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Application of Indirect Linkage Analysis for Carrier Detection of Hemophilia A in Kurdistan Region of Iraq: Usefulness of Intron 18 *Bcl*I T>A, Intron 19 *Hind*III C>T, and IVS7 nt27 G>A Markers

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Abstract

Hemophilia A (HA) is the most common congenital X-linked coagulopathy caused by mutations in the *factor VIII* gene. One in 5000 to 10 000 male persons worldwide suffer from HA. It is the archetype of high-cost, low-volume disease. Therefore, identification of carriers is crucial to avoid the birth of affected males. Tracking of the defective X chromosome through indirect linkage analysis represents the most practical method for screening for carriers in developing countries. In this study, 227 individuals from 41 families with HA and 100 normal participants were recruited from the Kurdistan region of Iraq and evaluated for intron 18 *Bcl*I, intron 19 *Hind*III, and IVS7 nt 27 markers by polymerase chain reaction restriction fragment length polymorphism and direct sequencing. Among the studied women, 49%, 42%, and 14% were discovered to be heterozygous for *Bcl*I, *Hind*III, and IVS7 markers, respectively. Using *Bcl*I, *Hind*III, and IVS7 markers, 56%, 46%, and 17% of the families were informative, respectively. The combined informativity of these polymorphic sites reaches 66%. The current study illustrates the effectiveness of the *Bcl*I and *Hind*III markers for the diagnosis of HA carriers among the Iraqi Kurdish population.

Keywords

hemophilia A, *FVIII* gene polymorphism, indirect linkage analysis, PCR-RFLP, linkage disequilibrium, carrier detection

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Introduction

Hemophilia A (HA) is an X-linked recessive bleeding disorder that occurs as a result of various mutations in the clotting factor VIII (*FVIII*) gene, leading to partial or total deficiency of FVIII activity, which is fundamental for the propagation of the intrinsic coagulation pathway.[1] The incidence of HA is approximately 1 in 5000 to 10 000 live male births among all ethnic groups.[2] As a recessive X-linked disease, it is extremely rare in females.[3-5] Hemizygous males are affected, whereas heterozygous women (carriers) are generally asymptomatic, having normal or intermediate FVIII levels.[6] More than half of patients have an affected sibling or other male relatives, and there is an obvious inheritance pattern within the family.[7,8] However, about 30% of patients with HA are sporadic cases with a negative family history.[8,9] Therefore, the mother of a patient with HA could be a noncarrier (the patient having acquired new mutations), a carrier with a de novo mutation, or a classical carrier with an inherited mutation from her parents.[10] As a carrier, there is a 50% chance of transferring the mutated gene to the offspring.[11] Thus, carrier diagnosis is essential for preventing the birth of children with HA through genetic counseling and prenatal detection of an affected fetus.

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Although the direct identification of *FVIII* gene mutations is the gold standard for molecular diagnosis of HA,[12-14] and considered the best approach for carrier detection and prenatal diagnosis (PND), the large size and complexity of the *FVIII* gene (~186 kb length comprising 26 exons) and its highly mutational heterogeneity make direct analysis of the mutations challenging in underresourced molecular diagnostic laboratories.[15-17] In addition, in approximately 2% to 5% of patients with severe HA, the responsible mutation is not identified in the *FVIII* gene.[18] Considering these difficulties associated with the direct detection of FVIII mutations, the indirect analysis of DNA markers linked to the FVIII locus seems to be a more practical approach for the detection of HA carriers in developing countries, including Iraq.

Polymorphic markers are minor DNA sequence variations commonly found in noncoding sites of a gene in the population. The informativity of each polymorphic marker—in other words, the heterozygosity rate of each polymorphic site—should be determined in the same population for effective tracing of the chromosome bearing the defective gene.[15,19,20] Particular DNA markers at the FVIII locus used for the identification of carrier women were reported by the World Health Organization in 1993.[15] As a result of genetic disparity among diverse populations, the genetic informativity varies between different ethnic groups.[21] Therefore, it is fundamental to find informative genetic markers linked to the *FVIII* gene in each particular population.

