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Diagnosis of Hemophilia B Carriers, Using Taq I and Xmn I Polymorphisms of the FactorIX Gene in Iranian Individuals

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Abstract
Hemophilia B is factor IX deficiency and is inherited as X-linked recessive disorder. The subject of carrier detection in hemophilias has received new impetus in the last several years. Analysis of factor IX gene polymorphisms is considered the best approach for prenatal diagnosis and hemophilia B carrier detection, if the identification of the gene mutation is possible. Allele frequencies of two intragenic RFLP (Xmn I and Taq I) was investigated in 100 Iranian families. For some families, carrier's detection was done using this method. The results indicated that RFLP segregation analyses provide a useful method for carrier detection in hemophilia B.

Keywords: *Hemophilia B, X-link recessive, Polymorphism carrier detection, Iran*

Introduction

Hemophilia B (Christmas disease) is an X-linked recessive bleeding disorder caused by a deficiency or an abnormality of coagulation factor IX. Its birth frequency is about 1 male in every 30000 (1). According to the latest statistical figures, there are approximately 700 hemophilia B affected individuals in Iran (2). A total of eight intragenic RFLP within the factor IX gene have been characterized (3). Hemophilia B carrier detection using both intragenic and extragenic RFLP have been performed (3). A prenatal diagnosis of hemophilia B using intragenic Taq I site has been carried out (4). In families where the mutation has been characterized, direct detection of the gene defect can be applied to carrier and prenatal diagnosis (5, 6). Hemophilia B is less frequent than hemophilia A and is about 1 in 6. Eight-percent factor IX gene mutations are sporadic. The location of the factor IX gene has been narrowed down to the distal portion of band Xq27 (7) (Fig. 1). The human factor IX gene consists of eight exons and seven introns, spaced out over some 33 kb of chromosomal DNA, which is transcribed to a mRNA of 2803bp (7). Eight intragenic RFLPs which are BamH1, Hhal, Ddel, XmnI, Taq1, MnII, MspI, MseI which are located across a 20 kb region between exons I and VI (3) (Fig. 2). These RFLPs are used for carrier detection and prenatal diangosis (8). The Xmn I have been shown to be in strong linkage disequilibrium with the Taq I polymorphism (8). The frequencies of these intragenic RFLP are high in European populations but low in non-European populations. The second polymorphic sites have been used for carrier detections and prenatal diagnosis, have been performed in Russia (9) using Taq I and Ddel RFLP and also carrier detection are done in Japan using two dinucleotide RFLPs (F9 – 793, F9 – 192) and Hhal RFLP (10). This study presents the results of RFLP analysis for two intragenic polymorphisms in the Iranian hemophilia B families. This is the first investigation of RFLP analysis and carrier detection for hemophilia B in Iran.

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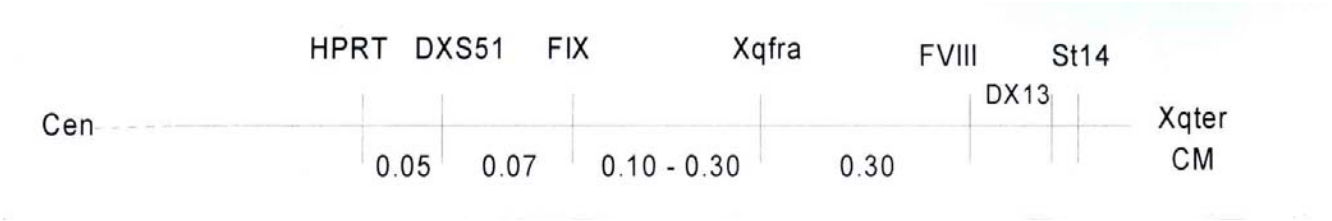

Fig. 1: Genetic map of the long arm of X chromosome has shown with genetic distances in centimorgans. HPRT, DXS51, FIX, FVIII, St14, DX13 and fragile X site are shown.

