

## Molecular Basis of Hemophilia B in Pakistan: Identification of Two Novel Mutations

*Nazia Nawaz, Rashid Hussain, Khalid Masood and Gulzar Niazi*

Centre of Excellence in Molecular Biology, University of Punjab, Lahore, Pakistan

**Abstract:** Hemophilia B, an X-linked recessive bleeding disorder is caused by deficiency of clotting factor IX protein. In an attempt to understand the molecular basis of hemophilia B which is a rare disease in Pakistan, we analyzed amplified genomic DNA from 20 patients (ages 3-36 years) with different ethnic back-grounds. Eleven mutations were identified in 15 out of 20 patients with mutation detection efficacy of 75%. Out of these 11 mutations, 9 have been described earlier and these included: 7 missense and 2 nonsense mutations. The remaining 2 mutations are novel and are not listed in the hemophilia B mutation databases. These are: a 6bp insertion (CCTGCT) (Ser-Lys) in exon5 at codon110 in the second epidermal growth factor (EGF2) domain, which increases the length of domain by a little; and the other is a deletion of nucleotide #946(AAA-AA) (-Lys) in exon8 at codon316, located in one of highly variable surface loops resulting in frame-shift in catalytic domain of the protein. Five SNPs in exons 6, 7 and 8, which are only one of its kinds, were present in Pakistani hemophilia B patients.

**Key words:** Hemophilia B • Molecular studies • Coagulation disorders • Pakistan

### INTRODUCTION

Hemophilia B (MIM # 306900) [1] is a hereditary X-linked bleeding disorder of coagulation caused by mutation in factor IX (F IX) gene. Mutations in F IX gene cause deficiency or dysfunction of clotting factor IX, a vitamin K dependant single chain glycoprotein (mol. wt 57000) whose presence is essential for the efficient progression of blood coagulation [2]. Hemophilia B affects 1 in every 100,000 male newborns [3]. The severity of the disease is directly related to the degree of FIX deficiency. Based on the level of F IX activity, hemophilia B has been classified into three groups: severe (FIX activity < than 1%); moderate (2-5%) and mild (6-30%) of the normal. This variability in clinical manifestations is due to heterogeneity of molecular defects present with each mutation resulting in a specific pattern of alteration of FIX activity. Structural and functional defects in FIX are either due to gene alterations, including large and small deletions, insertions or splice junction alterations, single base substitutions or nonsense mutations. More than 800 mutations in FIX gene have been reported from all over the world [3] but no molecular studies have been carried out on Pakistani

hemophilia B patients, where the true incidence of the disease is unknown but it is a rare blood coagulation disorder.

The main objective of this study was to identify the mutations that produce mild to severe form of hemophilia B disease in Pakistani patients and to establish the relationship between cause and effect.

### MATERIALS AND METHODS

Twenty blood samples of Hemophilia B patients from different areas of Pakistan were collected in a 5 ml EDTA tubes. All the available data including laboratory investigations (e.g. PT, APTT, CBC) and relevant clinical information were collected on specially designed database form. Hemophilia B was diagnosed either by factor IX assay or by correcting with aged plasma. Genomic DNA was extracted by Qiagen Maxi blood extraction kits. Quality and quantity of DNA was estimated by 0.8% agarose gel electrophoreses and spectrophotometry. As a part of the study all the 8 exons of the Factor IX were selected and oligonucleotide primers were synthesized in primer designing laboratory of CEMB. PCR was carried out with 25 ng DNA samples, 10 pm primer dilution, 10X

**Corresponding Author:** Dr. Gulzar A. Niazi, Medical Genetics Laboratory,  
Center of Excellence in Molecular Biology (CEMB),  
87 West Canal Bank, Thokar Niaz Beg, Lahore, Pakistan

20 mM buffer, 3mM dNTPs, 1U Taq Poly and ddH<sub>2</sub>O [4] Desired bands of product DNA were estimated by 1.5% agarose gel electrophoresis and these were precipitated with 80% ethanol followed by centrifugation at 13,000 rpm. Sequencing PCR was conducted for each oligonucleotide primer on ABI 3100 sequencer using manufacturer's sequencing kit (ABI BigDye Terminator v3.1 Cycle sequencing Kit). The data obtained from the sequencing was visualized and examined on Chromas 3 and BLAST tool on NCBI web site [5]. 3D structure of the mutations was obtained by using bioinformatics tools Swissprot which is accessible via the ExpASy web server [6] or from the program DeepView (Swiss PDB-Viewer). By using this server protein models were made. Heavy chain structure was based on these templates. For the light chain structure 1pfx C from PDB was used. Model was retrieved from Swiss-Model server as submitting in the form of project.

## RESULTS AND DISCUSSION

Hemophilia B is a pan-ethnic disorder without predilection but it is less commonly encountered disorder in Pakistan. Many different FIX mutations have been identified in patients with hemophilia B; but in contrast to frequent partial inversion of FVIII in hemophilia A, a common F IX mutation has not been identified for hemophilia B. Point mutations (single nucleotide substitutions) are the most common gene defect and are present in approximately 90% of Hemophilia B patients, followed by deletions in nearly 5–10% of patients. Insertions and rearrangements / inversions are less common within the hemophilia B population [7].