The population of Iraq consists of different ethnic groups, with the Iraqi Kurdish people representing the major ethnic group in the northern part of the country. The aim of the current study was to evaluate the usefulness of 3 intragenic DNA markers (intron 18/*Bcl*1 T/A, intron 19/*Hind*III C/T, and IVS7 nt 27 G/A) in linkage analysis for genetic counseling and carrier detection of HA, in particular for the Kurdistan region of Iraq.

Materials and Methods

Patients and Family Members

This study was conducted following approval by the local institutional ethical committee (approval number 55 on September 7, 2017). Informed consent was collected from patients and other family members. The study group consisted of 227 individuals from 41 HA families, of whom 63 were patients with HA, 82 nonhemophilic males (fathers and brothers), and 31 obligate and 51 probable female carriers (mothers and sisters). The study also included 100 normal participants (76 males and 24 females) from the same population to investigate the informativity of the markers. The categorization of the investigated women as obligate or probable carriers was done on the basis of pedigree analysis using Bayesian rule.[15,22] The patients were registered in the local hemophilia treatment centers, and the diagnosis of HA was founded on detailed personal and family history of bleedings, physical examination, prolonged activated partial thromboplastin time, and reduced FVIII bioassay. A positive family history of HA was determined in 31 (75.6%) families, while 10 (24.4%) families were isolated with the propand being the only person with HA. Depending on FVIII levels, 20.6% (13/63) of the patients had severe (FVIII: C < 1%), 54% (34/63) had moderate (FVIII: C 1%-5%), and 25.4% (16/63) had mild deficiency (FVIII: C > 5%-30%).

DNA Extraction and FVIII Gene Analysis

High-molecular-weight genomic DNA was extracted using 5 to 10 mL peripheral blood samples anticoagulated in 0.5 M K_2EDTA using a salting-out procedure.[23] The quantity and quality of the isolated DNA samples were determined using spectrophotometry. Three intragenic markers were analyzed using polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) for intron 18/*Bcl*I T/A and intron 19/*Hind*III C/T polymorphisms and using direct sequencing of the PCR product for IVS7 nt 27 G/A polymorphism. Amplification was achieved in a 25 μL final volume containing 0.1 to 0.5 μg of genomic DNA, 0.4 μM of each primer (SinaClon Company, Tehran, Iran), a 0.2 mM of each dNTP (Gen Fanavaran Co, Tehran, Iran), 1.5 U of Super Taq DNA polymerase (Gen Fanavaran Co), 1× PCR buffer, 2 μL of dimethyl sulfoxide ≥99.9% (Sigma Chemical Co, St. Louis, Missouri), and 1.4 mM $MgCl_2$ (Gen Fanavaran Co). The sequences of the oligonucleotide primers, PCR conditions, and the size of the PCR products are given in Table 1. Following PCR, amplification of the target sequence was affirmed using 2% agarose gel electrophoresis. The PCR products were then digested using the restriction enzyme *Bcl*I and *Hind*III (Thermo Fisher Scientific, Waltham, Massachusetts) for the intron 18/*Bcl*I T/A and intron 19/*Hind*III C/T markers, respectively. The digestion reaction was based on a 25 μL reaction volume with 7 μL of PCR product, 10 U of the respective enzyme, and 1× digestion buffer. The digested PCR product was then incubated for 3 hours at 55°C and 37°C for *Bcl*I and *Hind*III, respectively. Following incubation, the digested PCR products were electrophoresed on a 4% agarose gel. Concerning the intron 18/*Bcl*I T/A marker, the indigestible A allele produced 1 fragment (142 bp), while the digestible T allele produced 2 fragments (99 + 43 bp). For the intron 19/*Hind*III C/T marker, a common (470 bp) fragment besides 2 fragments (154 + 79 bp) was produced for the digestible C allele, while the indigestible T allele produced 2 fragments (470 + 233 bp). The IVS7 nt 27 G/A polymorphism was detected by direct sequencing of the 365 bp PCR product. The amplified fragments were sequenced commercially using an ABI 3130 XL sequencer (Applied Biosystems, Foster City, California). A sequence analysis software package (FinchTV; Geospiza, Inc, Espoo, Finland) was used for sequence reading and analysis.

