*Hin*dIII-based restriction fragment length polymorphism in hemophilic and nonhemophilic patients

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Abstract

Hemophilia A is most common recessively inherited bleeding disorder, which affect one in five thousand male births throughout the world. In most of the hemophilic A patients, no common mutation is easily identifiable. This limitation has been overcome by the use of polymorphic DNA marker, i.e., restriction fragment length polymorphism (RFLP). This marker of polymorphism could only be detected by amplifying the polymorphic region and digestion the polymerase chain reaction (PCR) product with the restriction enzyme (PCR−RFLP), i.e., *Hin*dIII. The polymorphic region of *Hin*dIII is 608 bp in length and after the restriction digestion, different sizes of fragments, i.e., 427 and 181 bp were, respectively, obtained. However, in homozygous (+/+) condition three bands of 427, 100, and 81 bp were obtained and in the other negative allelic homozygous condition (-/-) two bands of 427 and 181 bp were obtained. Similarly fragments of different sizes, i.e., 427, 181, 100, and 81 bp were obtained in heterozygous conditions. Therefore, in this study, we have analyzed the factor VIII gene in the 17 different families using restriction enzyme *Hin*dIII-based RFLP molecular marker technique. Out of these, the observed heterozygosity for *Hin*dIII was found 47.5%, whereas, for positive allele it was 26%, and for negative allele the frequency was 74%.

Key words: Gene, hemophilia, polymerase chain reaction, restriction enzyme, restriction fragment length polymorphism

INTRODUCTION

Restriction enzyme, also known as restriction endonucleases, recognizes the specific base sequences in double stranded (ds) DNA and cleave both of its strands at the specific places. Approximately 3000 restriction enzymes, spreading over 230 different DNA sequences, have been discovered. They are mostly found in bacteria, but can also be isolated from viruses, archea, and eukaryotes.[1]

*Hin*dIII is a kind of restriction enzyme, commonly isolated from the *Haemophilus influenzae d* bacteria.[2] It possesses the polymorphic restriction site on to the intron 19 of factor VIII gene.[3] This factor is found as an important factor for the blood-coagulation procedure. The gene of factor VIII is found to be located on the long arm of X-chromosome at Xq^{28} locus. It has a length of 186 kb; possess 26 exons and 25 introns. Out of which, exon 14 and 26 are the largest in their length, i.e., 3106 and 1958 bp, respectively, whereas, the other 24 exon ranges in size between 69 and 262 bp.[4,5]

The translation product of the factor VIII gene results in a synthesis of mature protein of 2332 amino acids with a molecular weight of 265 kDa. Factor VIII protein is found in plasma as a part of complex glycoprotein (thromboplastin) and as an essential cofactor for the activation of factor X and XI. Factors X and XI are the most important amplifier for the cascade mechanism of blood clotting. Several heterogeneous mutations/changes, i.e., point mutations, deletions, insertions, and rearrangements/inversions[6] have been identified in the factor VIII gene, and these described mutations in the factor VIII gene are found to be responsible for causing the Hemophilia disease.[7]

Hemophilia disease also called "The Royal Disease" was discovered by Haldane in the royal families of Europe. The

main symptom of this disease is the lack of blood clotting. Ordinarily blood clotting takes place within 5–6 min at a bleeding wound in a person, so that further bleeding stops. But in persons suffering from the Hemophilia-A disease, blood clotting may take a time of 30 min–24 h and sometimes may cause the death of the patients due to the lack of the blood clotting, i.e., hemorrhage. This condition occurs due to the lack of the thromboplastin protein in blood plasma and the nonactivation of factor X and factor XI because of the mutations in the factor VIII gene.

Factor VIII gene mutations/changes can be detected using the restriction fragment length polymorphism (RFLP) molecular-marker technique. RFLPs are the first type of DNA-based marker, which have been recognized to track the hemophilic inheritance.[8] It is a kind of molecular-marker technique, in which the DNA molecule produces the different set of fragments, due to cleavage with a restriction enzyme. These enzymes possess the specific restriction sites within a DNA molecule, and cut the ds DNA in to the different polymorphic fragments. These polymorphic fragments are found as a basis for the polymorphism. Similarly, the *Hin*dIII restriction enzyme possesses the restriction site in the intron 19 of the important blood-coagulating factor VIII gene, and can be used to find out the polymorphism in the different hemophilic and nonhemophilic individuals.[3,9]

Therefore, an attempt has been made to find out the polymorphism using *Hin*dIII-based upon the restriction-fragment length polymorphism molecular-marker technique in the hemophilic and non-hemophilic patients.

