Original Article

Iran J Ped Hematol Oncol. 2016, Vol6.No3, 182-189

Genotyping of Intron 22 and Intron 1 Inversions of Factor VIII Gene Using an Inverse-Shifting PCR Method in an Iranian Family with Severe Haemophilia A

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Received: 26 March 2016 **Accepted:** 10 July 2016

Abstract

Background: Haemophilia A (HA) is an X-linked bleeding disorder caused by the absence or reduced activity of coagulation factor VIII (FVIII). Coagulation factors are a group of related proteins that are essential for the formation of blood clots. The aim of this study was to genotype the coagulation factor VIII gene mutations using Inverse Shifting PCR (IS-PCR) in an Iranian family with severe Haemophilia A.

Material and Methods: Genomic DNA was extracted from blood of Iranian family members with severe hemophilia A and then was genotyped using specific primers by Inverse Shifting PCR method and was analyzed by sequencing for all FVIII exons.

Results: Sequence analysis of *F8* gene revealed two distinct mutations. The first mutation was a C-to-G transition at 3780 position in exon 14, which cause an Asp1240 Glu in the region encoding the B domain of FVIII. It seems that this mutation could be a polymorphism. The second mutation was a 2-bp AA deletion in exon 18 (nt. 5820-5823, del. AA). The patient's mother and sister were also heterozygous for 2bp AA deletion. This deletion caused a frame shift in exon 18 and terminated after 29 amino acids for a premature stop codon.

Conclusions: Based on the results, it can be concluded that two IS-PCR and genomic sequencing techniques are robust and low cost method that facilitates the analysis of HA patients and carrier detection.

Key words: Hemophilia A, Coagulation Factor VIII Gene, mutation

Introduction

Haemophilia A (HA) is an X-linked bleeding disorder caused by the absence or reduced activity of coagulation factor VIII (FVIII) (1). Coagulation factors are a group of related proteins that are essential for the formation of blood clots (2). Factor VIII deficiency is caused by broad spectrum mutations, which occurs along the entire length of the factor VIII gene. Mutation in F8 gene includes the intron 22 and intron 1 inversion, point mutations (nonsense and missense mutations), deletions, and insertions (3). The mutations lead to defect at the level of transcription or translation or to change of individual amino acids in factor VIII protein (3, 4). At the present, more than 1209 mutations within the F8 coding and un-translated regions have been identified and listed in the F8 HAMSTeRS mutation database. The most recent update of the HAMSTeRS database (30 November, 2012) listed more than 357 small deletions, 255 large deletions and more than 146 insertions as being causative for Hemophilia A. The recurrent insertions or deletion of a single A has also been

reported at shorter stretches of A nucleotides (5, 6). Inversions cause about half of the cases of severe hemophilia A. Inversion mutation reported in intron 22 in 48 to 52% in intron 1 in 4-5% of patients with severe hemophilia A. The intron 22 of the FVIII gene contains a 9.5 kb region; int22h1, which is present outside of the gene (7). Insertions and deletions that cause HA are classified into large (more than 50 bp) and small which usually span one or several nucleotides. Various types of repetitive sequences, including SINES and LINES, which are present in the factor VIII gene, may be involved (8).

Currently, the Inv22 can be detected either by Southern blot analysis or by long-distance PCR (LD-PCR) (9). Given the short comings of these detection methods, a new system for Inv22 analysis can be IS-PCR that is used for Inv22 and Inv1 genotyping (10, 11). The main objective of this study was to genotype the coagulation factor VIII gene mutations using inverse-shifting PCR in an Iranian family with severe Haemophilia A.

Materials and Methods

Patients

In this experimental research, an Iranian family member with severe hemophilia A was choice to investigation of Intron 22 and 1 Inversions of Factor VIII Gene. All participants provided written informed consent which was approved by the institutional review board before participation.