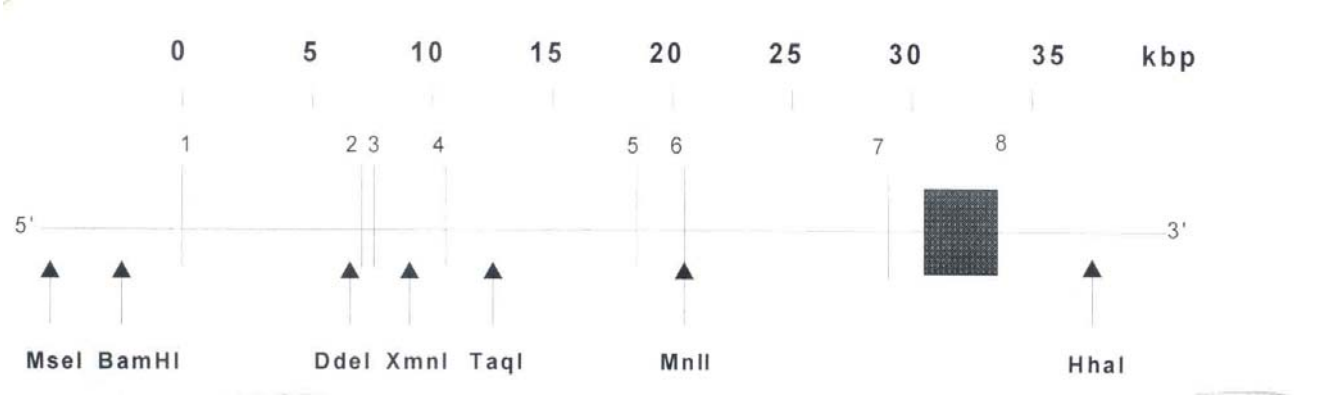

Fig. 2: Systematic diagram of the factor IX gene and its linked RFLPs. Numbers on top row, indicate distance in kbp. The numbers just above the gene, indicate exon number. Arrows below the, gene indicate sites of RFLP with the appropriate restriction enzyme.

Materials and Methods

Five ml of blood samples (in EDTA as coagulated) were collected from 100 mothers of patients with hemophilia B and were frozen at –20ºc until use. DNAs were extracted from white blood cells by boiling method (11). Briefly, 0.5ml of each blood sample was taken for DNA extraction. Each blood sample was washed with R-buffer, containing 0.32 mM sucrose, 10 mM Tris-HCl, 5mM Mgcl2, 1% 100 x triton, centrifuged at 10000 *g for 1 min and repeated until the pellet looked clear. One hundred µl of 50 mM NaOH was added to each pellet and placed in boiling water for 20 min. Then, 20 µl of 1 M Tris-HCl (pH 7.5) was added, vortexed and then centrifuged at 10000 *g for 20 seconds. The supernatant was transferred into fresh clear tubes and kept at - 20ºC until use.

Oligonucleotides primers were synthesized at Biotechnology Department, Pasteur Institute of Iran. The sequences of primers were as follows (12):

For Xmn I: primer 1: 5’ - CAG AGA CTG CTG ATT GAC TT-3’

Primer 2: 5’ – ACA GCC AGA TAA AGC CTC CA-3’

For Taq I: primer 1: 5’- TAT AAT GGG AAT TCT CCA CAT-3’

Primer 2: 5’ – AGT AGA AAG TGA ATT CCT CA-3’

For PCR reaction 8µL of a genomic DNA sample was added to 50µl of PCR mix (10 mN Tris-Cl, 50 mM Kcl, 1.5 mM MgCl2, 0.2mM dNTP, 1mM spermidine, dH20), 1µL of each primer and 0.3µl of recombinant Taq DNA polymerase (5 u/µl) (CinaGene co, Tehran, Iran). The mixture was overlaid with 20 µl of mineral oil and subjected to 30 cycles of amplification, which included denaturation for 2 min at 93ºC, annealing for 30s at 58ºC,