Statistical Analysis

The allele frequencies for the polymorphic markers were calculated depending on the number of the studied X chromosomes. The expected heterozygosity rates were estimated using the Hardy-Weinberg equation.[25] A χ^2 test was used to

Table 1. Sequences of Oligonucleotide Primers and PCR Conditions.

Marker	Primer Sequence	Amplification Conditions	Amplified Product (bp)	Reference
Intron18/ *Bcl*I T/A	5′TACTTACTTTAAATGGTCTAGGC3′ 5′TTCTATATCTGAAATTATCTTGTTC3′	94°C 4 minutes 28 cycles of: 94°C 1 minute 50°C 1 minute 72°C 1 minute 72°C 5 minutes	142	Newly designed[a]
Intron19/*Hind*III C/T	5′GGCGAGCATCTACATGCTGGGATGAGC3′ 5′GTCCAGAAGCCATTCCCAGGGGAGTCT3′	94°C 4 minutes 28 cycles of: 94°C 1 minute 68°C 1 minute 72°C 1 minute 72°C 10 minutes	703	Graham et al[24]
IVS7 nt27 G/A	5′TCACCTACCCCCATGATTGT3′ 5′GCAACTGAGCGAATTTGGAT3′	94°C 5 minutes 28 cycles of: 94°C 1 minute 60°C 1 minute 72°C 1 minute 72°C 10 minutes	365	Newly designed[a]

Abbreviation: PCR, polymerase chain reaction.
[a]The primers were designed using Primer3 based on the reference sequence (RefSeq accession no: NM_000132.3).

Table 2. Allele Frequency of Intron18/*Bcl*I T>A, Intron19/*Hind*III C/T, and IVS7 nt 27 G>A Markers in Iraqi Kurdish Population.[a]

Marker	Allele	Fragment Length (bp)	X Chromosomes	Frequency
Intron18/*Bcl*I T>A	+ (T)	99 + 43	258/433	0.60
	− (A)	142	175/433	0.40
Intron19/*Hind*III C/T	+ (C)	470 + 154 + 79	184/433	0.42
	− (T)	470 + 233	249/433	0.58
IVS7 nt 27 G>A	G allele	/	399/433	0.92
	A allele	/	34/433	0.08

[a]Total number of cases = 327 (221 males and 106 females).

analyze differences between observed and expected heterozygote frequencies and to compare the differences in the allele frequency and heterozygosity rate between different populations. A P value of .05 or less was considered statistically significant. The expected haplotype frequencies and the linkage disequilibrium (LD) between different loci were assessed using the Haploview (version 4.2) software. The polymorphism information content (PIC) was determined according to the method described by Botstein et al.[26]

Results

A total of 227 patients from 41 families with a history of HA were investigated and linkage analysis was performed using 3 biallelic markers—intron 18/*Bcl*I T/A, intron 19/*Hind*III C/T, and IVS7 nt 27 G/A polymorphisms—to detect carrier status by tracing the defective X chromosome in the family. Likewise, 100 normal individuals were involved in the analysis of the 3 polymorphisms. A total of 433 X chromosomes (221 males and 106 females) were evaluated. The allele frequencies of the "+" (digestible by *Bcl*I or T allele) and "−" (indigestible by *Bcl*I or A allele) alleles were shown to be around 0.60 and 0.40, respectively. As to the *Hind*III marker, the frequencies of the "+" (digestible or C allele) and "−" (indigestible or T allele) alleles were found to be about 0.42 and 0.58, respectively. The other intragenic polymorphism at IVS7 illustrates the highest frequency for the G allele, giving a value of 0.92 with the minor allele frequency of approximately 0.08 (Table 2).

