Genotyping of Intron Inversions and Point Mutations in Exon 14 of the *FVIII* Gene in Iranian Azeri Turkish Families with Hemophilia A

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Abstract Hemophilia A (HA) is an inherited X-linked bleeding disorder caused by a variety of mutations that are distributed throughout the large *FVIII* gene (F8). The most common mutations in studied populations with severe HA are introns 22 and 1 inversions, gross exon deletions and point mutations in exon 14. The aim of this study was to define the frequency of these common mutations in Iranian population of Azeri Turkish in North West of Iran. Fifty patients with severe HA and forty-three female potential carriers were genotyped by inverse shifting polymerase chain reaction (IS-PCR), long-range PCR, multiplex PCR, and sequencing methods for the detection of Intron 22 and 1 inversions, gross exon deletions, and exon 14 point mutations, respectively. *F8* intron 22 inversion was detected in 22 (44 %) out of 50 patients. Moreover, we detected one intron 1 inversion (2 %), and one point mutation in exon 14 (2 %). In this population, 52 % of the patients with hemophilia A did not show to carry a mutation in the analyzed regions by three mentioned methods. *F8* intron 22 inversion was the major causative mutation in nearly 50 % of severe HA cases in an Azerbaijani Turkish population, which is similar to the incidence of other populations. IS-PCR is a robust, rapid, efficient, and cost-effective method for the genetic analysis of patients with

severe HA and for HA carrier detection, especially in developing countries.

Keywords *FVIII* gene · Hemophilia A · Intron 22 inversion · IS-PCR · Long-range PCR

Introduction

Hemophilia A (HA) is, one of the most common bleeding disorders in humans, that is inherited in an X-linked pattern. This disorder caused by a variety of mutations in the coagulation factor VIII gene (*F8*). Among males at birth, the incidence of this disease is 1 per 5000 in most populations [1]. The *F8* is large and has a complex structure. It is 186 kb in length and includes 26 exons, and is located on the end of the long arm of the X chromosome at Xq28 [2].

The most common cause of severe HA is the large DNA inversion of intron 22 (Inv 22) of the *F8*, which occurs in approximately half of the patients with this mutation condition. This defect arises through homologous recombination between identical segmental duplications, or duplicons, that are inversely oriented within the *F8* (i.e., int22h). In other words, Inv 22 occurs when a 9.5-kb segment of intron 22 (int22h1) located in the *F8* locus recombines with either of its two identical sequence copies that lie telomerically within the *F8* [3]. Recombination between the intronic segment and the extragenic proximal segment (int22h3) results in Inv 22 type 1 (or proximal) inversion, and recombination of the intronic segment with the distal segment (int22h2) results in Inv 22 type 2 (or distal) inversion. These recombinations occur because of unequal crossing over between segments [4]. According to the findings reported by Rossiter et al. [5], unequal crossover in most cases happens in meiotic divisions of male

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germ cells, but there is a probability that the error might be in mitotic divisions as well as in female divisions.

Another, less common mutation, but one that is important in the occurrence of HA, is the inversion of intron 1 (Inv 1). Intron 1 of the *F8* contains a 1-kb region (int1h1) that has one other copy (int1h2) in the reverse orientation approximately 140 kb telomeric to the *F8*. Recombination between intronic and extragenic copies results in the Inv 1 mutation [6]. The other mutations, such as exon skipping, gross exon deletions, and point mutations, also have an important role in HA occurrence.

In almost all previously reported studies, the most common mutations in severe HA were Inv 22, Inv 1, gross exon deletions, and point mutations in exon 14. In these studies, carrier detection in families with HA was carried out based on genetic tests such as polymerase chain reaction (PCR)-amplified restriction fragment length polymorphism, and detection of Inv 22 and Inv 1 was performed with current methods such as Southern blot analysis [7] and long-range PCR [8]. Although Southern blot analysis is the gold standard method for detection of Inv 22 and long-distance PCR is a powerful tool, these methods present some difficulties in technique and results [9]. Efficient and cost-effective inverse shifting PCR (IS-PCR), as designed by Rossetti and colleagues, resolves all of these difficulties and provides a standardized method for detection of Inv 22 rearrangements [10].

In light of the heavy burden of HA on the health system in Iran, we focused on the genotyping of HA rearrangements and other common mutations in patients with severe HA in an Azeri Turkish population in the Northwest of Iran.