MATERIALS AND METHODS

Sample collection

Blood samples of 17 families were taken for the analysis. About 5–8 ml of peripheral blood of the different individuals (i.e., P, PM, PF, and PS) were taken in the vials with an anticoagulant Ethylenediamine tetra acetic acid (0.5 M) in the ratio of 1:9. Finally, the samples were stored at 4°C. The above samples were provided by the "Hemophilia Society," Lucknow.

Here,	P:	Patient
	PM:	Patient's mother
	PF:	Patient's father
	PS:	Patient's sister

DNA isolation

The DNA was extracted from the peripheral blood by using rapid lysis method.[10] In this method, the following reagents were used:

Buffers and other reagents

1. Lysis buffer consisting of 11% sucrose (w/v); 1% Triton X (v/v); 0.1% $MgCl_2$ (w/v); 0.15% Tris buffer (v/v) and autoclaved.
2. Proteinase buffer consisting of 5% NaCl (w/v); 9% EDTA (w/v, pH 8.0), stored at 4°C.
3. 10% SDS.
4. Phenol: Chloroform (4:1, v/v).
5. 70% ethanol (v/v); 100% ethanol or absolute alcohol (v/v).

Qualitative analysis of DNA

Quality and purity of the extracted DNA was checked by running the samples on 0.8% (w/v) agarose gel.

PCR amplification of the extracted DNA

PCR reactions were performed in a 17.5 μl volume containing 1.5 μl of 10× Taq buffer (100 mM Tris pH 9.0; 500 mM KCl; 15 mM $MgCl_2$; 0.1% gelatin), 200 μM (0.2 mM) of each dNTPs (Bangalore Genei™, Bangalore, India), 0.2 μM of each forward and reverse primer (Operon Biotechnologies, Germany), 1 unit of Taq DNA polymerase (Bangalore, India), and 10 ng of DNA for the optimum amplification.

The PCR conditions were as follows: First, the samples were incubated for 30 cycles of denaturation at 94°C for 1 min, annealing at 50–60°C for 1 min, and extension at 72°C for 2 min. Then, reactions were completed by incubating the samples at final extension for 10 min at 72°C. PCR reactions were conducted in a Mastercycler Gradient PCR system (Eppendorf, Hamburg, Germany). The amplified PCR products were resolved by electrophoresis on 2% (w/v) agarose gel stained with ehtidium bromide (0.5 μg/ml) and run in 1× Tris Boric acid EDTA buffer (89 mM Tris, 89 mM Boric acid, and 2.24 mM Na_2 EDTA, pH 8.0) for 2–3 h at 60 V. Finally, the resolved DNA bands were observed under the UV transilluminator chamber.

Restriction digestion of the amplified products

For the RFLP methodology, good quality of amplified PCR products were selected. The restriction digestion of the amplified PCR products were performed in a 20.0 μl volume containing 2.0 μl of 10× digestion buffer, 0.5 μl *Hin*dIII restriction enzyme (Bangalore, India), 8.0 μl of amplified PCR product and 9.5 μl of HPLC water. Then the samples were incubated for the digestion at 37°C in a water bath for 4–5 h. After the restriction digestion of the PCR products, they were loaded on to the 2.5% (w/v) agarose gel and 8% (w/v) polyacryl amide gel electrophoresis (PAGE) gel[11] and run at 60 V for 1–2 hours. Finally, the resolved bands were observed under the ultraviolet light (UV) light and scored for their presence/

Table 1: Heterozygosity, infected, and uninfected hemophilic individuals in the studied 17 families

Family code	Sample code	*Hin*dIII restriction site
H-01	P	-
	PM	-/-
	PF	na
	PS1	na
H-02	P	+
	PM	±
	PF	-
H-03	P	-
	PM	-/-
	PF	na
H-04	P	-
	PM	±
	PF	-
H-05	P	+
	PM	±
	PF	+
	PS1	±
H-06	P	-
	PM	-/-
	PF	na
H-07	P	-
	PM	-/-
H-08	P	-
	PM	-/-
	PF	na
	PS1	na
H-09	P	+
	PM	±
	PF	na
H-10	P	-
	PM	-/-
	PF	na
H-11	P	-
	PM	-/-
	PF	na
	PS	na
H-12	P	+
	PM	±
	PF	-
	PS	-/-
H-13	P	-
	PM	±
	PF	-
	PS1	±
	PS2	±
	PS3	±
H-14	P	+
	PM	+/+
	PF	na
	PS3	na
H-15	P	-
	PM	-/-
	PF	-
	PS1	-/-
	PS2	-/-
	PS3	-/-
H-16	P	-
	PM	±
	PF	na
H-17	P	-
	PM	±
	PF	-
	PS3	-/-
	PS4	-/-