polymerization for 30s at 72ºC and final extension for 3 min at 72ºC (13). A total of 30µl of each amplified sample was digested with 15 u of restriction endonuclease, either at 37ºC (Xmn I) or at 65ºC (Taql) in a 35µl reaction volume incubated overnight after the completion of digestions, 30 µl of each sample was electrophoresed on a 2% agarose gel for Taql and 2.5% agarose gel or PAGE 15% for Xmnl. The gel was stained with EtBr, and visualized under UV transilluminator and analysis was done on pictures taken. Factor IX DNA haplotypes were generated by analyzing 200 X chromosomes in which linkage could be ascertained with PCR-mediated assays for two polymorphic sites: FIX Xmn I (X1/X2)and FIX Taq I (T1/T2). These 200 X chromosomes were from 100 Iranian females with hemophilia B in their families. The association of these sites was analyzed using the chi-square test as previously reported (14). The disequlibrium coefficient (D) was calculated by $D= (P11P22 – P12P21)/n^2$, where P11, P22, P12, and P21 were the number of chromosomes bearing the four possible haplotypes and *n* was the total number of chromosomes. The null hypothesis of D=0 (linkage equilibrium) was tested by $X^2 = nD^2/(p_a q_a p^b q^b)$, with one degree of freedom, where p_a, q_a, p_b and q^b were the four gene frequencies (15). Intragenic Taq I and Xmn I RFLPs were studied in Iranian families with hemophilia B. It was found that these two RFLPs are located at intron 3 and 4 of factor IX which gene could be used for Iranian hemophilia B carrier diagnosis. The essential characteristics of the PCR products are shown in table 1. The locations of the enzyme-restriction site, the region amplified and the sizes of the digested fragments were noted. The patterns specific for the Xmn I and Taq I polymorphisms are shown in figure 3. These patterns were simple, but in the absence of an external control the (-) and (-/-) genotypes might sometimes be difficult to distinguish from nondigested amplified product. So in every gel, positive control samples were used (+), (+/+), or (+/-) to control the digestibility of the sample tested.

Table 1: Comparison of PCR Polymorphisms of FIX

Polymorphism Name, Gene Bank Location	Region Amplified	Size Of Fragments (bp)
Xmnl, 10041-10051 bp	9979- 10199	220, 153, 67
Taql, 14076 – 14079 bp	13980- 14279	299, 203, 96

Allele frequencies and heterozygosity values for Taq I and Xmn I polymorphisms for 200 chromosomes are shown in Table 2. The predicted heterozygosity rates were 37% for Xmn I and 38% for Taq I. From these results, the estimated frequency of female heterozygous (informative) using all two polymophisms was 46% in Iranian subjects. No statistical difference was detected between observed and expected heterozygosity ($X^2 = 0.3$, $p>0.05$ for both polymorphisms) (Table 3). Figure 4 demonstrates typical carrier detection in Iranian hemophilia B families using Taq I polymorphism.

Table 2: Allele frequencies values for Taq1 and Xmnl

Polymorphisms	Allele	Allele Frequency	Heterozygosity Rate (%)	Not of Examined X Chromosomes
Taq I	T1 (+) T2 (-)	0.25 0.75	37	200
Xmn I	X1 (+) X2 (-)	0.21 0.78	38	200

Table 3: Diagnostic value of two polymorphisms of factor IX gene

Polymorphic site	Informative (%)		
	No. of informative	Observed	Expected
Taq I	37	37	38
Xmn I	38	38	34
Taq & Xmn I	46	46	41

Strong allelic association was known to exist between the two intragenic RFLPs. Association between these sites being 4 kb apart was examined in this study (P<0.001). Also, strong linkage disequilibrium was observed between these two sites among Iranian families studied (P<0.001) (Table 4). The observed values were similar to expected values. Combined power discrimination or informativeness for these two sites was close to 50%.

Table 4: Allelic association between the two polymorphisms of factor IX gene in Iranian families

Association	No. of Chromosome	D	X2	P
Taq I & Xmnl	200	0.126	102.07	<0.001

The haplotypes for the two polymorphisms of 100 granian women are shown in table 4. Four haplotypes were characterized for them. Haplotype IV was (-) for the two polymorphisms (73%) and had the highest percentage among the others (Table 5). Haplotype I was (+) for them and was 35%, but haplotypes II and III that were (-) and (+) for Xmn I and Taq I sites were 12% and 7%. The percentage of haplotype II [(+) for Taq I and (-) for Xmnl] was more than type III, but it was not very different. So, it was shown that Taq I site was more polymorphic than Xmn I.