Material and Method

Samples and DNA Extraction

A total of 83 Azeri Turkish subjects from 50 unrelated families with HA, including 50 patients with severe HA and 33 female potential carriers were studied. They were referred from hemophilia centers in the Northwest of Iran to the outpatient clinic of Tabriz University of Medical Sciences. Following approval of the study by the ethics committee of Tabriz University of Medical Sciences, informed consent was obtained from every participating patient and family members. Clinical evaluation and coagulation and immunologic assays to measure F8 coagulant activity of patients with HA were performed at the referring centers. The results of these examinations were categorized according to a standard protocol, with the F8 activity classified as mild (>5 %), moderate (1–5 %), or severe (<1 %). Whole blood in 5–7-ml aliquots was

collected from the subjects and stored at 4 °C until being processed [11].

Genotyping of int22h-Related Rearrangements by IS-PCR

For detection of Inv 22 rearrangements, the IS-PCR protocol of Rossetti and colleagues was applied [10]. This method is a variant of inverse PCR [12]. In brief, genomic DNA was subjected to *Bcl*I restriction enzyme digestion for 4 h in a 50-μL reaction. Digested DNA was isolated using phenol–chloroform and precipitated with ethanol. Digested DNA fragments were treated with 1.5 μL of T4 DNA ligase in 100 μL ligation Mix containing 90 μL water, 10 μL T4 DNA ligase buffer at 15 °C overnight. Purification of circularized fragments was performed using equal volumes of a phenol–chloroform mixture; the upper aqueous phase was removed; and ethanol-precipitated DNA was resolved in 30 μL of distilled water. Primers were designed as previously described by Rossetti and colleagues [10]. The PCR thermal program for detection comprised 30 cycles of denaturation at 94 °C for 30 s, annealing at 56.5 °C for 45 s, and an extension at 72 °C for 1 min. Cycling was initiated with the first denaturation at 94 °C for 2 min and ended with a final extension at 72 °C for 5 min. IS-PCR products were analyzed by 1.5 % agarose gel electrophoresis [10].

Genotyping of int1h-Related Rearrangements by Long-Range PCR

Inv 1 was detected by using a PCR method previously reported by Bangall and coworkers [13]. In brief, the PCR final reaction volume of 20 μL containing approximately 100 ng of genomic DNA, 5 pmol/L of each primer, 2.5 μL of 10 × PCR buffer, 0.25 mM of each deoxynucleotide (dNTP), 1.5 mM MgCl₂, and 4 U of SmarTaq DNA polymerase (CinnaGen, Tehran, Iran) in the following conditions: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 40 s, annealing at 56 °C for 40 s, an extension at 72 °C for 2 min, and a final extension at 72 °C for 10 min. Analysis of the PCR product was performed using 1 % agarose gel.

Gross Exon Deletion Analysis by Multiplex PCR

All 26 exons and exon–intron junctions were analyzed for gross exon deletions mutation by performing eight multiplex PCR assays. All of the primers (Table 1), amplification conditions, annealing temperatures, and product sizes have been published previously by Hwang et al. [14].

