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Indirect Genetic Analysis of Pakistani Hemophilia a Pedigrees by using BclI and HindIII Polymorphic Markers

***Memoona Rasheed*[1]**
***Naghmi Asif*[2]**
***Asfa Zawar*[3]**
***Khalid Hassan*[4]**
***Tazheen Anwar*[5]**

[1] Molecular Biologist, Department of Pathology, Islamabad Medical and Dental College
[2] Professor/Head of Department, Department of Pathology, Islamabad Medical and Dental College
[3] Demonstrator, Department of Pathology, Islamabad Medical and Dental College
[4] Chief Pathologist, Department of Pathology, Dr. Akbar Niazi Teaching Hospital.
[5] In-charge Thalassemia Center, Pakistan Institute of Medical Sciences

Address for Correspondence
Dr. Memoona Rasheed
Islamabad Medical and Dental College
memoona_rasheed@hotmail.com

Abstract

Objective: The current study was aimed at analyzing the utility of intragenic polymorphic restriction sites (*Hind*III and *Bcl*I) in screening of haemophilia A patients and their immediate family members.

Methodology: Thirteen families having one or more affected child were recruited from Pakistan Institute of Medical Sciences. For Genomic DNA extraction, phenol-chloroform method was used. Genomic regions containing the RFLP site were amplified by using specific primers designer from flanking regions. The PCR products were digested using *Hind*III and *Bcl*I and the products were resolved on 6% Polyacrylamide gel electrophoresis and visualized by silver staining technique.

Results: A total of 100 X-chromosomes were investigated in this study (36 males, 32 females). The minor allele frequency for Intron 18/*Bcl*I T>A was observed to be 0.68 while for Intron 19/*HindIII* C>T it was observed to be 0.55 in our study population. The Intron 18/*Bcl*I T>A marker was informative in four of the families while Intron 19/*Hind*III C>T was informative for 8 families. The PIC was calculated to be 0.34 for *Bcl*I marker and 0.37 for *Hind*III.

Conclusion: The rate of heterozygosity and the PIC values obtained for the two markers suggests that *Hind*III marker is more informative as compared to *Bcl*I in our study population. The two markers were decisive in haplotyping 8 (66.6%) families.

Keywords: Hemophilia A, linkage Analysis, *Hind*III, *Bcl*I

Introduction

Hemophilia A is a rare single gene disorder that occurs due to deficiency of plasma levels of coagulation factor VIII. Carrier screening through indirect linkage analysis can be a cost effective alternate for molecular diagnosis. Hemophilia A (OMIM 306700) is an X-Linked recessive bleeding disorder that affects approximately 1/5000 males.[1] The disease results due to deficiency of plasma levels of coagulation factor VIII which is encoded by *F8* gene located on long arm of X- chromosome. According to National patient registry of hemophilia foundation, Pakistan (www.hemophilia.org.pk), a total 1,998 cases of hemophilia A are registered in Pakistan which makes its prevalence of 0.92/100,000 individuals. The phenotypic severity of the disease is characterized as severe, moderate and mild depending upon the circulating plasma levels of coagulation factor VIII of <1%, 1-5% and 5-30% respectively.[2] Patients with hemophilia can present with joint swelling and damage, organ damage or intracranial hemorrhage leading to increased morbidity and mortality. [3] A number of disease causing mutations have been reported in *F8* gene but among them only two, intron 22 inversion (int22h-1/int22h-2.3) and intron 1 inversion (int1h-1/int1h-2) occur more often with prevalence of 40-50% and 5-10% in cases with severe form of haemophilia A.[4] Beside this, almost 3735 different mutations have been reported in CDC Hemophilia a Mutation Project (CHAMP). [5]

Genetic diagnosis of the disease involves two strategies, direct identification of pathogenic mutation in *F8* gene by inversion specific PCR or Sanger/Next generation sequencing or alternatively indirect linkage analysis of polymorphic intragenic or extragenic markers. [6,7] Direct mutation analysis is the most suitable option for carrier detection and prenatal diagnosis but due to the size constrain of the F8 gene and mutational heterogeneity, it often becomes difficult. Linkage analysis by using restriction fragment length polymorphisms (RFLPs) and short tandem repeats (STR) markers is helpful in carrier screening in families of affected

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individuals.[8,9] The heterozygosity rates of these markers differ in various ethnic groups, it is important to have data about informatively of these markers. This study is focused to analyze utility of two polymorphic sites in intron 18 and 19 of the F8 gene in individuals affected with haemophilia A and their immediate family members.

Methodology

Subjects: The research was approved from ethical review board of Islamabad Medical and Dental College, Islamabad, Pakistan. The families were recruited from Thalassemia Center of Pakistan Institute of Medical Sciences (PIMS) after taking an informed written consent. Information such as age at diagnosis, family history of disease, parental consanguinity and site of bleeding were recorded in a specially designed performa.