Genotyping

Five ml of peripheral blood was collected in tubes containing EDTA. Genomic DNA was extracted by commercial DNA extraction kit (Qiagen, Germany). Then quality and quantity of extracted DNA were assessed by nanodrop and agarose gel electrophoresis, respectively. Molecular analysis was performed using the following strategy. Patients with severe HA (FVIII: C< 1%) were first screened for intron 22 (Inv22) and intron 1 (Inv1) inversions using an Inverse Shifting Polymeras chain reaction (IS-PCR) method. Mutations were compared to those previously described on the HAMSTeRS mutation register. Briefly, 2 µg of genomic DNA was digested with 20 units BclI enzyme (Fermentas, Canada) for 4 h in a 50 µl reaction buffer. Self-ligation of digested fragments was performed overnight at 15°C in a total volume of 400 µl with three units of T4 DNA Ligase (Fermentas, Canada). Fragmented DNA was extracted once in phenol–chloroform and once in chloroform–isoamyl alcohol (24:1 by volume), precipitated in 0.3 mol/L NaCl with 2 volumes of ethanol, and re-suspended in 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 enzyme (Cinnagen, Iran), 200 µM dNTP, 1.5 mM MgCl2, 10× PCR buffer with the following step: a denaturing at 94°C for 2 min, 32 cycle at; 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 90s and a final extension step at 72°C for 5 min.. The IS-PCR products were then separated on a 2% agarose gel elecrophoresis and visualized under UV light. The primers characteristics are given in Table 1. Exons amplification of the *F8* gene was done with a standard primer set of 37 primers related to regions of the *FVIII (F8)* gene under the same thermal cycling conditions. The primers for each PCR have a common tail to allow the use of one complementary sequencing primer in all forward sequencing reactions. PCR reactions for exon amplication region was performed under identical conditions: 95 °C for 2 minutes followed by 30 cycles of 94 °C for 30 S, 59 °C for 45 S and 72 °C for 90 S, and final extension at 72 C for 5 min. All PCR products were separated electrophoretically in 1% agarose gel.

Results

In this study, inversions in the *FVIII (F8)* gene intron 1 and 22 of prob and and her

sister (Figure 1) by IS-PCR molecular diagnostic method. However, results showed that they were normal for introns 1 and 22 (Figure 2). Sequence analysis revealed a 2 bp AA deletion in exon 18 and a missense mutation in exon 14. These mutations were confirmed in patient's mother and sisters. The 2 bp AA deletion in exon 18 was a novel mutation that has not been reported yet. The mutation in exon 18 was a new mutation that has not been reported yet. This mutation caused a stop codon after 87 nucleotides that mean the protein sequence terminated after 29 amino acids and produced an incomplete protein. In addition, the identified mutation in exon 14 was a missense mutation, C-to-G transition at position 3780 at the exon. Figure 3 and 4 show IS-PCR gel electrophoresis and figure 5 and 6 demonstrate the DNA sequence analysis of the exons.

Table I: The characteristic of primers used in this study

Primers	Sequence 5'-3'	Product size (bp)
Intron 1		
1-IU	GCCGATTGCTTATTTATATC	304
1-ID	TCTGCAACTGGTACTCATC	304
1-ED	GCCTTTACAATCCAACACT	244
Intron 22		
ID	ACATACGGTTTAGTCACAAGT	487
1U	CCTTTCAACTCCATCTCCAT	487
2U	ACGTGTCTTTTGGAGAAGTC	333
3U	CTCACATTGTGTTCTTGTAGTC	385

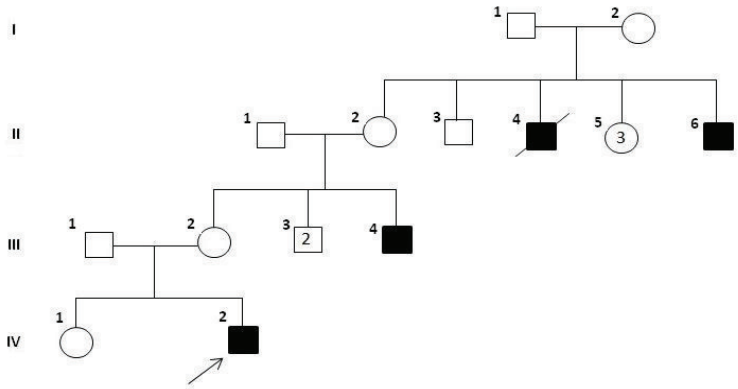

Figure 1**.** Pedigree of patient with severe Haemophilia A, his factor VIII clotting activity is lower than 1%.