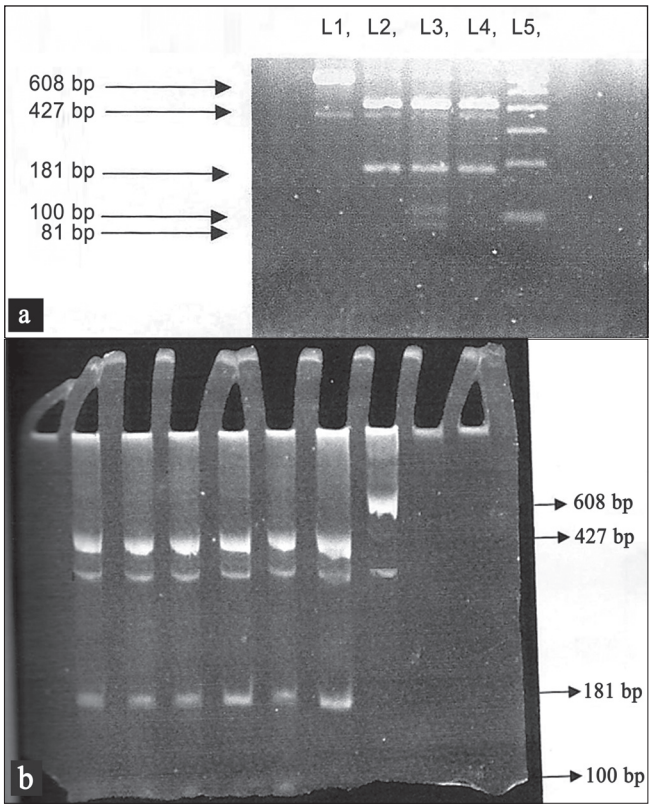

Figure 1: (a) *Hin*dIII PCR–RFLP products on agarose gel: Lane 1 showing the undigested sample, lane 2 showing the case of negative allele (uninfected mother); lane 3 showing the heterozygosity ± (informative mother) and lane 4 showing the positive allele (infected mother). Lane 5 is 100 bp DNA ladder. (b) *Hin*dIII PCR–RFLP products on 8% PAGE gel: Lane 2 showing the undigested sample; lane 3 showing the case of negative allele (uninfected mother); lane 4, 6, 7, and 8 showing the heterozygosity ± (informative mother) and lane 5 showing the positive allele (infected mother). Lane 1 is 100 bp DNA ladder.

absence variation in different individuals with respect of the *Hin*dIII polymorphism.

RESULTS AND DISCUSSION

In this study, a total of 17 families were analyzed for the hemophilic and nonhemophilic variation using *Hin*dIII-based RFLP technique. The variation between the hemophilic and nonhemophilic specifically male patients were find out on the basis of the patient mother (female) as a carrier/informative (heterozygosity), infected (positive allelic) and uninfected (negative allelic) [Table 1]. Out of these 17 families, 8 mothers were informative (±) and rest of them were found to be in noninformative in condition. The observed heterozygosity rate was found to be 47.5% and positive allele frequency (infected) was 26%, whereas negative allele frequency (uninfected) was 74%. The heterozygosity and infected/uninfected conditions were observed on the basis of *Hin*dIII restriction digestion of the amplified region of the intron 19 of the factor VIII

gene. The gene of factor VIII polymorphism site intron 19 is 608 bp long. After digestion with *Hin*dIII RFLP markers, the gene frequency is found as: for positive allele, it is 427, 100, and 81 bp; for negative allele, it is 427 and 181 bp; and for heterozygosity, it is 427, 181, 100, and 81 bp [Figures 1a and b].

In this study, female patients were used as a base for the variation between the hemophilic and nonhemophilic male patients due to the fact that only these act as a carrier for it. Hemophilia A is the most common cause of X-linked inherited-bleeding disorder resulting from a defect in factor VIII gene located on long arm of X-chromosome. This disorder affects about 1 out of 5000 male births throughout the world. Due to the inadequate medical facility for management of the disease, the DNA-based genetic diagnosis has assumed a great importance. In fact, a direct detection of the mutation is the most accurate and reliable approach for carrier detection and parental diagnosis. The limitation has overcome by the use of a linkage-based RFLP analysis in factor VIII gene. Therefore, in this study, we had used *Hin*dIII-based RFLP technique to differentiate the hemophilic and nonhemophilic male patients, which is based on the female X-linked variation either as a carrier or infected or uninfected mothers.

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