The observed heterozygosity (ie, the number of heterozygous genotypes divided by the total number of genotypes examined) in females (n = 106) for the *Bcl*I marker demonstrates the highest frequency of all the 3 polymorphic sites, giving a rate of 0.49. Of the 41 studied families, 23 (56%) were informative using this marker alone. Among the 41 evaluated sisters (probable carriers), 23 (56%) were diagnosed, and carrier status could be excluded in 14 (14/23) females, while it was detected in 9 (9/23) females using the *Bcl*I marker. The observed heterozygosity rate for the *Hind*III marker was found to be 0.42, and 46% (19/41) of the families were informative. Using this marker alone, 19 probable carriers were categorized as carriers (7/19) and noncarriers (12/19). On the other hand, the IVS7 nt 27 G>A showed the lowest heterozygosity rate

Table 3. Allele Frequency and Heterozygosity Rate of Intron18/*Bcl*I T>A, Intron19/*Hind*III C/T, and IVS7 nt 27 G>A Markers in 106 Females in the Study Group.

Marker	Allele	X Chromosome	Allele Frequency[a]	*H* (Observed)	*H* (Expected)[b]	Informative Families (N) %	PIC[d]	*P* Value
Intron18/*Bcl*I T>A	+ (T)	130/212	0.61	0.49	0.47	(23/41) 56	0.36	.924
	− (A)	82/212	0.39					
Intron19/*Hind*III C/T	+ (C)	89/212	0.42	0.42	0.49	(19/41) 46	0.37	.239
	− (T)	123/212	0.58					
IVS7 nt 27 G>A	G allele	191/212	0.90	0.14	0.18	(7/41) 17	0.14	.119
	A allele	21/212	0.10					
Total[c]						(27/41) 66		

Abbreviations: H, heterozygosity; MAF, minor allele frequency.
[a]Allele frequency refers to the number of allele detected divided by the total number of allele analyzed.
[b]Expected heterozygosity rate is calculated according to the equation: $1 - (p^2 + q^2)$, where p is the positive allele frequency and q is the negative allele frequency.
[c]Total refers to the cumulative informativity rate using one or more of the 3 markers.
[d]PIC refers to polymorphism information content, $PIC = 1 - \{MAF^2 + (1 - MAF)^2\} - \{2 \times MAF^2 \times (1 - MAF)^2\}$.

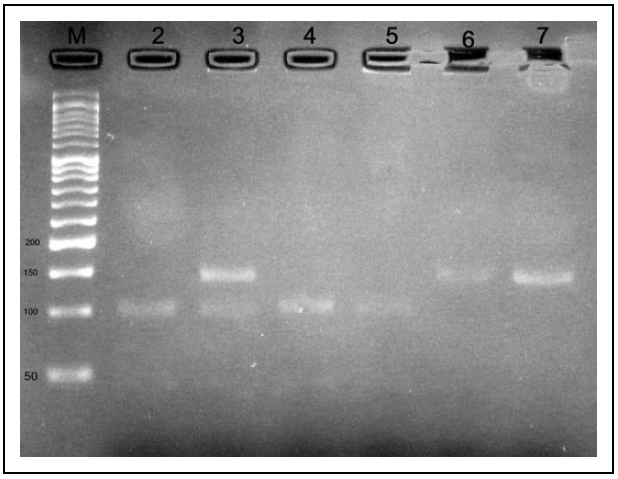

Figure 1. Agarose gel illustrating the alleles of intrageneic *Bcl*I RFLP in an informative family. Lane 1: 50-bp ladder, lane 2: father (T), lane 3: mother (A/T), lane 4: carrier sister (T/T), lane 5: hemophilic son (T), lanes 6, 7: unaffected brothers (A).

