MODIFIED INVERSE SHIFTING-PCR (IS-PCR) TO INVESTIGATE INTRON 22 INVERSION

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INTRODUCTION

It is well known that F8 intron 22 inversion (Inv 22) is the most important causative mutation in approximately 45% of severe haemophilia A patients. Inv 22 occurs as a result of homologous recombination between copies of a repeated DNA sequence, the intron 22 homologous region (int22h), one copy located within the F8 intron 22 (int22h-1), and the other two extragenic distal inversely-oriented copies (int22h-2 and int22h-3). The frequency of this chromosomal rearrangement suggests that in families with severe haemophilia A, the affected male(s) should first be tested for the presence of the inversion. Since 1993, when Lakich described for the first time this mutation, many methods have been developed to identify it. The inversion is detectable by Southern Blotting or by Long PCR; more recently Rossetti (JTH 2008) developed an Inverse-Shifting PCR method (IS-PCR) that has proved to be reliable in a diagnostic setting (Fig. 1). It comprises BclI restriction enzyme digestion of genomic DNA, followed by self ligations of restriction fragments and multiplex-PCR analysis. Products are then visualised by standard gel electrophoresis.

METHODS

IS-PCR method for molecular diagnosis of Inv 22 was described by Rossetti; in this study we described a modified protocol by Acquila e Bicocchi. DNA was extracted with Gentra Puregene Blood Kit (Qiagen). The starting amount of DNA for each patient is 5-6 µg (minimal quantity 2-3 µg). DNA concentratrion was measured with Thermo Scientific NanoDrop spectrophotometer. DNA was digested with fastBCLI (fermentas) according with producter's instructions. The digested product was purified by an ETOH/NaAc precipitation. The precipitated DNA was resuspended in 20 µl of water. Subsequently, for every patient were conducted two separated reactions : one refered to screening test and one for complementary test. In two different tubes 5-6 µl of digested DNA were ligated with T4 ligase (Roche). The ligated DNA was purified with phenol/chloroform extraction and again with an ETOH/NaAc precipitation. Finally, PCR reactions were assembled directly in ligase tubes. Diagnostic PCR reaction was performed with IU, 2U, 3U, ID primers (12,5pmol/ µl); complementary PCR reaction was prepared with IU, 2U, 3U, ED primers (12,5pmol/ µl). The DNA was amplified with AmpliTaqGOLD DNA polymerase (Applied Biosystems). Thermalcycler conditions were different: we performed a Touch-Down PCR for diagnostic Test and a common PCR for complementary test. Finally, the amplified fragments were analysed in agarose gel 2% (run Time 2-3h 70-80V) with standard electrophoresis (fig.2).

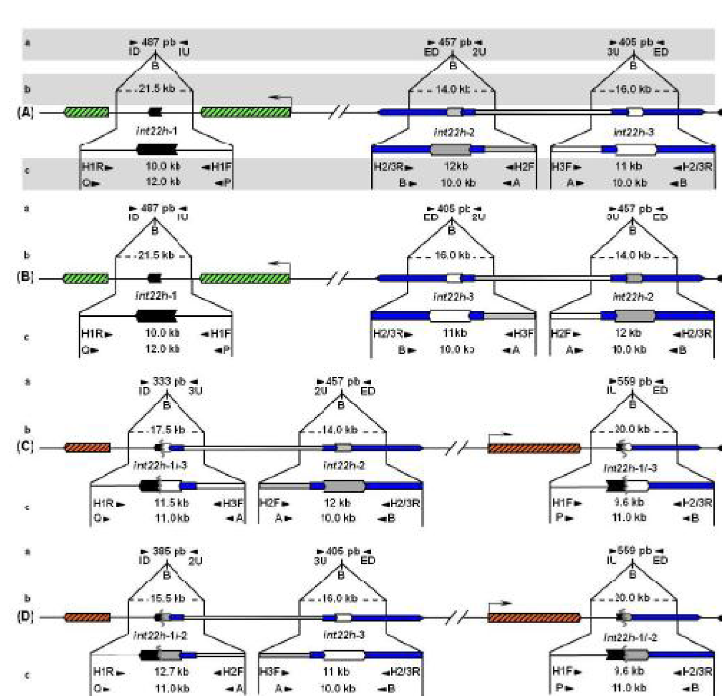

Fig.1: Schematic view of the *F8 int22h* normal gene regions (A, B) and *int22h*-related recombination variants (C-D).
Rossetti et al. *Int. J. Mol. Sci.* **2011**, *12*, 7271-7285

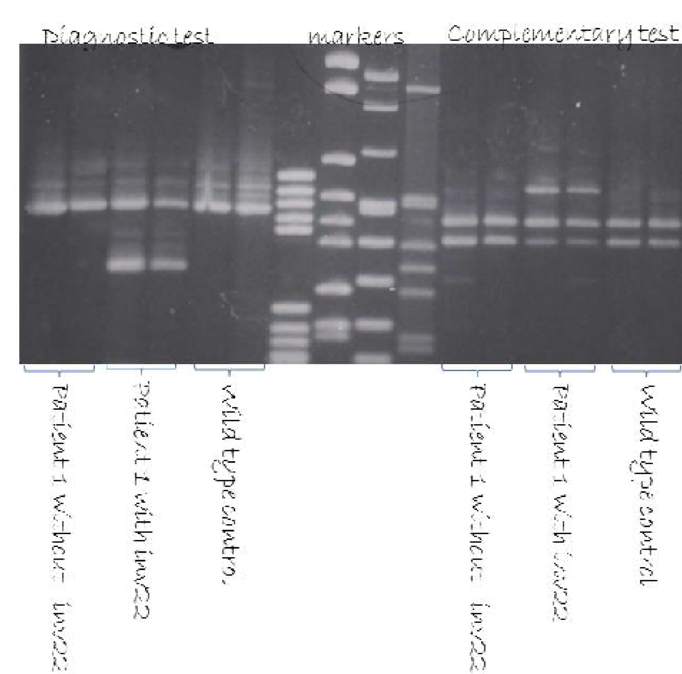

Fig.2: IS-PCR gel electrophoresis

RESULTS AND CONCLUSIONS

We tested IS-PCR modifications on ten new cases of severe affected Haemophilia A patients; in four of them we identified Inv22. We also tested DNA samples extracted from old-aged blood samples conserved at -80°C for at least two years but with unsuccessful results. We performed the protocol also on four suspected carriers: two were confirmed with inv22 in heterozygosis. Inv22 IS-PCR diagnostic tests have proven to be a rapid, robust and reliable technique and could represent the method of choice at first line in severe HA cases.

Rossetti LC, Radic CP, Larripa IB, De Brasi CD. Developing a new generation of tests for genotyping hemophilia-causative rearrangements involving int22h and int1h hotspots in the factor VIII gene. J Thromb Haemost. 2008 May;6(5):830-6

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