Table 1 Used primers for detection of gross exon deletion in F8

Set	Primer	Exon	Product size (bp) ^a	Primer sequence (forward)	Primer sequence (reverse)
Set 1	1	20	228	TTTGAGAAGCTGAATTTTGTGC	GAAGCATGGAGATGGATTTCATTA
	2	22	287	TCAGGAGGTAGCACATACAT	GTCCAATATCTGAAATCTGC
	3	11	362	CCCTTGCAACAACAACATGA	TTTCTTCAGGTTATAAGGGGACA
	4	08	484	CACCATGCTTCCCATATAGC	ATGGCTTCAGGATTGTGTTGG
	5	14 B	599	GATCCATCACCTGGAGCAAT	GGGCCATCAATGTGAGTCTT
Set 2	6	26 E	230	CCCCAAAGGTGATATGGTTTT	TCAGTGTTCACATTTTTATTTC
	7	02	290	CATTACTTCCAGCTGCTTTTTG	TTTGGCAGCTGCACCTTTTTA
	8	18	362	TGGTGGAGTGGAGAGAAAGAA	AGCATGGAGCTTGTCTGCTT
	9	26 A	557	CTGTGCTTTGCAGTGACCAT	TTCTACAACAGAGGAAGTGGTGA
Set 3	10	19	248	AACCAATGTATCTCATGCTCATTTT	GGAAGAAAGCTGTAAAGAAGTAGGC
	11	23	294	TTGACAGAAATTGCTTTTTACTCTG	TCCCCCAGTCTCAGGATAACT
	12	13	364	CATGACAATCACAATCCAAAATA	CATGTGAGCTAGTGGGCAAA
	13	14 A	567	CTGGGAATGGGAGAGAACCT	ATGTCCCCACTGTGATGGAG
Set 4	14	21	261	CCACAGCTTAGATTAACCCCTCA	TGAGCTTGCAAGAGGAATAAGTAA
	15	25	300	TGGGAATTTCTGGGAGTAAATG	AAGCTCTAGGAGAGGTGGTATTTTT
	16	01	480	TAGCAGCCTCCCTTTTGCTA	CTAACCCGATGCTGACCT
	17	26 B	596	GGAGAAACCTGCATGAAAGC	TTGGCCATCACAAATTTCAA
Set 5	18	10	250	TTCTTGTTGATCCTAGTCGTTTT	GCTGGAGAA AGGACCAACATA
	19	12	298	TGCTAGCTCCTACCTGACAACA	CATTCAATTATCTGGACATCACTTTG
	20	04	372	CATGTTTCTTTGAGTGTACAGTG G	TTCAGGTGAAGGAACACAAATG
	21	14 E	570	GGATGACACCTCAACCCAGT	CCTTCCACGAGATCCAGATG
Set 6	22	24	250	ACTGAGGCTGAAGCATGTCC	CCCAACCACTGCTCTGAGTC
	23	03	299	GCATGCTTCTCCACTGTGAC	GCCACCATTACAAAGCACAC
	24	17	397	AGGTTGGACTGGCATAAAAA	CCCTGGATCAAGTCTCATTTG
	25	26 C	580	TGCAAATGTGCATTTTCTGA	CCTCCAGCCCCCTTTACTAT
	26	14 C	695	AGCTCATGGACCTGCTTTGT	CATTCTCTGGATTAATGTTTCCT
Set 7	27	06	258	GCGGTCATTATGAGACACA	CCGAGCTGTTTGTGAAGTGA
	28	15	299	TGAGGCATTTCTACCCACTTG	CCAAAAGTGGGAATACATTATAGTCA
	29	07	400	TGTCCTAGCAAGTGTTTTCCATT	AATGTCCCCCTCAGCAACAC
	30	26 D	580	CCACCCCCATAAGATTGTGA	CTGAAGAAACCAGCAGGAAAA
	31	14 F	686	TCCCTACGGAACTAGCAATG	TCACAAGAGCAGAGCAAAGG
Set 8	32	05	259	TCTCCTCCTAGTGACAATTTCC	CCATCTCCTTCATTCTCTGA
	33	09	300	TTTGAGCCTACCTAGAATTTTCTTC	GGTATTTTAGAACTCAAACTCTCC
	34	16	468	CAGCATCCATCTTCTGTACCA	AAAGCTTCTTATGCACGTAGG
	35	14 D	595	TCCAAGCAGCAGAAACCTATT	AGTAATGGCCCCTTTCTCCT

^a Base pair

Analysis of Exon 14 Point Mutation by PCR-Sequencing

Exon 14 is the largest exon of the *F8*. Its large size can cause many mutations. Mutational analysis of this exon was performed using sequencing of six primer pairs (Table 2). PCR amplification was performed at a final reaction volume of 30 μ L containing approximately 100 ng of genomic DNA, 4 pmol/L of each primer, 3 μ L of

10 $\times$ PCR buffer, 0.25 mM of each dNTP, 1.5 mM $MgCl_2$, and 2 U of Taq DNA polymerase (CinnaGen) under the following conditions: initial denaturation at 94 $^{\circ}$ C for 5 min, followed by 30 cycles of denaturation at 95 $^{\circ}$ C for 40 s, annealing at 60 $^{\circ}$ C for 30 s, an extension at 72 $^{\circ}$ C for 1 min, and a final extension at 72 $^{\circ}$ C for 5 min. Sequencing was performed using a BigDye Terminator v3.1 cycle sequencing kit on an Applied Biosystems 3730xl DNA Analyzer (Life Technologies, Carlsbad, CA).

