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RESEARCH LETTER

Inverse shifting PCR based prenatal diagnosis of hemophilia-causative inversions involving *int22h* and *int1h* hotspots from chorionic villus samples

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Hemophilia A (HA) is an X-linked recessive bleeding disorder affecting one in 5000 human males worldwide, which arises from deleterious mutations in the coagulation factor VIII gene (*F*8). Inversions of *F*8-intron 22 (Inv22) and Inversions of *F*8-intron 1 (Inv1) are responsible for almost half of the cases with severe disease (Lakich *et al*., 1993; Naylor *et al*., 1993; Bagnall *et al*., 2002).

Both, intron 22 and intron 1 inversions result from homologous recombination between DNA repeats located within *F*8 intron 22 and intron 1, termed *int22h*-1 and *int1h*-1, respectively, with their more telomeric *F*8-extragenic copies, which have been shown to be in opposite orientation. Moreover, Inv22 and Inv1 originate from male germ cells by intrachromosomal pairing followed by nonallelic crossing over between the homologous repeats resulting in the complete disruption of the gene. These recombined *F*8 genes are presumably unable to produce and secrete any FVIII protein.

Currently, prenatal molecular detection of Inv22 has been achieved by use of Southern blot (Lakich *et al*., 1993) or long range-polymerase chain reaction (PCR) (Liu *et al*., 1998; Belvini *et al*., 2001), while Inv1, by using double-PCR (Bagnall *et al*., 2002). In order to improve the prenatal diagnosis (PND) of Inv22; and in second level for Inv1, which was properly resolved by the standard method of double-PCR (Salviato *et al*., 2007; Liang *et al*., 2008); we propose the use of a new genotyping system based on a version of inverse shifting-polymerase chain reaction (IS-PCR). This method has been enhanced in order to differentiate in two diagnostic tests: (1) Inv22 type I and type II inversions, *int22h*-induced deletions and duplications, and (2) *int1h*-related rearrangements (Rossetti *et al*., 2008). The use of this method will facilitate analysis of both inversions, although side-by-side analysis of Inv22 and Inv1 from the fetus sample is restricted to exceptional circumstances (outlined below).

The aim of this report is to analyze the ability of the method of IS-PCR for PND on DNA extracted from chorionic villus (CV) samples. Because human genome sequences are openly available in the web, specific IS-PCR approaches can be easily designed for genotyping virtually any full-defined structural variants, thus providing a powerful tool to investigate other disease causative rearrangements from CV samples.

In 2002, a pregnant carrier of HA, mother of a severe HA patient, whose Inv22 mutation had been identified in our laboratory, came to our attention for PND. During her twelfth week of gestation, a CV sample was obtained and gender analyzed by karyotyping, indicating that the fetus was a female. The direct mutation identification was then attempted by Southern blotting (Lakich *et al*., 1993), the former Inv22 analysis available at the time in our laboratory, with no success probably because of the poor quality of the CV-extracted DNA.

Recently, we presented an IS-PCR based method that performs robustly yielding proper results over a wide spectrum of DNA substrate qualities and concentrations (Rossetti *et al*., 2008), whereas long range-PCR methods (Liu *et al*., 1998; and its derivative techniques) often fail. IS-PCR was originally designed to be integrated at first line in the algorithm for analysis of *F*8 in severe HA patients because it provides a convenient analysis of *F*8 inversions, as Inv22 and Inv1 diagnostic tests may be performed side-by-side from the same processed substrate (Rossetti *et al*., 2008).

Genomic DNA was obtained from CV sample by phenol–chloroform and ethanol precipitation (Sambrook *et al*., 1989). A CV sample from a male fetus of a family with no history of hemophilia was received from a genetic laboratory and used as no-inversion tissue-specific control. DNA controls obtained from peripheral blood leukocytes were previously genotyped for Inv22 and Inv1 by IS-PCR analysis and included seven cases: one normal control, one Inv22 type I, one Inv22 type

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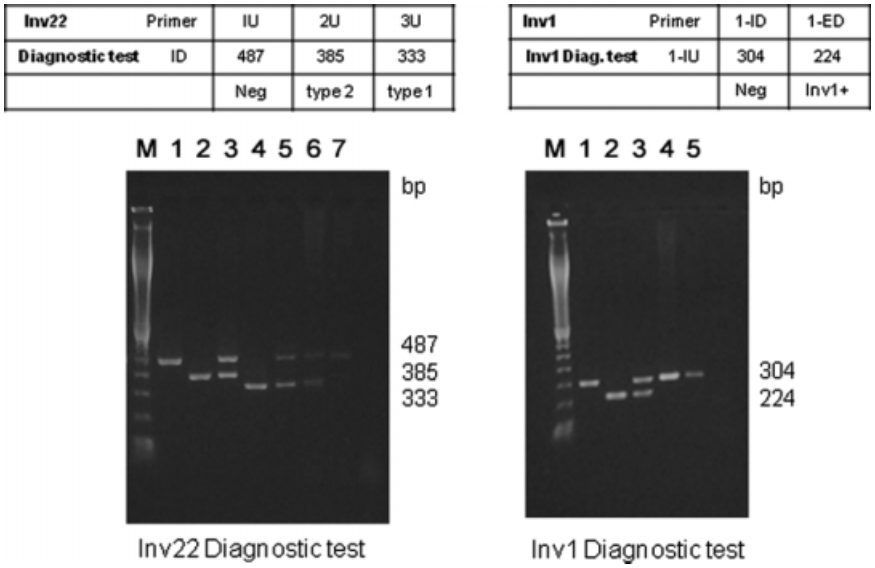

