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Evaluation of intron 22 and intron 1 inversions of the *factor 8* gene using an inverse shifting PCR method in severe haemophilia A patients

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ABSTRACT: Haemophilia A (HA) is an X-linked recessive bleeding disorder caused by several mutations in the *F8* gene, whose inversion causes most of the mutation in 40% of severe HA patients. Methods such as long distance PCR and southern blot have been exploited to analyse intron 22 inversion in the *F8* gene. For this purpose, we used an inverse shifting PCR assay, a novel genetic diagnostic method, for the detection of intron 22 inversion in severe HA patients. A screen for the presence of intron 22 and intron 1 inversions at the *F8* gene in 30 Iranian severe HA patients revealed that 47% of patients showed intron 22 inversion (40% type 1 and 7% type 2), while 7% of patients had intron 1 inversion. Among the patients carrying the inversion, 12% developed inhibitors. Inverse shifting PCR is a precise method for assessment of rearrangements related to int 22h and int 1h in patients and carriers of haemophilia A. Since the previous methods of assessing inversion are time-consuming and demanding, this method can be a good replacement.

KEYWORDS: *F8*, DNA inversions, mutation

INTRODUCTION

Haemophilia A (HA) is a hereditary bleeding disorder caused by heterogeneous mutations in the *F8* gene leading to deficiency of the activity of blood coagulation factor VIII (FVIII). HA affects 1 in 5000 males[1–3]. Generally males are affected and females are carriers of the disease. All daughters of an affected father are carriers but his sons are normal. For a carrier mother, there is a 50% chance to have a haemophiliac son and 50% chance to have a carrier daughter[4]. Laboratory diagnosis of haemophilia A is performed by clinical tests, in which activated prothrombin thromboplastin time (aPTT) is prolonged, prothrombin time is normal, and the factor VIII antigen level is reduced or normal[5]. The severity of the disease depends on the level of normal F8 activity and is classified as severe cases (<1%), moderate (1–5%), and mild (>5–30%). Among all the HA patients, about 50% have severe form of the disease, while 10% are moderate and 40% are mild[1–4]. The type of bleeding symptoms is related to the severity of the disease. Patients with severe haemophilia A have spontaneous bleeding, but patients with moderate disorder experience infrequent spontaneous bleeding and their bleeding is typically after trauma or surgery. Mild patients have bleeding after surgery or trauma[3,4,6,7]. Anti-FVIII antibodies are the most serious complication that arises in response to infusion of factor VIII concentrates[3,4]. The frequency of inhibitor development in severe patients (20–40%) is higher than mild or moderate (1–13%) HA cases. HA patients with inhibitor formation can use bypassing agents such as recombinant activated factor VII ($rFVII_a$) or activated prothrombin complex concentrate (aPCC) as therapeutic agents[8–11].

The gene encoding factor VIII coagulation protein has 186 kilo base length and is located on chromosome Xq28 and consists of 26 exons and the resulting mature F8 mRNA is approximately 9 kb. Domain organization of the primary translated product of *F8* gene is (NH_2) A_1-A_2-B-A_3-C_1-C_2 (COOH). This single polypeptide chain of 2332 amino acids has a molecular weigh of 300 kDa. The FVIII protein is secreted as a heterodimer with heavy (A_1-A_2-B domains) and light (A_3-C_1-C_2 domains) chains which are related to each other by divalent metal ions[4,12–14].

Several different mutations in *F8* gene cause HA

such as deletion (del), gene inversion (inv), point mutations (missense and nonsense), and translocation[7,15]; with 30–35% of severe HA patients possess point mutations and deletion/insertion playing a minor role. Inversion of intron (int) 22 is the most HA causative mutation in 40–45% of severe haemophiliacs and 2–5% of severe HA cases are affected by intron 1 inversion[4,16,17].

Intrachromosomal recombination between intron 22h-1 within the *F8* gene (9.5 kb) and its duplicons int 22h-2 and int 22h-3 which lie 500 kb and 600 kb distal to *F8* gene, respectively, causes *F8* gene inversions. Int 22h-2 involved in proximal type (type II) of inversion 22 and int 22h-3 involved in distal type (type I) of inversion 22. Inv 22 type 1 has higher frequency than Inv 22 type 2. Similar recombination between 1041 bp int 1h-1 of *F8* gene and its inverted copy, int 1h-2 (140 kb distal), leads to Inv 1[4,18–20].