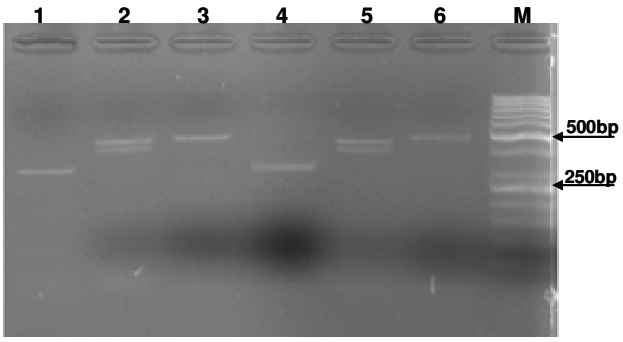

Figure 2. The IS-PCR products from a severe HA patient and his family using 1-ID and 1-IU primers. Line 4-6: Patient, Lines 1-3: Her sister; Line 1 and 4: Show 304bp band (1-ID and 1-IU primers) that indicate the normal band for intron 1 and 22. Line 2 and 5, 487bp band that is normal for both introns. Line 3 and 6 show a fragment of 457bp (2U and ED primer) and 405bp (ED and 3U), respectively that are normal for intron 22. Line 7: DNA ladder (50bp).

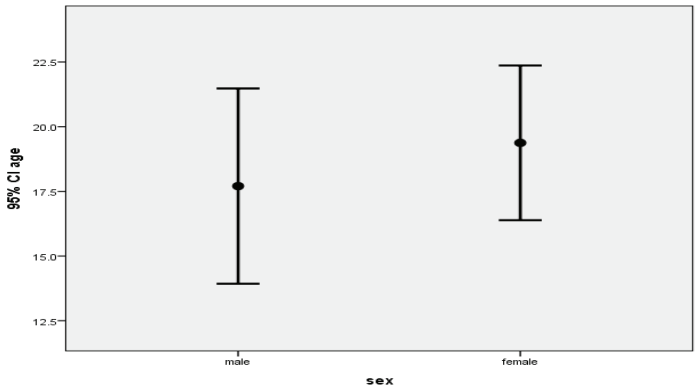

Figure 2. Average age according to sex (Error Bar Accordigto the confidence interval

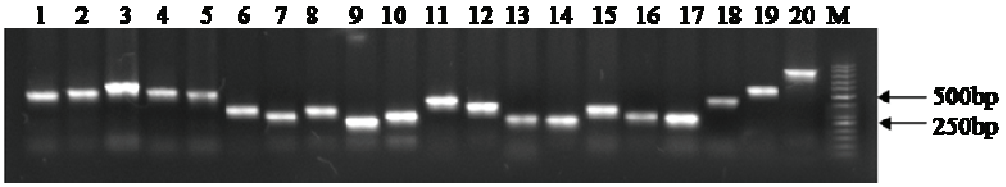

Figure 3. One percent agaros gel electrophoresis of PCR products. line 1-20 show exons 14E, 14D, 15C, 14B, 14A, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1 , promote 1 and promoter 2, respectively. The sizes of these exons are 570,595, 695, 599, 567, 364, 298, 361, 250, 300, 448, 400, 258, 259, 372, 299, 290, 480, 600, and 896 bp, respectively. In the last line loaded DNA ladder 50bp. The last line is DNA ladder 50bp.

