Implications of gene sequencing in the direct diagnosis of hemophilia by restriction fragment length polymorphism analysis of polymerase chain reaction products

We present an example to illustrate how restriction fragment length polymorphism analysis of polymerase chain reaction products (PCR-RFLP), commonly used in indirect diagnosis by linkage analysis, can also be applied to the direct molecular diagnosis of hemophilia in a significant number of affected families once the causative mutation has been identified. Restriction mapping of 33 mutations responsible for hemophilia indicates that this procedure could be extended to nearly 75% of families.

Multiple benefits derived from mutation identification are promoting the development of methods to detect the gene defect in hemophilia, ranging from a variety of screening techniques to the direct sequencing approach.[1] We present here an illustrative example of how the knowledge of the causative mutation permits the design of specific PCR-RFLP protocols to determine carrier status and perform prenatal studies in affected families in a made-to-measure manner.

The propositus, a 12-year old boy of Spanish origin, was a sporadic case of severe hemophilia A with factor VIII:C lower than 1% and no detected inhibitors. Fourteen relatives were studied, including the father, the maternal grandfather and 12 women at risk. The patient was negative for the presence of the intron 22 inversion, as demonstrated by Southern blot and confirmed by a long-fragment PCR method.[2] Previous linkage analysis studies to determine the inheritance pattern of the affected chromosome had been unsuccessful because both the extragenic and intragenic markers used were uninformative. We have recently described a direct factor VIII gene sequencing procedure[3] that was used to characterize the causative mutation. The mutation was identified in the propositus as a deletion in exon 14 (4272delC) resulting in a frameshift at codon position 1405. In order to clarify whether this was a *de novo* mutation or whether it was already present in previous generations, we proceeded to sequence the corresponding factor VIII gene region in the mother, revealing her to be a carrier. To go deeply into the origin of the mutation we then proceeded to analyze 11 women (see relationships in Figure 1), the maternal grandfather and the father of the propositus (both included as normal controls). Before initiating the study of these family members we decided to look for the presence of restriction endonuclease (RE) targets that could be used in a PCR-RFLP diagnosis-based approach. In this direction we analyzed a 1061 bp DNA fragment from exon 14 containing mutation 4272delC (1062 bp length in control). RE mapping revealed that the cytosine deletion destroys one of the two *Bst*NI targets present in the normal sequence and, at the same time, creates a new *Tth*111I restriction site. The expected *Bst*NI restriction pattern consists of three DNA fragments of 763, 169 and 130 bp in a normal control and only two bands of 931 and 130 bp when the mutation is present. The expected *Tth*111I restriction pattern consists of two fragments of 763 and 298 bp in the mutated allele and a single band of 1062 bp (no restriction site) in the wild-type allele. Figure 1 shows an agarose gel with the restriction pattern obtained after enzymatic digestion of the PCR products from the patient and his relatives with *Bst*NI and *Tth*111I.

This example illustrates the applicability of PCR-RFLP in genetic counseling and prenatal diagnosis once the mutation has been identified, and it should not be contemplated as an exceptional case. In our experience mutations frequently create and/or remove a commercially available restriction specificity: an in-depth analysis of 33 mutations recently described by us as

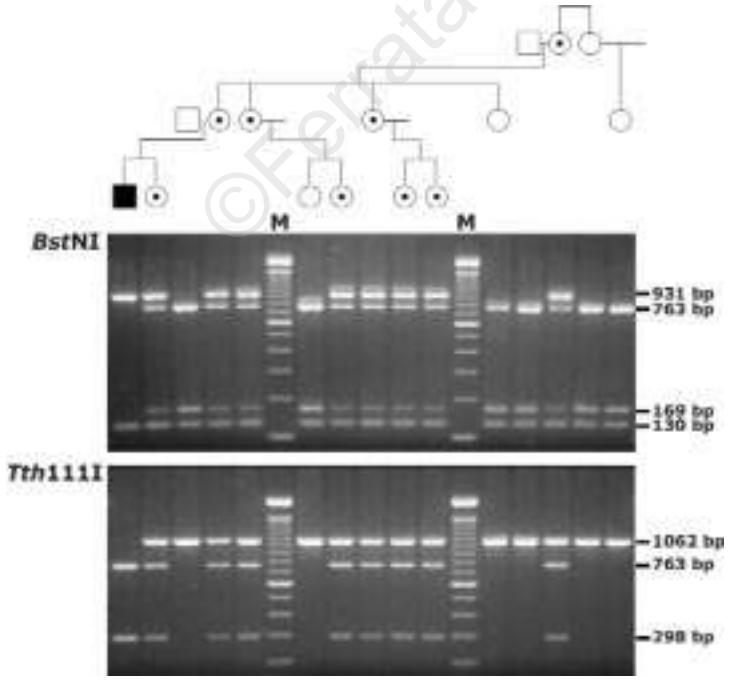

Figure 1. Agarose gel electrophoresis showing the PCR-RFLP family analysis of a severe hemophilia A patient caused by mutation 4272delC. A PCR fragment from exon 14 of the factor VIII gene containing the point of deletion was digested in the presence of *Bst*NI (upper panel) or *Tth*111I (lower panel). Digestion patterns reveal the presence or absence of the target mutation. Besides the propositus (first lane), a total of 14 relatives were analyzed including 2 healthy men who served as normal controls. From the 12 females at risk, 8 resulted to be carriers for the mutation (pointed circle). In the family tree, members are located above their corresponding PCR-RFLP lane. M: 100 bp DNA ladder.