Genetic methods for mutations analysis in HA patients are divided into direct and indirect tests. In indirect analysis (Linkage Analysis) intragenic and extragenic polymorphisms of factor *VIII* gene are tested. This method is simple but DNA samples or profiles of all family members of patients required for Linkage Analysis, is not always available.

Direct analysis includes southern blot, polymerase chain reaction (PCR) and sequence analysis. Due to the fact that about 45% of sever HA cases is caused by inversion of *F8* gene intron 22 so usually for molecular analysis of this type of patients, inversion mutation must be firstly detected. Therefore, among useful techniques, PCR and southern blot analysis are frequently used to detect inversion, but each of them had their own problems[4]. Rossetti et al[18] had used inverse shifting PCR (IS-PCR) for evaluation of 3 groups. The first group includes patients previously analysed by southern blot and the second group includes patients previously screened by IS-PCR[21]. The obtained results from IS-PCR analysis in both group agreed with previous findings, also inversion 22 detection by IS-PCR in patients of group 3 which had not been studied previously, was done. IS-PCR can be used for molecular diagnosis of inversion 22 type 1, type 2, inv 1, del 22, and duplication (dup) 22. This method can also assess carriers[18].

In this study we used IS-PCR for rapid and precise detection of inversion mutation in severe haemophilia A in patients from Isfahan also to show the probability of inhibitor development in severe type versus other types of HA, inhibitor development in the picked severe HA patients on the basis of the laboratory previously reported results was also assessed.

MATERIALS AND METHODS

Samples and DNA extraction

To evaluate the efficiency of IS-PCR test in determining intron 22 inversion, 30 severe HA patients ($<$1% *FVIII* activity) from Isfahan, Iran were included in the study. After obtaining the informed consent from the patients, 5–10 ml of peripheral blood were collected in tubes containing EDTA. Genomic DNA was extracted from peripheral blood leukocytes by salting-out procedure[22]. DNA quality and quantity were assessed by UV-spectrophotometry (260 nm and 280 nm) or agarose gel electrophoresis. Clinical and laboratory findings including the presence of autoantibody (inhibitor) were gathered from clinical files.

IS-PCR amplification

To analyse introns 22 and 1 inversion, IS-PCR was used as described by Rossetti et al[18], briefly, the genomic DNA was incubated with *Bcl*I enzyme, the digested fragments were self-ligated to provide cyclic DNA and finally, PCR analysis was performed using specific primers. To provide cyclic DNA, 2 µg of genomic DNA was digested with 20 units of *Bcl*I (Fermentas, Canada) for 4–5 h in a 50 µl reaction. Fragmented DNA was extracted using phenol-chloroform and ethanol precipitation. Self-ligation of DNA fragments was performed overnight at 15 °C in a total volume of 400 µl with 3 units of T4 DNA Ligase (Fermentas, Canada) and the ligation buffer and ddH_2O. Ligated fragments were purified using phenol-chloroform or chromatography columns and then the ethanol-precipitated DNA was recovered in 15–30 µl of distilled water. PCR was performed in a volume of 25 µl containing 3 µl and 6 µl of circularized DNA for assessing Inv1 and Inv 22, respectively, 0.6 µM of each primer, 0.5 U of *Taq* DNA polymerase (Cinnagen, Iran), 200 µM dNTP, 1.5 mM $MgCl_2$, 10$\times$ PCR buffer (KCl 10 mM, Tris-Cl 67 mM). The primers characteristics are given in Table 1.

Thermocycling program for inv 22 and Inv 1 IS-PCR amplification was: 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 1.30 min, in 32 cycles, Initial denaturation at 94 °C for 2 min and final extension at 72 °C for 5 min. The IS-PCR products were then electrophoresed on a 1.5% agarose gel and visualized under UV light. Positive Inv 22 samples (type I and II) previously analysed by southern blot method, were used as a positive control.

RESULTS

To evaluate the prevalence of *factor VIII* gene inversion, a group of 30 severe HA patients were analysed

Table 1 The primer characteristics of Inv 1 and Inv 22.

