The Frequencies of three Factor IX-Linked Restriction Fragment Length Polymorphisms in Iranian Patients with Hemophilia B

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Abstract

Background: Hemophilia B is an X-linked recessive coagulation disorder caused by factor IX deficiency. Analysis of factor IX gene polymorphisms is considered the best approach for prenatal diagnosis and carrier detection of hemophilia B where the identification of gene mutation is not easily possible.

Objective: To study the frequency of three factor IX-linked restriction fragment length polymorphisms (RFLPs) in Iranian women.

Methods: 50 normal women and 50 mothers of patients with hemophilia B entered this study. RFLP/Polymerase chain reaction (PCR) techniques for detection of BamHI, HhaI and MnlI sites were used.

Results: The frequencies of DNA polymorphisms were 0.50/0.50 for HhaI; 0.24/0.76 for MnlI; and 0.02/0.98 for BamHI sites. Among 9 different haplotypes, 2 major haplotypes were predominated.

Conclusion: HhaI and MnlI RFLPs can be used for carrier detection and prenatal diagnosis of hemophilia B.
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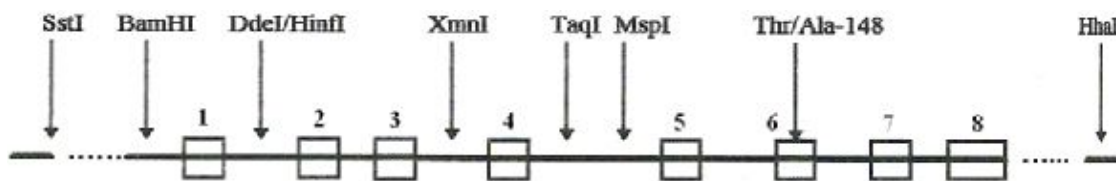**Keywords** • Hemophilia B • Restriction fragment length polymorphisms • Factor IX

Introduction

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Hemophilia B, also known as Christmas disease, is an X-linked recessive coagulopathy caused by a deficiency of or an abnormality in coagulation factor IX. Its frequency is about one in every 30,000 males at birth.[1] According to the latest statistical data given by Iranian Hemophilia Society, there are almost 900 patients with hemophilia B in Iran (personal communication). In Iran, we spend at least $US 7.5 million per year for importing factor IX that is transferred to $US 8,330 for each person per year. Since, for the time being, there is no permanent or long-term cure for the disease, carrier detection and prenatal diagnosis seem to be the best method for preventing birth of the familial cases of hemophilia B. Clinical manifestations of hemophilia B are

Fig 1: Location of DNA polymorphisms in relation to the factor IX gene

similar to hemophilia A, however, it occurs less frequently. In 8% of cases it occurs sporadically.[1,2]

The human factor IX gene consisting of eight exons and seven introns, spaced out over some 34 kb of chromosomal DNA in the long arm of the X chromosome. It is transcribed into an mRNA of 2,803 bp.[1,3,4] Due to the nature of this disorder, several hundred mutations have so far been deposited in the database.[1] There are, however, no common mutations in a given population. In order to provide prenatal diagnosis (PND) and/or carrier detection, restriction fragment length polymorphisms (RFLP) sites have been identified which are linked to factor IX gene. Both extragenic and intragenic factor IX RFLPs (Fig 1) have been used for carrier detection and prenatal diagnosis in non-sporadic hemophilia B families.[1] The factor IX genomic DNA is large in size (>30 kb) and this makes the direct mutation detection very time-consuming and expensive. On the other hand, the reverse transcriptase (RT) polymerase chain reaction (PCR) analysis of the mRNA is not so simple since liver cells are the main sources of factor IX mRNA. The mRNA approach is facing similar limitations and can only be performed in well-equipped centers. DNA polymorphic analysis, on the other hand, is quick, accurate and simple to perform. It is important to point out that it can only be used for families where the disease is familial. The limitation in using polymorphism site is that, in most cases, the frequencies of each DNA polymorphism should be studied in a given population prior to being used for carrier detection, particularly for prenatal diagnosis (PND). For this purpose we used three previously reported[5,6] factor IX-linked RFLPs (*i.e.,* BamHI, HhaI and MnlI) to study 200 chromosomes taken from 100 Iranian women.

