However, these assays can at best detect 90% of the carriers (5), and prenatal diagnosis involves fetal blood sampling and is technically demanding.

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1. *Abbreviations used in this paper:* DBM, diazobenzyloxymethyl; FIX, Factor IX; FIX:Ag, Factor IX antigen; FIX:C, Factor IX procoagulant activity; RFLP, restriction fragment length polymorphism.

With the recent isolation of DNA sequences corresponding to coagulation FIX gene (6–8), it is now possible to analyze Hemophilia B mutations at the molecular level. DNA analysis can be applied to genetic screening in different ways, as shown over the past few years for the hemoglobinopathies (9). The DNA probe for FIX can be used to detect gene deletions in Hemophilia B patients. However, a first analysis (10) has suggested that such deletions are very rare, and might be common only in patients who develop antibodies towards FIX during the course of replacement therapy. The direct detection of point mutations that is now possible for sickle cell anemia (11–13) or certain β-thalassemias (14–15) is unlikely to be feasible on a general basis because of heterogeneity of Hemophilia B mutations (2). Only for Hemophilia B Chapell Hill has the actual mutation been identified at the protein sequence level (16). To use this approach in the other cases would require the isolation of the mutant gene in each family at risk, the analysis of the functional defect, and sequence determination of the appropriate regions. This would clearly be a major effort because of the gene's large size (≥20 kb), and because the possibilities of analysis of the functional defect in the patient cells are limited, due to the restriction of the expression of the gene to the liver.

A third, indirect approach can be used fruitfully, again as illustrated in the case of some hemoglobinopathies. DNA probes allow the detection of a new class of genetic markers, the restriction fragment length polymorphisms (RFLP), which are, in general, selectively neutral. If these markers are in or near the region of the gene that is affected in a particular disease, the analysis of their segregation in families at risk can be used for genetic screening (17–20). We have recently described a restriction site polymorphism in the FIX gene region (21), and we show here its use in carrier detection in a Hemophilia B family, and its potential application for prenatal diagnosis. This is the first instance that carriers for a Hemophilia gene have been identified by DNA analysis.

Methods

Coagulation and immunological assays of FIX. Detection and classification of patients with FIX abnormality in the family studied were done according to standard methods (22) using global coagulation tests, activated partial thromboplastin time, prothrombin time, ox-brain prothrombin time, and specific one-stage assay of FIX:C based on the activated partial thromboplastin time. The antigenic activity of Factor IX (FIX:Ag) was measured by an immunoenzymatic method using the reagents of a commercial kit (Asserachrom FIX:Ag, Stago, Paris) and micro-immunoenzymatic plates (Nunc, Denmark).

Leukocyte DNA preparation. DNA was prepared from 10 to 20 ml of whole blood that had been kept frozen (−80° or −30°C) for periods of up to several months. After thawing, the blood was diluted with 2 vol of 20 mM Tris, pH 7.5, 5 mM EDTA (TE 20-5), and centrifuged at 4,000 rpm for 5 min. The pellet was resuspended in TE 20-5 and centrifuged again in the same conditions. The cycle was generally repeated once until a pale pellet was obtained. The pellet was well resuspended by efficient vortexing in 0.7–1 vol (with respect to the volume of starting blood) of TE 20-5. SDS (0.4% final) was added only when all clumps had disappeared. Proteinase K digestion (70–100 μg/ml) was performed for 16 h at 37°C. DNA was further purified by two cycles of extractions with phenol (equilibrated with 0.1 M Tris, pH 8, 0.1% 8 hydroxyquinoleine)-chloroform-isoamyl alcohol (25-24-1), then two cycles of chloroform/isoamyl alcohol extraction, and finally by two successive ethanol precipitations. DNA was then dissolved in 20 mM Tris, pH 7.5, 1 mM EDTA, and kept at 4°C.

Digestion of DNA samples with restriction enzyme Taq I, gel electrophoresis, blotting onto diazobenzyloxymethyl (DBM) paper (23), and hybridization to a FIX cDNA probe were performed as previously described (21).