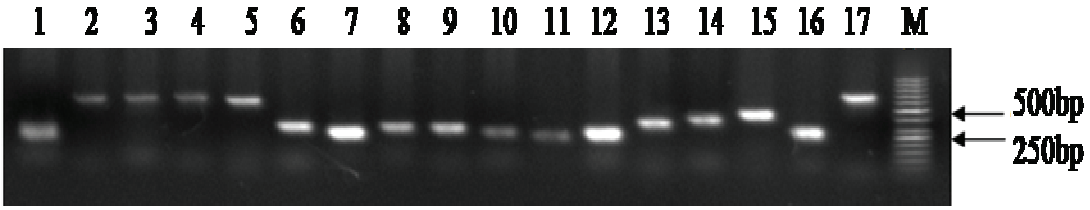

Figure 4. One percent agaros gel electrophoresis of PCR products. line 1-17 show exons 26E, 26D,26C,26B, 26A, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14F, respectively. The sizes of these exons are 230, 580, 580, 596, 557, 300, 250, 294, 287, 261, 228, 248, 262, 397, 468, 299, and 686 bp, respectively. The last line is DNA ladder 50bp.

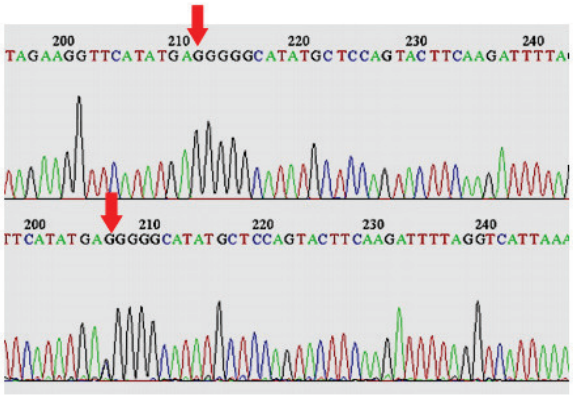

Figure 5. Exon 14 sequence analysis by reverse primer. A: patient. B: her mother. Red arrows show the mutation sites. The mother was heterozygote (c.3780C>G).

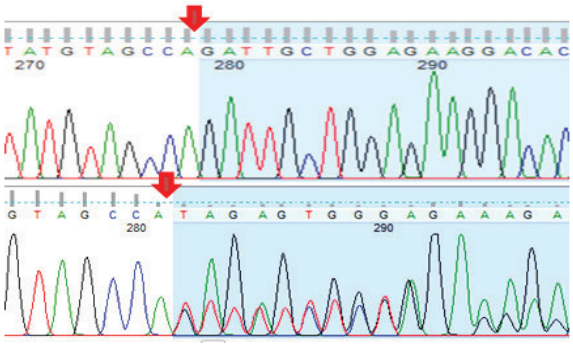

Figure 6. Exon 18 sequence analysis by reverse primer. A: patient. B: her sister. The red arrows show mutation location.

Discussion

The human *F8* gene was cloned during1982–1984. However, the identification of the mutations, the causal agents of hemophilia was performed in 1993 years (10, 11). To date, wide diversity of practical approaches including Southern blot, distance PCR (LD-PCR), and inverse shifting-PCR have been used for genotyping the Inv22. LD-PCR permits the analysis of long sequences of DNA and can analyze the inversion, deletion, and translocation in the genome. Recently, IS-PCR method has been developed and applied for the detection of int 22 inversion in different studies. Therefore, the genome sequencing, southern blot analysis, LD-PCR, and IS-PCR techniques for Inv22 genotyping are interesting example of evolution in order to resolve challenging molecular diagnostic problems (10).

IS-PCR method can identify all DNA structural rearrangements related to intron 22 and intron 1 including large deletion, inversion, translocation, and duplication. In this study, IS-PCR assay was used to evaluate the presence of int 22 and int 1 inversions in severe HA patients family. Intron 22 inversions are estimated to be the cause of 40% of severe cases of hemophilia A (12-14). It seems that Intron 22 inversions occur at a 10 fold higher rate in male germ cells. Intron 22 contains 32 kilobases (kb) and is composed of two nested genes; namely, F8A and F8B. Furthermore, intron 22 contains a 9.5kb region which is also present outside of the *FVIII* gene, near two extragenic copies located 400kb 5' to the *FVIII* gene (15, 16).