In present study, eleven mutations (1 insertion, 1 deletion and 9 point mutations) were identified in 15 patients (Table 1). Out of these, an insertion and one deletion is novel which was detected in two unrelated patients and these are probably loss-of-function mutations. It is well known that most recessive mutations which are due to loss function of gene products can occur from a variety of different causes including failure of gene to be transcribed or translated and failure of translated gene products to function correctly.

One patient from Islamabad had novel insertion of six nucleotides (CCTGCT) in exon5 at codon110 which code for two additional amino acids, serine and cystine in the second epidermal growth factor EGF2 domain. This domain is important for the assembly of factor X (FX) activating complex on phospholipid vesicles [8]. Upon comparative modeling it was observed that the domain

length was increased by a little, due to which inter-molecular interactions are affected, impairing the attachment of Ca<sup>++</sup>. Insertions within FIX are although rare but have been reported in many ethnic groups [9].

An Urdu speaking patient from Karachi had a deletion (AAA-AA) (-Lys) in exon8 at codon316. F IX residue Lys316 is located in one of highly variable surface loops, which contributes to characteristic substrate of each individual enzyme. [10]. The Lys316 specifically determines the reactivity of FIXa towards its natural substrate FX. The catalytic portion of factor IX is a typical triad serine protease which is responsible for cleaving factor X to factor Xa, thus predicting a severe phenotypic expression of hemophilia B. Comparative modeling suggests that the deletion of single nucleotide# 946 (AAA-AA) results in frame shift in catalytic domain of protein. However the possibility of premature termination of protein chain can not be ruled out. A similar deletion of Lys residue at codon 316 was reported by Wulff in 2001(unpublished), in which nucleotide #947(A-A) was deleted but in our patient the deletion is of nucleotide #946(-AA).Based on the fact that the deletion of adenosine in two different patients is at two different sites, we consider that deletion of AAA-AA in our patient makes it a novel mutation although in both cases it is the lysine residue that has been deleted.

The majority of point mutations that have been reported in the hemophilia B patients are missense and nonsense. In our study we have identified 9 point mutations (7 missense and 2 nonsense mutations). All of these have been reported previously in other ethnic groups. Seven missense mutations identified in Pakistani hemophilia B patients are (a) in exon1 (GTT → ATT) Val → Ileu at codon-17 in pre-pro ladder sequence of protein and (b) CGG→TGG nucleotide change (Arg→ Trp) is present in exon2 at codon-4 (pre-pro ladder sequence of protein) in three patients from Karachi including carrier;the pro peptide of human coagulation factor IX (FIX) directs the c-carboxylation of the first 12 glutamic acid residues of the mature protein into c-carboxyglutamic acid (Glu) residues. This pro peptide is normally removed before secretion of FIX into the blood. However, mutation of Arg at codon-4 in the pro-peptide abolishes pro-peptide cleavage and results in circulating pro factor IX in the blood [11] (c) two missense mutations at codon95 (TGC → TAC) Cys → Tyr and other in codon99 (TGT → TAT) Cys → Tyr in exon5 were detected. Both of these point mutations are located in growth factor domain of protein. Comparative modeling results showed that the point mutations of Cys of residue numbers 95 and 99 make structure unstable because

disulfide bond is present between 95 &109 and 99 & 88. Substitution of Cys→Tyr results in elimination of disulfide bond. The residues 88-109 are important for platelet binding affinity, stoichiometry and assembly of the FX activating complex [12] (d) in exon6, a mutation (CGG→TGG) Arg→Trp at codon180 was identified which is the last amino acid of activation peptide in protein sequence. (e) a mutation in exon7 (GGA → GAA) Gly → Glu at codon207 is present in the catalytic domain of protein which is a typically serine protease. This mutation at codon 207 makes structure unstable due to absence of hydrogen bonding in 3D structure (f) the last mutation (GGG→AGG) (Gy→Arg) is in exon 8 at codon 317, which is located in catalytic domain of protein. Glycine is non polar neutral amino acid and it has been replaced by Arg which is a strongly basic. Homology modeling showed that this substitution produces an unstable structure because of absence of hydrogen bonding. This change in catalytic domain affects the structure and also catalytic activity.

Two nonsense mutations were identified. One mutation was in exon2 and second in exon8. In both cases, the mutation is (CGA → TGA) Arg → stop codon.

The first nonsense point mutation at codon 29 is in GLA domain of protein. The outcome of this mutation is expected to be a truncated protein molecule. Truncation is likely to make it defunct. It causes severe hemophilia B [13]. Second nonsense point mutation which is also a Arg→stop codon mutation but it is at codon 333 which is located in catalytic domain of protein. This mutation was detected in two brothers from Peshawar. The alpha-helix region333-339 plays a prominent role in Factor VIIIa-dependent stimulation of Factor X activation, but they do not contribute to the high-affinity interaction with Factor VIIIa light chain. It is suggested that complex assembly between Factor IXa and Factor VIIIa involves multiple interactive sites that are located on different domains of these proteins [14]. Nonsense mutation at codon 333 show a truncated protein.