Results

We have studied a large, four generation family where a severe form of Hemophilia B segregates (Fig. 1). Coagulation studies performed on two affected males (II8, III5) demonstrated that they carry a Hemophilia B^+ mutation with normal levels of

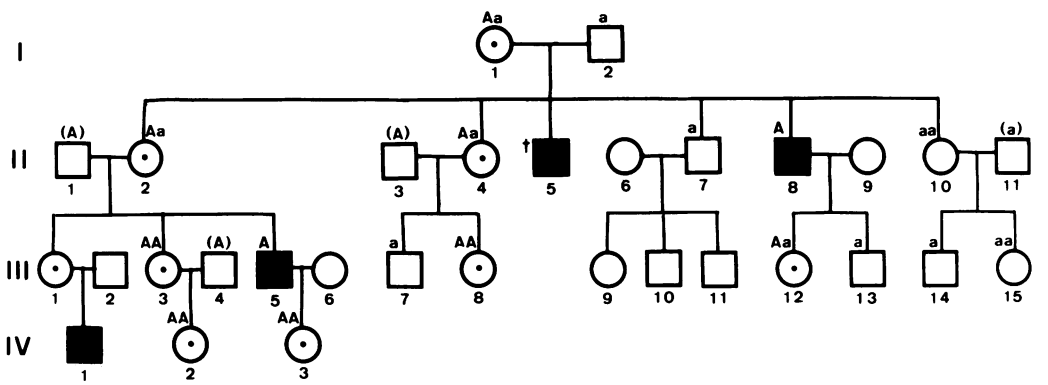

Figure 1. Pedigree of family M. . . Carrier females (⊙) are either obligatory carriers (mothers or daughters of affected males) or those whose status has been established by functional assays and/or DNA analysis. The genotype defined by the Taq I polymorphism is indicated for all analyzed subjects. A: 1.8-kb fragment; a: 1.3-kb fragment. Genotypes indicated in brackets are those that were deduced from the genotype of the children, but that were not analyzed directly.

FIX:Ag. The ox-brain thromboplastin time was normal, indicating that it is not a B^M variant (2). Determination of FIX:C and of FIX:Ag levels were carried in 10 women from the family, four of them being obligatory carriers (I1, II2, III12, and IV3), the other six being potential carriers (II4, II10, III3, III8, III15, and IV2). All four obligatory carriers had levels of FIX:C below 0.5 U/ml. In particular, II2 had a very low activity (0.09 U/ml) that was associated with mild hemorragic manifestations. This probaby results from an extreme lyonization, with a high proportion of FIX-producing cells having the active X chromosome carrying the mutation. Among the six potential carriers, four had FIX:C levels ranging from 0.33 to 0.42 U/ml and are almost certainly carriers. The mother and daughter II10 and III15 on the other hand had FIX:C levels of 0.67 and 0.81 U/ml, the former value being at the lower limit of the normal range. Such a value would also be found in a carrier if X chromosome inactivation had favored the expression of the non-mutated gene.

Analysis of the segregation of a RFLP detected with a FIX cDNA probe was carried out for 17 members of the family. Leukocyte DNA was digested with the enzyme Taq I, subjected to agarose gel electrophoresis, and transferred onto DBM paper. Hybridization to a FIX cDNA probe allowed the detection of four constant fragments and two allelic fragments of 1.8 kb (allele A) and 1.3 kb (allele a) (Fig. 2). In the first generation, the obligate carrier II was heterozygous for the Taq I RFLP. One affected son (II8) carried the A allele, while a normal son (II7) had the a allele. Thus the A allele segregates with the Hemophilia B mutation. This is further demonstrated by the fact that the obligatory carrier II2 had inherited the A allele from her mother, and that she transmitted it to her hemophiliac son (III5). It is thus possible to follow the chromosome bearing the Hemophilia B mutation in the rest of the family. Such an analysis demonstrates that II4, III3, and III8 are Hemophilia B carriers, because they inherited the A allele associated with the mutated gene, confirming definitively the results of FIX:C measurements. However, for IV2, which also had low FIX:C, the genotype analysis is uninformative, since her mother is homozygous AA. On the other hand, the genotype analysis allows one to exclude II10 and III15 as carriers, since they carry the a allele only. The ambiguity due to the relatively low level of FIX:C in II10 (0.67 U/ml) is thus resolved. It is worth noting that, of the eight carriers analyzed, four have the genotype Aa that would be required for prenatal diagnosis by linkage to the Taq I polymorphism.

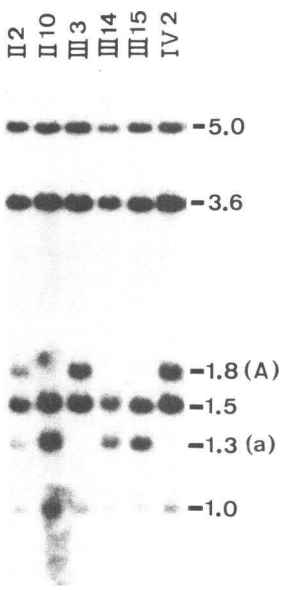

Figure 2. Southern blot analysis of Taq I-digested DNAs from members of family M. . . The DNAs were prepared, digested with Taq I, transferred onto DBM paper, and hybridized, as described previously (21, 23). The FIX cDNA probe used corresponds to the 5′ half of the cloned sequence, and does not hybridize to the 2.7-kb Taq I fragment of the gene (21). Numbering of lanes corresponds to pedigree numbers in Fig. 1. The size of DNA fragments is given in kilobases.