Inv 1			Inv 22		
Primer	Sequence	NC_000023.9	Primer	Sequence	NC_000023.9
1-IU	GCCGATTGCTTATTTATATC	153 899 635–54	ID	ACATACGGTTTAGTCACAAGT	153 758 587–608
1-ID	TCTGCAACTGGTACTCATC	153 886 959–77	IU	CCTTTCAACTCCATCTCCAT	153 779 730–50
1-ED	GCCTTTACAATCCAACACT	154 030 453–71	2U	ACGTGTCTTTTGGAGAAGTC	154 270 775–95
			3U	CTCACATTGTGTTCTTGTAGTC	154 333 426–48

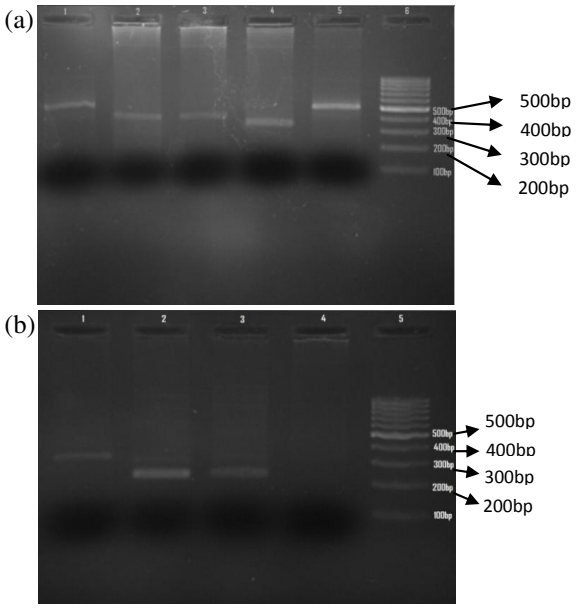

Fig. 1 Agarose gel electrophoresis of IS-PCR. (a) Inv 22 diagnostic test; lanes 1 and 5: normal control; lanes 2 and 3: Inv 22 type 2; lane 4: Inv22 type 1. (b) Inv 1 diagnostic test; lane 1: normal control; lanes 2 and 3: Inv 1 positive sample; lane 4: negative control without template.

by IS-PCR molecular diagnostic method. Among the patients studied, 14 patients (47%) had inversion of intron 22 (12 Inv 22 type 1 and 2 Inv 22 type 2) (Fig. 1a). The remaining 16 severe HA patients were screened for intron 1 inversion. The frequency of intron 1 inversion in this study was 2/30 (7%) (Fig. 1b). Two patients (12%) of positive inversion cases had developed inhibitors.

DISCUSSION

Severe HA is a life-threatening form of the disease occurs in 45% of patients and is accompanied with the risk of formation of autoantibody inhibitors. In addition, 20–40% of severe HA patients[9] and 30% of patients with inversion, large deletions, and nonsense mutations in *F8* gene[4,23] may develop inhibitors.

Inversion of *F8* intron 22 and intron 1 is the cause of HA in 50% of severe forms of the disease. Therefore, the evaluation of the inversion is the first step in mutation analysis in families with severe HA. Different methods have been developed to detect inversions, including PCR and southern blotting[4]. Southern blot analysis can evaluate carriers and detects intron 22 and intron 1 inversion but this method is slow and is labour-intensive and dangerous because the use of radiochemicals. Besides, southern blot require larger amount of DNA than PCR analysis[4,21,24].

Long distance PCR (LD-PCR) permits the analysis of long sequences of DNA and can analyse the inversion, deletion, and translocation in the genome. LD-PCR is sensitive to the quality of template DNA but there is no such sensitivity in IS-PCR method. DNA amplification and slow electrophoresis in LD-PCR technique requires long time. IS-PCR avoids PCR amplification through long and GC rich sequences of *F8* gene duplicons associated with intron 22h and intron 1h that these sequences may be amplified in LD-PCR[18,21].

Recently, IS-PCR method has been developed[18] and applied for the detection of int 22 inversion in different studies[25,26]. In this study we used IS-PCR assay to evaluate the presence of int 22 and int 1 inversion in severe HA patients from a single centre in Isfahan, Iran. In this study, we found 12 (40%) Inv 22 type 1 and 2 (7%) Inv 22 type 2 among 30 severe HA patients, indicating that the frequency of Inv 22 distal type is higher than that of the proximal type. Overall the prevalence of Inv 22 is 47% that is in agreement with the previous studies[19,27–30]. In the study performed by Polakova et al, the inversion mutation was identified in 50% of patients with severe HA. The inversions of distal type were more frequent (83%) than those of proximal type (17%)[29].

To validate inv 22 detection in the studied severe HA patients, selected patients were previously analysed by southern blot and concordance between the results obtained from both methods were seen[18].

IS-PCR method can identify all DNA structural rearrangements related to int 22h and int 1h like large deletion, inversion, translocation and duplica-

tion. This technique is a robust and low cost method that facilitates the analysis of HA patients and carrier detection. This method can also be used in prenatal diagnosis of the disease.

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