The human FIX gene contains several SNPs. Ethnic variations in FIX gene polymorphisms are well documented. In our study we have identified five SNPS in exons 6, 7 and 8, which are only one of its kinds (Table 2). In exon6 SNP ambiguity R: A/ G is observed in all the 20 samples out of these G is present in 5 and in other 15 A is present. In exon7 SNP ambiguity is Y: T/ C but in all the

Table 1: List of Mutations identified in Pakistani Hemophilia B patients

Patient	Age(yr)	Exon	Codon	Nucleotide change	A.A change	Comments
M.Q (karachi)	15	E1	-17	G → A	Val → Ileu	Giannel <i>et al.</i> J. Clin. Invest, 1994, 8:1619
J.Z (karachi)	30yr					
K (karachi)	35yr (F)					
H (karachi)	14yr	E2	-4	C → T	Arg → Trp	Ginnel <i>et al.</i> Nuc. Acid Res., 1994,22,3534
Z b/o H (Karachi)	3yr					
S-Ur-R (Karachi)	20yr	E2	29	C → T	Arg → Stop codon	Giannel <i>et al.</i> Nuc. Acid Res., 1994,22:3534
Unknown (Islamabad)	17 y	E5	95	G → A	Cys → Tyr	Giannel <i>et al.</i> Nuc. Acid Res., 1994:22:3534
Zee (Peshawar)	8yr	E5	99	G → A	Cys → Tyr	Attai <i>et al.</i> J. Thromb Haemost., 1999;82:1437
T (Islamabad)	12yr	E5	110	insertion (CCTGCT)	Ser-Cys	Not Reported
A.A.Y (Karachi)	25yr	E6	180	C → T	Arg → Trp	Ludwig <i>et al.</i> Am. J. HumGenet,1992;50:164
N.R (Karachi)	11yr	E7	207	G → A	Gly → Glu	Giannel <i>et al.</i> Nucl. Acid Res., 1994;22,3534
A.A.A (Karachi)	36yr	E8a	316	-A	-Lys (frame shift)	Not Reported
A K (Multan)	10yr	E8a	317	G → A		
				Gly → Arg		Giannel <i>et al.</i> Nuc. Acid Res., 1994; 82:1437
H.S (Peshawar)	15					
A b/o of HS (Peshawar)	12	E8a	333	C → T	Arg → stop codon	Giannel <i>et al.</i> Nuc. Acid Res., 1994:22,3534

Table 2: SNPs identified in Pakistani Hemophilia B Patients

Patient	Exon	SNP ambiguity	SNP	Codon	Codon #	Comments
A.AA	E6	R:A/G	G	ACT	194	rs6048 (dbSNP127) www.ensembl.org
M.Q	E6	R:A/G	G	ACT	194	rs6048 (dbSNP127) /www.ensembl.org
R.S	E6	R:A/G	G	ACT	194	rs6048 (dbSNP127) /www.ensembl.org
H.S	E6	R:A/G	G	ACT	194	rs6048 (dbSNP127) /www.ensembl.org
Z	E6	R:A/G	G	ACT	194	rs6048 (dbSNP127) /www.ensembl.org
In all other patients	E6	R:A/G	A	ACT	194	rs6048 (dbSNP127) /www.ensembl.org
In all 20 patients	E7	Y: T/C	T	GTT	273	rs1800455 (dbSNP127) /www.ensembl.org
In all 20 patients	E8	Y: C/T	C	CAC	303	rs1801202 (dbSNP127) /www.ensembl.org
In all 20 patients	E8	R: G/A	G	CAG	371	rs34698779 (dbSNP127) /www.ensembl.org

20 samples only T is present. In exon8 SNP ambiguity is Y: T/C but in all the 20 samples only C is present. Also in same exon8 at codon371 ambiguity is R: G /A, but in all the 20 samples only G was present. Our results have shown that in Pakistani population only one kind of SNP was present in exon7 and exon8 and both A and G were present in exon6.

The results of this study have shown that all the mutations identified in hemophilia B patients are heterogeneously distributed in Pakistani population and inter and intra ethnic variations in mutations exist (Table 1). In present study we have attempted to illustrate some of the possible effects on phenotypic expression by 2 novel and 9 reported mutations of FIX. We can expect severe phenotypic expression when FIX mutation is either a stop codon or frame shift, whereas a missense mutation, which causes a minor disruption of FIX protein can lead to less severe phenotype. In conclusion, the novel and other mutations identified in this study have contributed significantly to our understanding about the molecular pathology of hemophilia B in Pakistan and this can form the basis of further studies. Once these studies are carried out in depth, it will be possible to offer pre-and postnatal diagnosis and genetic counseling to affected families with hemophilia B disorder.

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