Discussion

Detection of heterozygous female carriers is a particularly important goal for X-linked recessive diseases. Here we have approached this problem in a large Hemophilia B family in two different ways. Coagulation assays and measurement of immunologically crossreacting material allowed an analysis at the level of the gene product. The segregation of the mutation was followed at the gene level using as an indirect marker a restriction site polymorphism in the FIX gene. On the basis of functional assays four women at risk were found to have a high probability of being carriers. Analysis at the DNA level was informative in three of them and allowed us to definitively establish their carrier status. Two other women at risk in the same family had low probability of being carriers, based on the coagulation assay. Their genotype demonstrated that they had not inherited the Hemophilia B mutation. Thus the results obtained using both methodologies were in agreement in this family study.

What are the respective advantages and disadvantages of these two approaches? Because of the broad range of values obtained by the functional and immunological assays in normal and heterozygous females, there is always some uncertainty in the diagnosis of carrier status (3, 5). There are several reasons for this variability: the biological variability of FIX levels and the difficulties inherent in the determination of clotting activity, which is less reproducible than the determination of the antigen levels. Because the FIX gene is X linked, phenotypic expression in carriers may vary according to the proportion of active X chromosome carrying the mutation in liver cells that synthesize FIX. Thus some carrier females might have either very low levels of FIX (see for instance subject II2, Table I), or have values that are within the normal range. In addition, in Hemophilia B^+ mutations (such as in family M . . .), the determination of FIX:Ag antigen cannot be used to determine the carrier status. However the functional assays can be applied to all subjects at risk, and is not dependent on the study of other members of the family (analysis of one affected male is, however, necessary to establish the nature [B^+ or B^-] of the mutation). Finally, these functional assays can be used for prenatal diagnosis ~20–22 wk of gestation, after fetal blood sampling (4).

Table I. FIX:C and Antigen and FIX Alleles in Family M . . . with Hemophilia B⁺

Patient	Age	FIX		Taq I genotype	Carrier status
		FIX:C (0.65–1.40)	FIX:Ag (0.60–1.30)		
	yr	*U/ml*	*U/ml*		
I_1	71	0.50	0.87	Aa	Obligatory carrier
I_2	81	1.18	0.94	a	
II_2	51	0.09	0.92	Aa	Obligatory carrier
II_4	37	0.38	0.81	Aa	Carrier
II_7	48	0.99	0.92	a	
II_8	45	0.01	1	A	
II_{10}	49	0.67	0.93	aa	Non carrier
III_3	27	0.36	1.12	AA	Carrier
III_5	28	0.01	0.76	A	
III_7	5	1.06	0.81	a	
III_8	3	0.42	0.68	AA	Carrier
III_{12}	17	0.29	0.78	AA	Obligatory carrier
III_{13}	14	0.79	0.84	a	
III_{14}	20	0.92	1.13	a	
III_{15}	17	0.81	1.05	aa	Non carrier
IV_2	2	0.33	1.85	AA	Carrier*
IV_3	2	0.23	0.62	AA	Obligatory carrier

* Determined from the coagulation assay only. FIX:C and FIX:Ag were determined as described in Methods and the normal range (mean±2 [SD]) is indicated in brackets. The Taq I allele associated in this family to the abnormal FIX gene is underlined.

For the analysis at the DNA level using linked genetic markers, some family study is mandatory (for instance, for the determination of carrier status, knowledge of the genotype of an affected proband is always needed, and depending on the cases, analysis of one or both parents of the potential carrier will also be required). The diagnosis can be performed only in families informative for the particular marker. Given the frequency of the Taq I polymorphism used here (21), ~40% of the cases would benefit from an analysis with this marker. On the other hand, the diagnosis of carrier status can be established with certainty. An additional advantage for prenatal diagnosis is that RFLP analysis can be performed on any type of fetal cells. This could allow very early prenatal diagnosis (8th–10th wk), using the recently developed biopsy of trophoblast villi (24, 25). It is thus essential to search for additional polymorphisms in the FIX gene region, which would render the analysis at the gene level applicable to a greater proportion of cases.

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Note added in proof. Since this manuscript was submitted, a report (26) has appeared also reporting the detection of Hemophelia B carriers by RFLP analysis.

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