Table 2 Designed primers for amplification of exon 14 of *F8*

Primer name	Sequence	Primer size (bp) ^a	TM (°C) ^b	Product size (bp)
Exo14F-seg 1	CTGGGAATGGGAGAGAACCT	20	60.5	637
Exo14R-seg 1	CTCTGTTGCTGCAGTTGTCC	20	60.5	
Exo14F-seg 2	TCACCTGGAGCAATAGACAGTAATA	25	62.5	695
Exo14R-seg 2	TAAAGCATTCTGTCATGAATCAAAGG	26	61.6	
Exo14F-seg 3	GCAACTAATAGAAAGACTCACATTG	25	60.9	616
Exo14R-seg 3	GAGGCAAACTACATTCTCTTGGA	24	61.8	
Exo14F-seg 4	GAGATGGTTTTCCAAGCAGCAGAA	25	64.2	658
Exo14R-seg 4	TTGCTTGAGGGATGCTATGACTC	23	62.9	
Exo14F-seg 5	ATGAAACATTGACCCCGAGCACC	24	65.3	664
Exo14R-seg 5	GAGGATCCAATAGCTTGGAGGGAGT	25	67.4	
Exo14F-seg 6	ATCAGAGGACCTATTCCCTACG	22	62.1	747
Exo14R-seg 6	ATCCTCTAACTCTCATTGTTGGTG	24	61.8	

^a Base pair^b Melting temperature**Table 3** Distribution of investigated mutations in this study

Investigated mutation	Count (n = 50)	Percentage (%)
Intron 22 inversion type 1 (Inv22-1)	22	44
Intron 1 inversion	1	2
Gross exon deletions	0	0.0
Point mutation in exon 14	1	2
No mutations	26	52
Total	50	100

Results

Genomic DNA from 50 patients with severe HA was analyzed for mutations in the *F8* using IS-PCR, long-range PCR, multiplex PCR, and sequencing methods. By studying of mentioned regions in *F8*, we identified causative mutation in 24 patients. In this group, we identified three different *F8* mutations. The mutation detection rate was 48 %.

Screening of the *F8* Intron Inversion

In this study, IS-PCR analysis of the *F8* revealed that 22 patients with HA had Inv 22. No intron deletions or duplications were detected, and all Inv 22 were Inv 22 type 1. Only one patient (2 %) had Inv 1 in this study. This means that 44 % of patients with severe HA had Inv 22 mutations and 2 % had Inv 1 mutations (Table 3). As Inv 22 and Inv 1 are well-recognized causative mutations in HA [3], there was no need to search for other mutations in the coding regions in these families.

Fig. 1 Representative gel for detection of gross exon deletion by multiplex polymerase chain reaction (PCR). *M*, 100-bp size marker; *lane 1*, PCR products of multiplex 1 (exons 20, 22, 11, and 8 and segment 14B); *lane 2*, PCR products of multiplex 2 (exons 2 and 18 and segments 26E and 26A); *lane 3*, PCR products of multiplex 3 (exons 19, 23, and 13 and segment 14A); *lane 4*, PCR products of multiplex 4 (exons 21, 25, and 1 and segment 26B); *lane 5*, PCR products of multiplex 5 (exons 10, 12, and 4 and segment 14E); *lane 6*, PCR products of multiplex 6 (exons 24, 3, and 17 and segments 26C and 14C); *lane 7*, PCR products of multiplex 7 exons 6, 15, and 7 and segments 26D and 14F); *lane 8*, PCR products of multiplex 8 (exons 5, 9, and 16 and segment 14D). The primer sequences and product sizes are given in Table 1

Screening of *F8* Gross Exon Deletions by Multiplex PCR

All 26 exons were analyzed for gross exon deletions mutations in eight multiplex PCR assays (Fig. 1). None of the studied cases showed an gross exon deletions.

Screening for Point Mutations in Exon 14 of the *F8* by Direct Sequencing

We performed sequencing analysis of exon 14 for mutation screening in those families who did not have Inv 1 or Inv

22. By sequencing analysis, we found that one patient had an insertion of adenine at position 2943.

Carrier Identification

It is possible to identify carriers of HA directly if the responsible mutation is present in carrier families. A total of 33 potential carrier females (including 24 mothers and 9 sisters of patients found to have mutations) were analyzed. Using the above mentioned methods for studying critical regions of *F8*, we were able to identify seven carrier mothers (six for Inv 22 and one for point mutation), and four carrier sisters (two for Inv 22 and two for point mutation).