Material and Methods

Source of DNA

Five ml of blood samples (in EDTA, 0.5 M, pH 8, as anticoagulant) were collected from 50 normal women and 50 mothers of hemophilia B patients. Samples were kept at -20 °C until analysis. One-half ml of blood was taken for DNA extraction. DNA was extracted from white blood cells by boiling method with minor modifications[7]. The blood samples were washed with 1 ml of lysis buffer (0.32 M sucrose, 10 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$ and 1% Triton X-100) and centrifuged at 10,000 rpm for 2 min. The supernatant containing genomic DNA was discarded and the procedure was repeated until the pellet looked clear. One hundred μL of 50 mM NaOH was added to each pellet and the tube was placed in boiling water for 20 minutes. Subsequently, 20 μL of 1 M Tris-HCl (pH 7.5) was added, vortexed and then centrifuged for 30 sec at 12,000 rpm. The supernatant was transferred into a tube and kept at -20 °C until processs.

RFLP/PCR

Reported oligonucleotide primers[5,6] were synthesized internally using an ABI sequencing machine. For each PCR reaction, 8 μL of genomic DNA sample was added to 50 μL of PCR mix (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP and 1 mM spermidine), followed by the addition of 1 μL of each primers (148 ng/μL) and 1.5 units of recombinant Taq DNA polymerase (Gibco BRL, Glasgow, UK). The mixture was overlaid with 20 μL of mineral oil and subjected to 25 cycles of amplification which included denaturation for 2 min at 93 °C, annealing for 1 min at 60 °C (68 °C for HhaI), extention for 1 min at 72 °C and final extension for 3 min at 72 °C.[5] Thirty

Table 1: Allele frequencies of three RFLP loci in normal women and hemophilia B carriers

	Carrier +/-	Normal +/-	Total +/-
BamHI	0.045/0.955	0/1.0	0.024/0.976
HhaI	0.49/0.51	0.51/0.49	0.50/0.50
MnlI	0.23/0.77	0.24/0.76	0.235/0.765

Table 2: Diagnostic value of two polymorphisms of factor IX gene

RFLP	Expected Heterozygoity (2pq)	Observed Heterozygosity
HhaI	50	50
MnlI	35	30
HhaI & MnlI	68	66
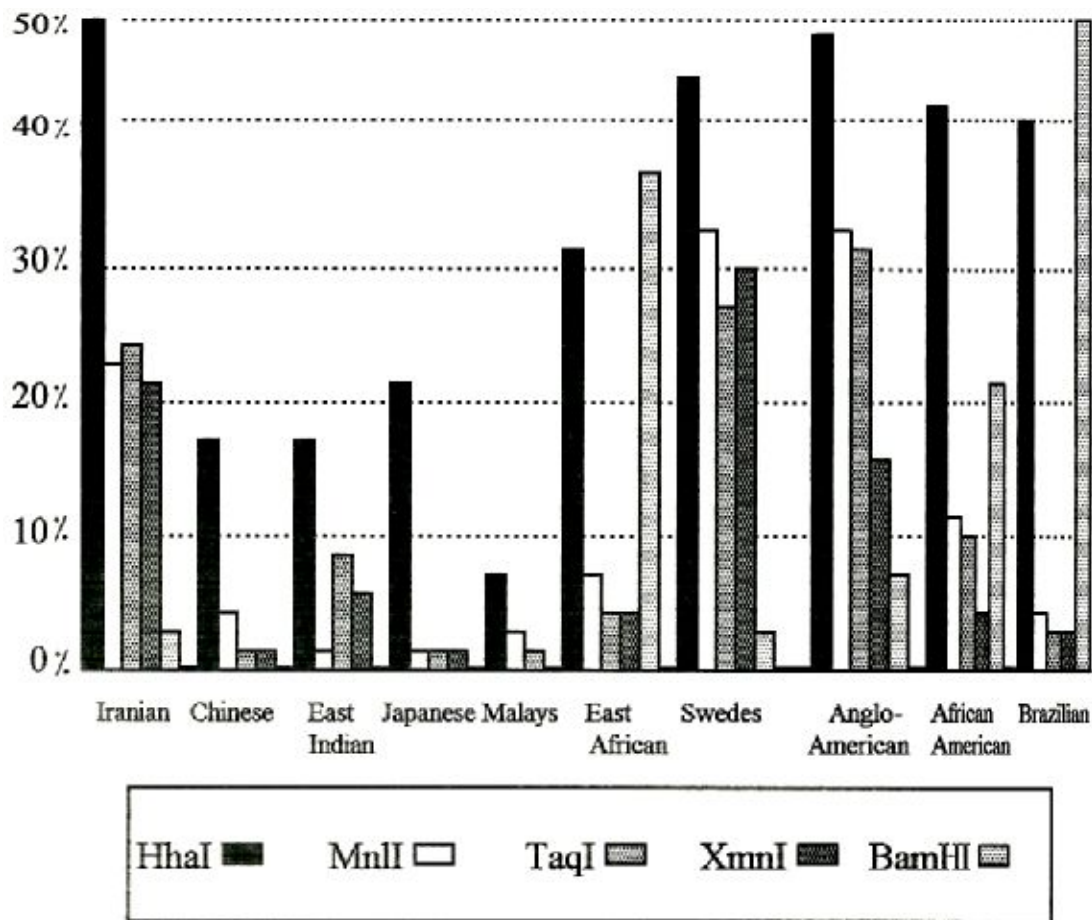
Fig 2: Comparisons for the rarer alleles frequencies for each RFLP sites in different populations (6,8-12)