Results of present study showed that the family members with HA were normal for introns 1 and 22. In addition, a new missense mutation at position 3780 exon 14 that caused Aspartic acid to Glutamic acid (Asp to Glu) was found. The second mutation was a 2-bp deletion AA in exon 18 (nt. 5820-5823, del. AA). This deletion caused a frame shift in exon 18 and terminated after 29 amino acids for a premature stop codon. In this family, the proband's mother and sister were heterozygous for this deletion. In a study of 52 patients with unknown mutations, Traystman et al. (1990) Polymerase chain reaction technique and denaturing gradient gel electrophoresis (DGGE) was used to characterize single nucleotide substitutions in the exon 8, the 3-prime end of exon 14, exon 17, exon 18, and exon 24(16).) A deletion of a single nucleotide T within an A(8)TA(2) sequence of exon 14 of the F8 gene was found In Japanese family with mild to moderately severe hemophilia A (17). They have observed that the severity of the clinical phenotype did not correspond to that expected of a frame shift mutation. An insertion of L1 elements into exon 14 of the *F8* gene in 2 of 240 Iranian unrelated patients with severe hemophilia A was detected (18).

The results of this study indicate that IS-PCR system is an ideal diagnostic tool for rapid genotyping of Inv 2 in *F8* gene. Fujita et al., (2012) have reported IS-PCR Inv22 diagnostic tests revealed that these patients carried either type I or II Inv22. However, they showed a wild-type (WT) pattern in the IS-PCR Inv22 complementary tests (19). In another study, 30 Iranian severe HA patients for the presence of intron 22 and intron 1 inversions at the *F8* gene were screened. Their results 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 (20). A total of 50 HA patients (including 35 non- Inv22, 14 Inv22-1, and one Inv22-2 patients) and 7 HA carriers were diagnosed by using modified inverse-shifting polymerase chain reaction and

capillary gel electrophoresis approaches which showed these methods could be considered as an ideal diagnostic tool for rapid genotyping of Inv2 in *F8* gene (21).

Conclusion

Based on the obtained results, it can be concluded that two IS-PCR and genomic sequencing techniques are robust and low cost method that can facilitate the analysis of HA patients and carrier detection. In addition, a double mutation in the FVIII gene (F8) in an Iranian family with severe HA was found.

Acknowledgements

The authors have great thanks to all laboratory expert of Genetic Research Center of Meybod, Yazd, Iran.

Conflicts of Interest

All authors disclose any financial and personal relationships with other people or organizations and the authors declare that there is no potential conflict of interest.

References

1. Flood E, Pocoski J, Michaels LA, Bell JA, Valluri S, Sasanè R. Illustrating the impact of mild/moderate and severe haemophilia on health-related quality of life: hypothesised conceptual models. European Journal of Haematology. 2014; 93(75):9–18.

2- Berntorp, E., &Shapiro, A.D. Modern haemophilia care. The Lancet. 2012, 379(9824):1447-56.

3. Tantawy AG. Molecular genetics of hemophilia A: Clinical perspectives. Egyptian Journal of Medical Human Genetics, 2010 Nov 3;11(2):105-14.

4. Renault NK, Dyack S, Dobson MJ, Costa T, Lam WL, Greer WL. Heritable skewed X-chromosome inactivation leads to haemophilia A expression in heterozygous females. European Journal of Human Genetics, 2007 Jun;15(6):628-37.

5. Konkle BA, Josephson NC, Fletcher SN, Hemophilia A. GeneReviews [Internet]. 2014 Jun 5.

6. Kessler L, Adams R, Mighion L, Walther S, Ganguly A. Prenatal diagnosis in haemophilia A: experience of the genetic diagnostic laboratory. Haemophilia, 2014 Nov;20(6):384-91.