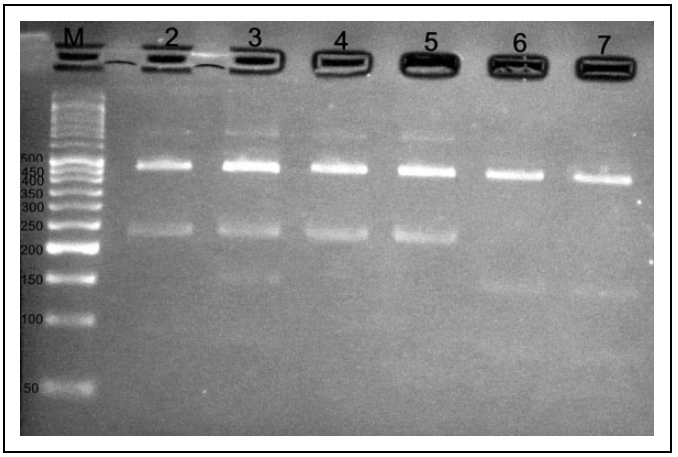

Figure 2. Agarose gel illustrating the alleles of intrageneic *Hind*III RFLP in an informative family. Lane 1: 50-bp ladder, lane 2: father (T), lane 3: mother (C/T), lane 4: carrier sister (T/T), lane 5: hemophilic son (T), lanes 6, 7: unaffected brothers (C).

among the studied females. The rate of heterozygous females was found to be 0.14, and only 17% (7/41) of the families were informative. Using this marker alone, 3 carrier and 4 noncarrier females could be detected of the 41 evaluated probable carriers. Considering the 3 markers together, 27 of the 41 families were found to be informative, giving a cumulative informativity rate of around 66% using one or more of the 3 markers. The expected heterozygosity rate was 0.47, 0.49, and 0.18 for the *Bcl*I, *Hind*III, and IVS7, respectively. No statistically significant difference between observed and expected heterozygosity of all 3 polymorphisms could be detected. Overall, the studied population was in Hardy-Weinberg equilibrium. The PIC value (that is the sum of frequency of each possible mating multiplied by the probability that an offspring will be informative) for the *Bcl*I and *Hind*III markers was 0.36 and 0.37, respectively, while the IVS7 marker showed a lower value of 0.14 (Table 3). Figures 1 and 2 illustrate the agarose gel of the *Bcl*I and *Hind*III RFLPs, respectively, in one of the informative families.

Of the 8 possible haplotypes identified by these 3 FVIII intragenic polymorphic sites, 6 haplotypes were generated by analyzing 221 hemizygous male patients in the study group (Table 4). The frequency of haplotype I (TTG) and haplotype II (ACG) was found to be the highest, accounting for 54.3% and 34.3%, respectively, while the other 4 haplotypes demonstrated low frequencies of less than 10%. Concerning the analysis of the 106 female patients in the study group, 11 different multilocus genotypes were observed (Table 4). The observed frequencies of multilocus genotype I (*Bcl*I T/T, *Hind*III T/T, IVS7 G/G) and genotype II (*Bcl*I T/A, *Hind*III C/T, IVS7 G/G) were found in more than 50% of the evaluated females, giving a value of 30.2% and 28.3%, respectively. More than half of IVS7 heterozygotes (9/15 or 60%) were seen in females who were heterozygotes for *Bcl*I and *Hind*III sites too. On the other hand, the *Bcl*I T/T genotype in 82% of the patients (32/39 of all the observed *Bcl*I T/T genotype) co-segregated with the *Hind*III T/T and IVS7 G/G genotypes. Accordingly, the expected haplotype frequencies were calculated by Haploview software using the females' genotypes as shown in Table 4. The

Table 4. Genotype and Haplotype Frequencies of Intron18/*Bcl*I T>A, Intron19/*Hind*III C/T, and IVS7 nt 27 G>A Markers.