µL of each amplified sample was digested with 15 units of restriction endonuclease (*i.e.*, BamHI, HhaI and MnII) in 3.5 µL of 10X buffer for each enzyme and was incubated at 37 °C overnight. After the completion of digestion, 30 µL of each sample was electrophoresed on a 2.5% agarose gel stained with ethidium bromide and visualized under UV trans-illuminator and photographed.

Results

In this study, we have determined the frequencies of the known factor IX gene linked RFLPs with BamhI, HhaI, and MnII restriction enzymes.

Restriction analysis of the fragments obtained by digesting PCR amplified products can be summarised as follows: Presence of BamHI RFLP site was very rare in our samples (2%) (Table 1). The result was similar in non-carriers and carrier females. The observed heterozygosity (the frequency of cases with or without the condition) was 2% (Table 2). The HhaI site presence or absence was similar (50% for each condition) (Table 1). There was no difference between carrier and non-carrier females. The observed heterozygosity for HhaI was 50% (Table 2). Allele frequencies of MnII site were 24% for its absence.(Table 1) The observed heterozygosity for MnII was 30%.

Our results also revealed that at least, 66% of samples showed heterozygosity for either HhaI or MnII (Table 2) with a combined heterozygosity value of 66% for these two sites.

Discussion

Factor IX gene is rather large (34 kb) and so far several hundred mutations have been reported worldwide, with no one being predominant.[1] The reason for this vast diversity of mutations is due to the nature of this X-linked disorder, that is, as the patients die new mutations occur and keep the balance. Because of the above reasons and also due to its easy application and lower expense, to study the
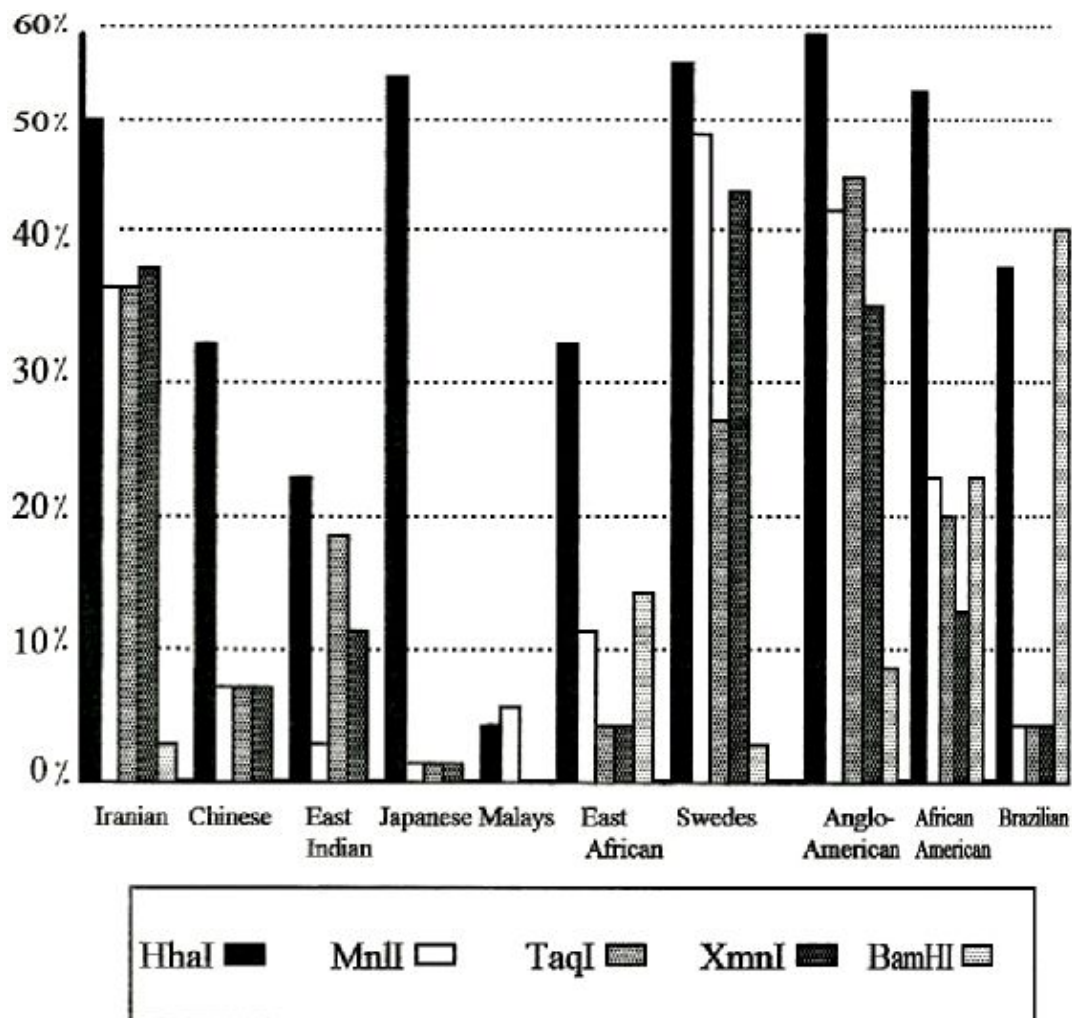
Fig 3: Comparisons in the percents of heterozygosity for each RFLP sites in different populations (6, 8-12)