7. Lam EPT. Clinical Applications of Molecular Technologies in Hematology. Journal of Medical Diagnostic Methods. 2013 Aug 16;4;2(4):2-6.

8. Andrikovics H, Klein I, Bors A, Nemes L, Marosi A, Váradi A, Tordai A. Analysis of large structural changes of the factor VIII gene, involving intron 1 and 22, in severe hemophilia A. Haematologica. 2003 Jul 21;88(7):778-84.

9. Rossetti LC, Radic CP, Abelleyro MM, Larripa IB, De Brasi CD. Eighteen years of molecular genotyping the hemophilia inversion hotspot: From southern blot to inverse shifting-PCR. International Journal of Molecular Science. 2011 Oct 24;12(10):7271-85

10. Rossetti LC, Radic CP, Larripa IB, De Brasi CD. Genotyping the hemophilia inversion hotspot by use of inverse PCR. Clinical Chemistry. 2005 Jul 3;51(7):1154-8.

11. Miyawaki Y, Suzuki A, Fujimori Y, Takagi A. Severe hemophilia A in a Japanese female caused by an F8-intron 22 inversion associated with skewed X chromosome inactivation. International Journal of Hematology. 2010 Sep 22;92(2):405-8.

12. Oldenburg J, Ananyeva NM, Saenko EL. Molecular basis of haemophilia A. Haemophilia. 2004 Oct 4;10 (4):133-9.

13. Sabatino DE, Nichols TC, Merricks E, Bellinger DA. Animal models of hemophilia. Progress in Molecular Biology and Translational Science. 2012;105:151-209.

14. El-Maarri O, Herbiniaux U, Graw J, Schröder J, Terzic A, Watzka M, et al.. Analysis of mRNA in hemophilia A patients with undetectable mutations

reveals normal splicing in the factor VIII gene. Journal of Thrombosis and Haemostosis. 2005 Feb 3;(2):332-9.
15. Bagnall RD, Waseem N, Green PM, Giannelli F. Recurrent inversion breaking intron 1 of the factor VIII gene is a frequent cause of severe hemophilia A. Blood. 2002 Jan 1;99(1):168-74.
16. Traystman MD, Higuchi M, Kasper CK, Antonarakis SE, Kazazian HH Jr. Use of denaturing gradient gel electrophoresis to detect point mutations in the factor VIII gene. Genomics. 1990 Feb 6;(2):293-301.
17. Young M, Inaba H, Hoyer LW, Higuchi M, Kazazian HH Jr, Antonarakis SE. Partial correction of a severe molecular defect in hemophilia A, because of errors during expression of the factor VIII gene. American Journal of Human Genetics. 1997 Mar 6;60(3):565-73.
18. Kazazian HH Jr, Wong C, Youssoufian H, Scott AF, Phillips DG, Antonarakis SE. Haemophilia A resulting from de novo insertion of L1 sequences represents a novel mechanism for mutation in man. Nature. 1988 Mar 10;332(6160):164-6.
19. Fujita J, Miyawaki Y, Suzuki A, Maki A, Okuyama E, Murata M, A possible mechanism for Inv22-related F8 large deletions in severe hemophilia A patients with high responding factor VIII inhibitors. Journal of Thrombosis and Haemostosis. 2012 Oct 10;(10):2099-2107.
20. Roozafzay N, Kokabee L, Zeinali S, Karimipoor M. Analysis of Intron 1 Inversion at F8 Gene in Severe Hemophilia A Patients by Inverse Shifting-PCR Referred from Isfahan Seyedolshohada Hospital. Journal of Ardabil University of Medical Science. 2013 May 13 (1):93-101.
21. Pan TY, Chiou SS, Wang CC, Wu SM. Separation of intron 22 inversion type 1 and 2 of hemophilia A by modified inverse-shifting polymerase chain reaction and capillary gel electrophoresis. Talanta. 2014 Dec 4;130:328-35.