Table 5: TX haplotype analysis of factor IX gene in 200 X chromosome

TX Haplotype	No. of Alleles/Observed (%)
T1X1	۳۵(17.5)
T1X2	12(6)
T2X1	7(3.5)
T2X2	146(73)

Discussion

Hemophilia B is an X-linked recessive disorder that is found equally among all ethnic groups (8). The cloning of the factor IX gene and the identification of intragenic factor IX DNA polymorphism have improved carrier testing and prenatal diagnosis in affected families. Direct sequencing of the factor IX gene, RFLP analysis by Southern blotting and PCR-based polymorphism analysis, are used for carrier detection but is expensive and time consuming (3). The direct sequencing of the factor IX gene is more accurate and is now generally preferred over the other procedures. However, PCR-based polymorphism analysis is still useful, because it is technically simpler, more rapid, and amenable to large-scale screening programs (16). The present study probably represents the most comprehensive investigation of two factor IX gene polymorphism in Iranian population. Based on our results it is possible to offer a systematic strategy for carrier detection and prenatal diagnosis of hemophilia B in Iranian population. The combined analysis of TaqI and Xmn I polymophisms should allow information in about 46% of females. The overall combined usefulness of the polymorphisms depends on the level of linkage disequilibrium between them (17). In haplotype analysis, almost strong linkage disequlibirum was found between TaqI and XmnI RFLPs. However, an increased frequency in heterozigosity was seen when Xmn I was combined with Taq I polymorphism. The difference between the observed (46%) and the estimated (41%) heterozygous frequencies using these two polymorphisms was minimal. One reason for this similarity could be the preferential association of the allele in Xmn I or Taq I with the common allele in the other locus. It has been suggested that the effect of linkage disequilibrium does not always reduce the level of observed heterozygous frequency, when multiple polymorphic markers are used (18). These results also allow a comparison between European and Iranian populations. The reported

gene frequencies for the '+' allele for TaqI polymorphism in Swedes, Basques, Caucasian and Russian Slavs are 0.26, 0.31, 0.35 and 0.29 respectively (9, 12). Also, the frequency of '+' allele for XmnI in Swedes, Basques and Caucasian are 0.30, 0.23, 0.29 respectively (12, 19). The Iranians show similar frequencies for XmnI (0.21) and TaqI (0.25) polymorphisms. For purposes of comparisons between populations and characterization of haplotypes, have obvious advantages over the determination of the allelic frequencies for individual polymorphisms. In another study, they determined factor IX haplotypes comparing five polymorphisms (5' BamHl, Xmn I, Taq I, Mnll and Hhal) in several ethnic groups (12). They identified three haplotypes for Xmn I and Taq I polymorphisms in Swedes and Basques. We also identified 4 haplotypes for these two polymorphisms associated with the factor IX gene in Iranians. However, this particular combination of polymorphism is inadequate for comparisons Iranian and European population because only two polymorphisms among the others were sutdied. Even with these limitations, significant similarity emerges from a simple comparison with our results. The haplotype (Xmn I and Taq I) - + in Swedes and Basques were found in 6% and 3% of chromosomes and respectively it was present in 5% of chromosomes in this study. On the other hand, the haplotype ++, which was present in 17.5% of chromosomes in Iranian, have been found in 12% and 25% of chromosomes in Swedes and Basques, respectively. In conclusion, this study allowed better genetic characterization of the Iranian population, detected carrier of hemophilia B and provided useful information for the familial study of this disorder in Iranian population by use of PCR-mediated Xmn I and Taq I polymorphisms rapidly and effectively. Also, It is suggested that the determination of factor IX gene baplotypes might be a powerful tool for the investigation of interrelationships between human populations.

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