Table 1. Susceptibility of hemophilia mutations characterized in our laboratory to PCR-RFLP detection. Restriction site identification prior and post mutation was done using the MacVector program (Oxford Molecular Ltd., UK) and a subset of REBASE (Restriction Enzyme Database) including only the commercially available type II restriction endonucleases.

Mutation	*Restriction site removed*	*Restriction site created*	*PC FLP feasibility*
Factor VIII gene			
209-212delTTGT	–	*Bfm*I	Yes
577G→A	*Nla*IV	–	Yes
1171C→T	–	–	No
1569G→T	–	*Mbo*II	Yes
1648C>T	*Aci*I; *Fau*I	*Bsp*MI	Yes
1834C→T	*Bsa*XI	–	Yes
3175A→T	–	–	No
3637delA	–	–	No
3994-3997delAGAG	–	*Hind*III	Yes
4272delC	*Bme*1390I; *Box*I; *Bsa*JI; *Bst*NI	*Tth*111I	Yes
4340insG	*Hgi*EI; *Nla*IV; *Sau*96I; *Sin*I	--	Yes
4757G→A	–	*Bpu*10I; *Dde*I	Yes
5123G→A	*Aci*I; *Bbv*I; *Fau*I; *Ita*I; *Tse*I	–	Yes
5345T→G	–	*Bsu*6I; *Mbo*II; *Sap*I	Yes
5615G→A	*Ava*II; *Nla*IV; Sau96I	*Xmn*I	Yes
6046C→T	*Aci*I; *Fau*I	–	Yes
6385A→T	*Mbo*II	*Fok*I	Yes
6494delC	–	–	No
6506G→A	–	–	No
6545G→A	*Mwo*I	–	Yes
6047G→C	*Aci*I; *Fau*I	*Bsr*FI; *Eae*I; *Hae*III; *Msp*I	Yes
IVS16-1 G→A	*Avr*II; *Bsi*YI; *Mae*I; *Bsa*JI; *Sty*I	–	Yes
IVS23+5 G→C	–	*Mae*III	Yes
Factor IX gene			
117G→A	–	–	No
6460C→T	*Bss*SI	*Nla*III	Yes
17783C→G	–	–	No
20413C→T	*Bse*LI	–	Yes
30864G→A	–	–	No
30875C→T	*Bst*BI; *Hinf*I; *Taq*I; *Tfi*I	–	Yes
30980C→A	–	–	No
31113-31115delTTG	*Hind*II	–	Yes
31326C→T	*Bae*I; BciVI; NciI; ScrFI	*Bsa*WI	Yes
IVS3-3 A→G	–	*Bse*MII; *Dde*I; *Hpy*188I	Yes

causative of hemophilia A or B[3,4] indicates that nearly 75% (24/33) of the studied cases comply with this condition (Table 1). These mutations include substitutions, short insertions and short deletions that can be subsequently detected through PCR-RFLP in relatives at risk. This high percentage is not surprising taking into account the rapid growth of REBASE, in which the number of commercially available RE has increased from 403 to 531 in the last 5 years and represent 200 of the 228 known type II RE specificities.[5]

Such an approach is of special relevance when the patient and his relatives come from small centers in which routine laboratories do not have the facilities and expertise required to carry out sequence technology. The PCR-RFLP technique avoids these limitations because only basic equipment and training are required. A more widespread involvement of laboratories could also facilitate the spread of genetic counseling and prenatal diagnosis to affected families, with the implicit benefits deriving from this situation.

Francisco Vidal, Elisenda Farssac,* Carme Altisent,° Lluís Puig,° Dominique Gallardo**

**Unitat de Recerca del Centre de Transfusió i Banc de Teixits de Barcelona; °Unitat d'Hemofília de l'Hospital General Vall d'Hebron, Barcelona, Spain*

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Correspondence: Dominique Gallardo, MD, Unitat de Recerca, Centre de Transfusió i Banc de Teixits, Passeig Vall d'Hebron 119-129, 08035 Barcelona, Spain.

Phone: international +34.9.32746725. Fax: international +34.9.32749027. E-mail: gallardo@hg.vhebron.es

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