frequency of this disease, linkage analysis seems to be more suited for less developed countries.

Literature review shows that BamHI and HhaI RFLP sites have independent informativeness. HhaI has shown to have a high heterozygouse value in most studied cases.(Table 2) BamHI site has not shown to be highly informative in most studied populations except in East Africans, Brazilians and African Americans (Fig 2).[6] However, BamHI site was absent in 98% of cases we studied. We did not study this site further due to its very little informativeness.

HhaI on the other hand, showed to be highly informative in Iranians. Its presence or absence was equal (50%) which is a perfect combination. This site has shown to be informative in Swedens and Angloamericans but less informative in Malays, Chinese, East Indi-

ans and Japanese. For MnlI RFLP site, we found that its presence was 24% and its absence 76%. This site was less informative in Iranians when compared to Europeans but was more informative than it is in East Asians and black Africans.[6]

Comparing allele frequencies between normal and carrier females did not show any significant difference (Table 1). We also derived the expected heterozygosity frequency using the Hardy-Weinberg equation; p^2+q^2+2pq where p denotes the presence of the site, q its absence and the product 2pq the expected heterozygosity frequency. Not much difference between expected and observed heterozygosity. (Table 2, Fig2, 3) was noticed. Our results for HhaI and MnlI together with a similar study on Iranians using TaqI and XmnI RFLP sites[9] can only show nine haplotypes out of 16 possible ones (Table 3).

Table 3: Haplotype analysis of factor IX gene in 54 chromosome

	Haplotype			Chromosome	%	
	XmnI	TaqI	MnlI	HhaI		
1	-	-	-	-	21	39
2	-	-	-	+	21	39
3	-	-	+	+	2	3.5
4	-	+	+	+	2	3.5
5	+	+	+	+	3	5.5
6	+	+	+	-	2	3.5
7	-	+	+	-	1	2
8	-	-	+	-	1	2
9	+	-	-	+	1	2

In order to show the hetrozygosity values for each RFLP site the following comparisons are given. Knowing the heterozygosities obtained in this study (*i.e.*, BamHI, 4.5%; HhaI, 50%; and MnlI, 37%) and those reported by Ghandil *et al*[9] (*i.e.*, TaqI 37%; and XmnI 38%) if we use HhaI and MnlI together, we will have 66% heterozygosity (*i.e.*, in 66% of families referred to us it will be informative) but if we use RFLP sites with lower heterozygosity values like TaqI, XmnI and MnlI combined, this value will be less (51%). This is equal if one uses only HhaI alone. If we use TaqI, XmnI, MnlI and HhaI all together then the heterozygosity will be increased to 80% (i.e. in 80% of cases the families will be informative. In another word we can perform carrier detection or PND for 80% of cases referred to us.

Acknowledgments

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