Observed Haplotype Frequency in 221 Males[a]						Observed Genotype Frequency in 106 Females					Expected Haplotype Frequency[b]				Linkage Disequilibrium Tab[c]	
Number	*BclI*	*HindIII*	*IVS7*	N	%	*BclI*	*HindIII*	*IVS7*	N	%	*BclI*	*HindIII*	*IVS7*	%	Association	*D′*
I	T	T	G	120	54.3	TT	TT	GG	32	30.2	T	T	G	53.8	***BclI*+*HindIII***	0.87
II	A	C	G	76	34.3	TA	CT	GG	30	28.3	A	C	G	27	**IVS7+*BclI***	0.80
III	A	C	A	12	5.4	TA	CT	GA	9	8.5	A	C	A	8.7	**IVS7+*HindIII***	0.78
IV	T	C	G	7	3.2	TA	CC	GG	8	7.5	T	C	G	6.3		
V	A	T	G	5	2.3	AA	CC	GG	7	6.6	A	T	G	3		
VI	T	T	A	1	0.5	TT	CT	GG	5	4.7	T	T	A	1.2		
VII	T	C	A	0	0	TA	TT	GG	5	4.7	T	C	A	0		
VIII	A	T	A	0	0	AA	CC	GA	4	3.8	A	T	A	0		
IX						AA	CC	AA	3	2.9						
X						TT	TT	GA	2	1.9						
XI						AA	CT	GG	1	0.9						
Total				221	100				106	100				100		

[a]Observed haplotypes in hemizygous male.
[b]Expected haplotype frequencies estimated by Haploview version 4.2 software.
[c]Linkage disequilibrium estimated by Haploview version 4.2 software.

expected haplotype frequencies demonstrated a similar trend with the observed haplotype frequencies. Linkage analysis identified strong, yet incomplete, allelic association between the 3 markers, with a high value of LD between *Bcl*I/*Hind*III and IVS7/*Bcl*I ($D' = 0.87$, $D' = 0.80$, respectively). Relatively weaker association of IVS7 and *Hind*III markers with a value of $D' = 0.78$ was estimated (Table 4).

Discussion

Recently, the therapeutic options for patients with HA have rapidly evolved, ranging from extended half-life concentrates and nonclotting factor concentrate products to many successful gene therapy trials.[27,28] Although these novel products will dramatically improve the management of HA in developed countries, in resource-constrained countries, the disease has no curative therapy and treatment involves replacement with exogenous FVIII, which is extremely costly and a major source of health-care-related concern. Thus, carrier detection and PND are alternative steps for prevention of this high-cost disease.[15]

Approaches to the molecular diagnosis of HA include indirect linkage analysis through tracing of the defective *FVIII* gene using polymorphic markers and direct detection of the disease-causing mutations. The direct identification of the causative mutation in HA is often difficult due to the large size, structural complexity, and high frequency of numerous types of mutations of the *FVIII* gene, rendering mutation detection labor intensive, time consuming, and expensive.[29] Therefore, carrier detection by indirect linkage analysis has been the best approach in many developing countries. Heterozygosities of different polymorphic loci linked to the *FVIII* gene have been commonly used to establish population-based strategies for carrier identification in HA. The current study was performed to investigate the role of 3 polymorphic markers (intron 18 *Bcl*1 T/A, intron 19 *Hind*III C/T, and IVS7 nt 27 G/A) separately and within haplotypes for indirect tracing of the defective *FVIII* gene among HA families from Iraqi Kurds. It represents the first such study on this population.

In the present study, the allele frequencies of the *Bcl*I "+" (T) and "−" (A) alleles were 0.60 and 0.40, respectively, while the allele frequencies of *Hind*III "+" (C) and "−" (T) alleles were 0.42 and 0.58, respectively. In addition, we observed a high frequency of the IVS7 nt 27 G allele (0.92/0.08) in our population. A comprehensive review of allelic frequency and a comparison between our study and other ethnic groups worldwide is detailed in Table 5.