Linkage Analysis: Genomic DNA (gDNA) was extracted from peripheral by using Phenol-Chloroform method. [10] Primers for Polymerase chain reactions were designed by using Primer 3 Input (version 0.4.0) by using genomic reference sequence flanking the polymorphic sites i.e. ChrX:154130143C>T (Disrupting a restriction site for HindIII) and chr23:154132090A>T (Disrupting a restriction site for BclI) (Table I). The primers were validated by using In-silico PCR function on UCSC genome browser. The restriction site on the reference and altered DNA was analyzed by using NEB Cutter V2.0. Standard PCR was performed on the recruited families followed by restriction digestion of the amplicon with HindIII (Cat. No. ER0501, Thermofisher Scientific) and BclI (Cat. No. ER0721, Thermofisher Scientific) according to manufacturer's protocol. Polyacrylamide gels (6%) were used to resolve the digested product and digital photographs were recorded. Allele frequencies were calculated by counting the major and minor alleles observed in restriction fragment length polymorphism analysis. The allele number was then divided by total number of chromosomes (number of males + [number of females x 2]). Hardy-Weinberg equilibrium (HWE) and observed and expected heterozygosities were calculated by using Popgene software package. The PIC values, which represents the sum of frequency of each possible mating multiplied by the probability that an offspring will be informative, were used to assess the genetic informative potential.

Results

Total 13 families affected with haemophilia A were recruited in the study. The mean age of affected individuals was 11.76 years (4-22 years). The family history of disease was reported in 4 families (30.7%) and parental consanguinity was present in 4 families (30.7%). Easy bruising, hemathrosis, purpura and joint involvement were the common presentations during previous visits to the centre (Table II). The total numbers of X-chromosomes were 100 (36 males + 32 females x 2). The minor allele frequency (MAF) for Intron 18/BclI T>A was 0.68 while for Intron 19/HindIII C>T was 0.55 in our selected families. Table III represents heterozygosity rate of selected markers in our families. The heterozygosity rate and polymorphism information content values suggest that HindIII marker is more informative as compared to BclI. The two markers were co-segregating with disease phenotype in 8 (66.6%) families (Figure 1).

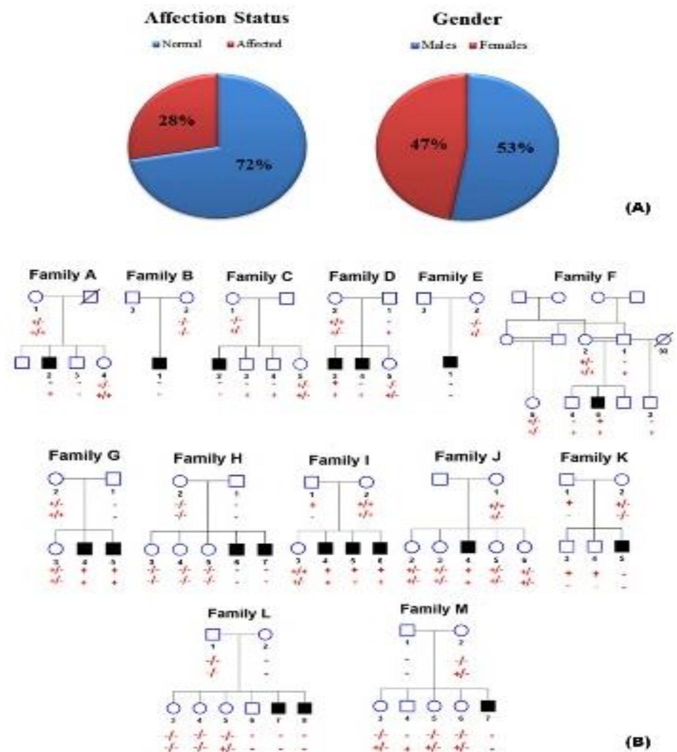

Table I: Primer Sequences and the amplicon size before and after restriction digestion.

Primer ID	Primer Sequence	Product Size	Product size after digestion
F8 In-18 (F)	TTCCCTAGGAAAGTACTGTGACAT	392 bp	163+229 bp
F8 In-18 (R)	TCAGGATCAAAGGATTCGATG		
F8 In-19 (F)	GTGCCAAGAACTGTACTCAACG	541 bp	313+228 bp
F8 In-19 (R)	GCATCTACATGCTGGGATGA		

Figure 1. Subjects and Genotype information. (A) Represents the affection status and gender ratio of the selected families. (B) Represents the Haplotype of the genotyped families. Positive sign represents major allele while minus sign represents minor allele. The marker is considered to be informative if the affected hemizygous male shows one of the genotype harboured by the heterozygous mother and the genotype of the affected individual is not shared by the phenotypically normal individuals of the family.