Discussion

This study revealed that mutation detection in about 50 % of patients with severe HA and carriers of HA is possible using an IS-PCR method. For the first time, to our knowledge, we report the analysis of the *F8* by direct mutation detection in patients with severe HA of Azerbaijani Turkish ethnicity from the Northwest of Iran. Our study almost confirms the prevalence in previous reports for the mutations found and with regard to their correlation with the severity of HA. Inv 22 and Inv 1 have been accepted as large structural changes in the *F8* that disturb the normal formation of full-length *F8* mRNA in patients with severe HA [15], and these two mutations are the first and second most common mutations, respectively, in almost all studied populations with HA (Table 4). In most previous studies, Inv 22 and Inv 1 mutations in patients

with severe HA have been reported in the ranges of 40–50 and 1–5 %, respectively.

The remaining cases of severe hemophilia A patients may have other mutations, such as point mutations in other exons or intron boundaries [4, 16]. Our findings confirm those of previous studies. The frequency of Inv 22 in our study was 44 %, which is similar to that in populations studied in the United Kingdom [13], Spain [17], Italy [18], Argentina [19, 20], India [21], Egypt [22], Iran [23], Mexico [24], Hungary [15], and Costa Rica [25]. On the basis of these results, we concluded that the incidence in different populations is similar. Our study confirms the results of previous studies in which researchers reported Inv 22 type 1 as more common than type 2 inversions.

The frequency of Inv 1 seems to be similar in the different populations. Our study showed that 1 (2 %) of 50 patients carried Inv 1. The incidence of Inv 1 in other populations has been reported as follows: 5.6 % in Spain [17], 1.71 % in Italy [26], 4.3 % in the Czech Republic [27], 4.8 % [13] and 1.85 % [6] in the United Kingdom, 0 % in Hungary [15], 0 % in Mexico [24], 0 % in Costa Rica [25], and 7 % in Iran [23].

In previous reports, gross exon deletions may be comprised of almost 4 % of mutations in patients with severe HA [28]. In the present study, all patients were normal for gross deletions. This result is different from findings in previous studies and might be due to the different ethnicities of the studied populations. Exon 14, with a length of 3 kb, accounts for approximately 43 % of the coding region of the *F8*, and many different mutations have been reported in this portion of the gene. Our findings explain the occurrence of a few mutations in exon 14, and our study has a little discrepancy with a previous report [28].

Table 4 Frequency and prevalence of introns 22 and 1 inversion in different populations

Population	Number of patients	Intron 22 inversion	Prevalence (%)	Intron 1 inversion	Prevalence (%)
United Kingdom [13] and [16]	209	94	44.9	10	4.8
	595	–	–	11	1.85
Spain [19]	102	42	41.1	–	–
Italy [18] and [28]	93	39	42	–	–
	20	–	–	1	5
Argentina [21] and [22]	34	14	41	–	–
	64	25	39	1	1.5
India [23]	80	35	43.75	3	3.75
Iran [25]	30	14	47	2	7
Mexican [24]	31	14	45	0.0	0.0
Hungarian [17]	104	54	52	0.0	0.0
Czech Republic [29]	162	–	–	7	4.3
Costa rica [27]	34	22	61.7	0.0	0.0
Iran (present study)	50	22	44	1	2

Identification of carriers in all families with HA is a very important issue and would improve the genetic counseling and quality of life of these families. In this study, 24 mothers and 9 sisters of patients with HA in whom mutations were found were analyzed for carrier potential. The mothers of seven patients were carriers, and we confirmed that two-thirds of HA mutated alleles were transmitted from normal females [29].

Conclusion

We found that Inv 22 is the major cause of severe HA in an Azeri Turkish population. Molecular study facilities for evaluation of the causes of HA disease is limited in developing countries, and HA remains a life threatening and often disabling condition. The IS-PCR method is a rapid, efficient, cost-effective, and robust method that may be a reliable, recommended tool for genetic analysis of *F8* Inv 22 in patients with severe HA and for carrier detection in families with severe HA.

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