The results of the current study also demonstrated that the *Bcl*I polymorphic site was the most informative among the studied markers, followed by the *Hind*III marker, while the IVS7 was the least informative, giving a value for heterozygosity rate of 0.49, 0.42, and 0.14, respectively. A comparison of heterozygosity rates of the 3 polymorphic markers between our population and different ethnic groups is shown in Table 6. The high discrepancy of these 3 polymorphic sites in various ethnic groups proves that indirect linkage analysis of *FVIII* gene is based on ethnicity.

Haplotype analysis was carried out, since the characterization of haplotypes has additional advantage over the estimation of allelic frequencies for individual markers and is hence a better mechanism for tracing of the defective *FVIII* gene, as reported earlier.[30,46] In the evaluation of LD, if a value of $D' > 0.33$ is considered as meaningful LD,[47] consequently our study showed significant LD between the alleles at the 3 polymorphic loci. The *Bcl*I+*Hind*III demonstrates the strongest association ($D' = 0.87$), followed by IVS7+*Bcl*I ($D' = 0.80$) and IVS7+*Hind*III ($D' = 0.78$). In spite of strong LD among the 3 polymorphic sites, they could still be used effectively for carrier identification in 66% of the families. Thus, although examination of polymorphic loci that are in high LD may not

Table 5. Comparison of Allele Frequency of *Bcl*I, *Hind*III, and IVS7 Markers Between Current Study and Different Ethnic Groups.

	*Bcl*I			*Hind*III			IVS7			
	Allele Frequency			Allele Frequency			Allele Frequency			
Ethnic Group	+ (T)	− (A)	*P* Value[a]	+ (C)	− (T)	*P* Value[a]	G	A	*P* Value[a]	References
Iraqi Kurds	0.60	0.40	[a]	0.42	0.58	[a]	0.92	0.08	[a]	Current Study
Iranian	0.52	0.48	.066	0.48	0.52	.135	0.88	0.12	.139	[30]
Indian (North)	0.57	0.43	.308	0.38	0.62	.234	/	/	/	[31]
Indian (Bihar)	0.68	0.32	.056	/	/	/	/	/	/	[32]
Indian (North)	0.43	0.57	<.0001	0.35	0.65	.088	/	/	/	[42]
Indian (Bihar)	0.45	0.55	.008	0.35	0.65	.088	/	/	/	[43]
Western European	0.54	0.46	.135	/	/	/	/	/	/	[33]
Italian	0.65	0.35	.172	/	/	/	/	/	/	[34]
Polynesian	0.57	0.43	.308	/	/	/	/	/	/	[35]
Caucasian	0.77	0.23	<.0001	0.26	0.74	<.0001	/	/	/	[15,36]
Japanese	0.84	0.16	<.0001	0.19	0.81	<.0001	/	/	/	[37]
Korean	0.81	0.19	<.0001	/	/	/	/	/	/	[38]
Korean	0.85	0.15	<.0001	/	/	/	/	/	/	[39]
Chinese	0.74	0.26	.002	0.24	0.76	<.0001	/	/	/	[24,40]
Brazilian	0.39	0.61	<.0001	0.42	0.58	.542	/	/	/	[41]
African American	0.20	0.80	<.0001	0.78	0.22	<.0001	/	/	/	[24]
Indian (South)	/	/	/	0.29	0.71	.004	0.97	0.03	.011	[44]
Azeri Turkish	0.69	0.31	.035	0.20	0.80	<.0001	/	/	/	[45]
The United Kingdom	/	/	/	/	/	/	0.88	0.12	.139	[19]

[a]Each *P* value indicated the difference in corresponding allele frequency between the current study and other ethnic groups.

Table 6. Comparison of Heterozygosity Rate of *Bcl*I, *Hind*III, and IVS7 Markers Between Current Study and Different Ethnic Groups.