Discussion

Antihemophiliac factor or FVIII is a central player of normal blood coagulation process. Damaging mutations in FVIII result in delayed clotting time which causes a minor cut in hemophiliac patient to bleed for several days. As males are hemizygous for X-chromosome, a single defective copy inherited from the carrier mother results in the disease expression. Therefore carrier detection of females is an important aspect for prophylactic measure especially in developing countries. Assessment of carrier status based on linkage analysis of polymorphic sites has been in use for decades and is cost effective procedure. Two types of

Table II: Clinical Profile of families

Family ID	A	B	C	D	E	F	G		H		I		J	K	L		M
Individual ID	2	1	2	3	1	6	4	5	6	7	4	5	4	5	7	8	7
Age (Years)	17	10	17	7	13	6	8	6	19	17	15	22	4	12	5	12	10
Family History	No	No	Yes	No	No	No	No	No	No	No	Yes	Yes	No	Yes	Yes	Yes	No
Parental Consanguinity	No	Yes	No	Yes	No	No	Yes	Yes	No	No	No	No	Yes	No	No	No	No
Age at diagnosis*	6 D	1.5 Y	2 M	6 Y	4 Y	6 M	2 M	2 Y	1 M	7 M	8 M	8 Y	1 Y	1 M	1 M	1 M	2 M
Symptoms																	
Easy bruising	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gingival bleeding	+			+	+	+			+	+	+			+			
Purpura				+	+		+	+	+	+	+	+	+			+	
Epistaxis		+			+				+	+		+		+			
GI bleeding																+	
Haematuria									+	+	+			+		+	
Melana										+					+	+	
Post circumcision bleeding	+	+	+			+				+	+	+	+	+		+	
Post-surgical/dental procedure bleeding	+		+		+												
Post Trauma Bleeding	+	+			+	+	+	+	+			+	+	+	+	+	
Hemathrosis		+	+		+	+	+	+	+	+	+	+	+	+	+	+	+
Cephalohematoma				+													
Bleeding from ear													+				
Joint involvement	+	+			+	+	+	+	+	+	+	+	+	+	+	+	

Table III: Allele frequencies, Heterozygosity rates, polymorphism information content and linkage disequilibrium of HindIII and BslI polymorphic marker.

Marker	Alleles	Allele Frequency	Heterozygosity (Observed)	Heterozygosity (Expected)	Informative families n(%)	PIC	P-Value
BclI	Major: T	0.32	0.18	0.43	4 (31)	0.34	0.00
	Minor: A	0.68					
HindIII	Major: C	0.45	0.27	0.49	8 (62)	0.37	0.327
	Minor: T	0.55					

polymorphic sites are used within the F8 gene i.e. single nucleotide polymorphism (SNP) and variable number tandem repeats.[11] Population based strategies for carrier identification rely on heterozygosities of different polymorphic loci in the *F8* gene. In present study, we have used restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) technology for detection of two SNPs i.e. Intron 18/*Bcl*I T>A and Intron 19/*Hind*III C>T in available affected and normal individuals of thirteen families having one or more affected male individual. The minor allele frequency obtained for *Bcl*I and *Hind*III were 0.68 and 0.55 respectively in our study population.

Another study from Pakistan reported the MAF of 0.67 for *Hind*III allele in hemophilia A families. Different linkage analysis studies from India have reported MAF for *Hind*III as 0.62, 0.63, 0.67 and 0.71.[12–15] A study on Iranian affected families reported minor allele frequency of 0.52 [16] which are very close to the frequency observed in our families however another study on Azeri-Turkish population of Iran showed MAF of 0.80. [8] A study from Iraq also showed frequency of 0.58 for *Hind*III polymorphic site. [9] Studies from Russia and Denmark have reported MAF of 0.71 and 0.70 respectively while a much higher MAF i.e. 0.81 for *Hind*III has been reported from studies based on Japanese population.[17]

The polymorphic site for *Bcl*I has not been previously studied in Pakistani population however the studies conducted in India showed MAF of 0.34, 0.43, 0.32 and 0.56.[11] Studies conducted on Iranian population showed frequencies of 0.48 and 0.31.[8,16] Studies on other populations such as Iraq, Polynesia and Italy showed MAF of 0.40, 0.45 and 0.65 respectively [9,18,19] while in rest of the populations the frequency was lower than <0.40.[11] The heterozygosity obtained in our study is much higher, 0.68, compared to the previously published data. This discrepancy in the data of these two polymorphic loci may be related to ethnicity. The cumulative informativeness of the two polymorphic sites for accessing inheritance of disease allele from haplotypes was 66.6% (n=8) in our study (Figure 1). A study conducted in India reported combined informativity of *Hind*III, *Bcl*I and intron 22 STR as 87.2% [20] while another study from India, the combined informativeness for *Bcl*I and *Hind*III was reported to be 66%.[21] The heterozygosity and PIC values for these 2 markers demonstrate that the *Hind*III marker is a better predictor in our families as compared to *Bcl*I polymorphic marker.

These finding propose that linkage analysis could be used to diagnose possible carriers of the disease trait in the family and pre-symptomatic males who could suffer bleeding following trauma. Addition of other polymorphic, intragenic and extragenic, SNP and VNTR markers in the diagnosis panel can help built diagnosis where the two markers were non-informative.

Conclusion

These finding propose that linkage analysis could be used to diagnose possible carriers of the disease trait in the family and pre-symptomatic males who could suffer bleeding following trauma. Addition of other polymorphic, intragenic and extragenic, SNP and VNTR markers in the diagnosis panel can help built diagnosis where the two markers were non-informative.

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