Figure 1—PND by IS-PCR. This system was designed for genotyping all possible *int22h*- and *int1h*-related rearrangements. Diagnostic tests for Inv22 and Inv1. IS-PCR amplicon length (bp) is determined by the function of a specific primer pair (small tables) (Rossetti *et al*., 2008). The lower panel shows the analysis of the IS-PCR products by standard 1.5% agarose gel electrophoresis. Inv22 diagnostic test: (1) non-Inv22 individual, (2) Inv22 type II hemophiliac, (3) Inv22 type II carrier, (4) Inv22 type I hemophiliac, (5) Inv22 type I carrier, (6) CV from the female index case that resulted Inv22 type I carrier and (7) CV from a non-Inv22 individual. Inv1 diagnostic test: (1) non-Inv1 individual, (2) Inv1 hemophiliac, (3) Inv1 carrier, (4) CV from the female index case that resulted non-Inv1 and (5) CV from a non-Inv1 individual. M indicates a marker of 100 bp ladder. CV (chorionic villus)

II, and carriers for both types of Inv22; and one Inv1 positive patient and his carrier mother. Informed consent was obtained in all cases.

IS-PCR PROTOCOL

Genomic DNA (2 μg) was digested with 20 units of *Bcl*I restriction enzyme according to the supplier's specifications (Fermentas, Maryland, USA) 4 h in a total volume of 50 μL. *Bcl*I-digested DNA was isolated using phenol–chloroform extraction and ethanol precipitation. DNA fragments were circularized with 3 units of T4 DNA ligase (Invitrogen, Buenos Aires, Argentina) in a volume of 400 μL (to facilitate self ligation of fragment-ends) at 15 °C overnight. Circularized DNA samples were then treated with an equal volume of phenol : chloroform mixture, ethanol-precipitated, and recovered in 30 μL of distilled water. Analytical PCR tests were performed in reactions containing 6 and 12 μL of circularized DNA for the analysis of Inv1 and Inv22, respectively, in the presence of 0.6 μM of each primer (sequence described in Rossetti *et al*. 2008), 0.5 U of Taq DNA Polymerase (Promega, Buenos Aires, Argentina) and additional standard PCR reagents in a total volume of 25 μL. Thermocycling involved 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 1 min and extension at 72 °C for 1.5 min; cycling was preceded by 94 °C for 2 min, and followed by 5 min at 72 °C. IS-PCR products were analyzed on ethidium bromide-stained 1.5% agarose gel electrophoresis and photographed.

It is noteworthy that because of the uneven quality of CV-extracted DNA some IS-PCR conditions, such as the final concentrations of ligated DNA and the thermocycling have been modified compared with what we referred in the previous article (Rossetti *et al*., 2008) (i.e. 3 and 6 μL of ligated DNA and 30 cycles) adjusted for DNA extracted from peripheral blood leukocytes.

Molecular analysis was carried out on two CV samples, one, a normal control male and the other one, corresponding to the female index case. IS-PCR Inv22 diagnostic test from a CV sample from the index case showed to be heterozygous for intron 22 inversion type I, and was diagnosed as carrier of severe HA. Results of Inv22 and Inv1 diagnostic tests are shown in Figure 1, including the index case, a nonhemophiliac male fetus and samples with all possible IS-PCR patterns.

In this study, we present an application of IS-PCR as an alternative to Southern blot analysis and long range-PCR-based methods, which is capable of genotyping all structural variants associated with *int22h* and *int1h* hotspots from CV-extracted DNA samples.

Waiting time between CV sampling and results can be exhausting and exposes women to significant psychological stress. Therefore, shortening this time as much as possible is very recommendable and possible even when HA-causative inversions (i.e. Inv22 and Inv1) are involved. Laboratories that routinely undertake *F*8 molecular analysis could save time by setting up both Inv22 and Inv1 diagnostic tests concurrently, using the same *Bcl*I-circles as substrate, PCR mix and thermocycling conditions, and performing analytical electrophoresis on the same gel.

Mainly for laboratories where IS-PCR is the technique of choice to investigate Inv22 and Inv1, IS-PCR may help in two different situations of PND. First,

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as in the case reported here, when one of the inversions (either Inv22 or Inv1) was diagnosed on a carrier mother by using IS-PCR, it should also be applied on fetus CV DNA because it rapidly and robustly provides the same precise information about the molecular rearrangement. Secondly, because of the high frequency of Inv22 and Inv1 in severe HA, simultaneous analysis of both inversions from CV samples may be useful in order to save time when samples from fetus and mother come to the laboratory together with no previous molecular information in a family with history of severe hemophilia. In addition, Inv22 or Inv1 IS-PCR may be used as backup information to confirm or reject any doubtful results obtained using other genotyping methods.

Prenatal diagnosis (PND) for Inv22 and Inv1 were performed without majors technical problems, demonstrating that IS-PCR is well optimized to provide molecular diagnosis from both, peripheral blood- and chorionic villus-extracted DNA.

In conclusion, Inv22/Inv1 IS-PCR provides an alternative approach to investigate *F*8 recurrent inversions from CV samples thus improving PND and genetic counseling for hemophilia-affected families.

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