Ethnic Group	*Bcl*I		*Hind*III		IVS7		
	Heterozygosity	*P* Value[a]	Heterozygosity	*P* Value[a]	Heterozygosity	*P* Value[a]	References
Iraqi Kurds	0.49	[a]	0.42	[a]	0.14	[a]	This study
Iran	0.48	.460	0.46	.242	0.22	.030	[30]
North India	0.54	.424	0.49	.097	/	/	[31]
Western Europe	0.50	.920	/	/	/	/	[33]
India (Bihar)	0.58	.043	0.43	.462	/	/	[32]
India (Bihar)	0.60	.017	0.63	<.0001	/	/	[43]
Italian	0.60	.017	/	/	/	/	[34]
Polynesia	0.49	.540	/	/	/	/	[35]
Japan	0.28	<.0001	0.30	.007	/	/	[37]
Korea	0.32	<.0001	/	/	/	/	[38]
Korea	0.21	<.0001	/	/	/	/	[39]
Caucasian	0.35	.003	0.38	.234	/	/	[15,36]
China	0.38	.016	0.37	.175	/	/	[24,40]
Brazil	0.47	.381	0.49	.097	/	/	[41]
North India	0.39	.027	0.57	.002	/	/	[42]
Azeri Turkey	0.47	.381	0.35	.088	/	/	[45]
South India	/	/	0.60	<.0001	0.06	.003	[44]

[a]Each *P* value indicated the difference in corresponding heterozygosity rate between the current study and other ethnic groups.

increase the informativity very much, they do provide confidence to the data obtained in the study and permit corroboration of the information observed from each locus.[48] The genetic informativity of the 3 studied markers was evaluated using PIC. The *Bcl*I and *Hind*III loci exhibited high PIC values of 0.36 and 0.37, respectively, while the IVS7 locus showed a lower value of 0.14. These findings were comparable to a report from India that gave a value of 0.35 for *Bcl*I and 0.36 for *Hind*III markers,[46] while Moharrami et al[45] illustrated a lower PIC value of 0.27 for the *Hind*III locus in the Azeri Turkish population of Iran. Accordingly, the *Bcl*I polymorphic site was useful for carrier detection in 56% of the families, the *Hind*III polymorphic site in 46%, and IVS7 in only 17% of the families. Taking into account the 3 polymorphic sites together, carrier

status could be categorized in 66% of the families, parallel to a study from India in which the combined informativity rate reached 77%.[31]

Polymorphism-based gene tracing has constitutional limitations. It may lead to incorrect diagnosis due to crossing-over and recombination events between the markers and mutation (1%-5% for intragenic and extragenic polymorphisms, respectively),[12] the requirement of all the key family numbers including the index case, noninformativeness of the markers, and the presence of somatic or germline mosaicism that has to be taken into consideration even during direct mutation detection. In addition, sporadic families comprise a special problem, as it is unknown whether the disease existed in preceding generations (so the carrier status of the mother is uncertain).[9,48] Furthermore, the usefulness of genetic markers for linkage analysis depends on the number of informative markers used and the number of alleles per marker (multiallelic would be a very good asset than biallelic).[9,48,49] On the other hand, the simplicity of the PCR-RFLP test and its applicability in developing counties make it a suitable alternative strategy for carrier detection and PND. Therefore, such data could be applied as a cornerstone for the establishment of carrier detection and PND program for patients with HA in our Kurdistan region of Iraq.

In conclusion, the heterozygosity and PIC values for these intragenic FVIII markers suggest that the *Bcl*I and *Hind*III are informative and can be used for identification of HA carriers in the studied population. Furthermore, using multiple markers together can increase the efficacy of carrier identification. The feasibility of the PCR-RFLP test and its suitability for implementation in underresourced laboratories make it a favorable screening procedure in underdeveloped countries like Iraq.

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