# Hemophilia B in a female caused by skewed inactivation of the normal X-chromosome

Carmen Espinós,* José I. Lorenzo,* Pilar Casaña,* Francisco Martínez,° José A. Aznar*

*Unidad de Coagulopatías Congénitas and °Unidad de Genética y Diagnóstico Prenatal. Hospital Universitario La Fe. Valencia, Spain*

## ABSTRACT

Hemophilia B (factor IX deficiency) is an X-linked recessive disorder with a prevalence of 1:30,000 male births, which rarely affects females. A missense mutation T38R (6488C>G) of the factor IX (FIX) gene was characterized in a young female with moderate-to-severe hemophilia B. She is heterozygous for this mutation, which she inherited from her carrier mother. Analysis of the methyl-sensitive *Hpa*II sites in the first exon of the human androgen-receptor locus indicated a *de novo* skewed X-chromosomal inactivation. This indicates that the paternal X chromosome carrying her normal FIX gene is the inactive one, which has led to the phenotypic expression of hemophilia B in this patient.
©2000, Ferrata Storti Foundation

Key words: hemophilia B, factor IX gene mutation, skewed X-inactivation, SSCP.

Hemophilia B (HB) is an X chromosome recessive disease caused by factor IX (FIX) deficiency. The estimated prevalence is 1:30,000 males. Females are rarely affected by this disease, although a few cases of females with HB have been reported.[1-4] The clinical manifestation of hemophilia in females could be due to homozygosity in the mutated gene, structural abnormalities of the X chromosomes, or skewed inactivation of the normal X chromosome. In this study, we report the results of genetic analyses in a young female with HB who is the only affected member of her family. The phenotypic expression of HB in this patient has been shown to be due to a mutation on the maternal FIX gene and an extreme inactivation of the paternal X chromosome.

## Design and Methods

### Case report

The patient was diagnosed in 1982, at the age of 3 months, as having moderately severe hemo-

philia B (factor IX between 1 and 5 IU/dL). She had frequent bleeding episodes, mainly joint bleeds and hematomas, which often required replacement therapy with prothrombin complex concentrates, and, more recently, with high purity FIX concentrates. She developed advanced hemophilic arthritis in her right knee. In 1988, she was diagnosed with pulmonary fibrosis (chronic interstitial pneumonitis). She has growth retardation, probably secondary to respiratory disease. In July 1999 a lung transplant was carried out successfully. Tests for FIX inhibitors have always been negative. She is anti-HBs, anti-HBe, anti-HBc and anti-HCV positive, but is not HIV infected. There is no history of hemophilia on the maternal side of the family. Her father, who died from alcoholic liver disease (cirrhosis), did not have hemophilia.

### Cytogenetic studies

Complete karyotype analysis was carried out using peripheral blood white cells, cultured in the presence of phytohemagglutinin for 72 h to induce mitosis. Standard GTG banding technique was used. At least 20 metaphases from the propositus were examined.

### DNA samples and RFLP analysis

Genomic DNA from the patient and her relatives was obtained by standard methods from peripheral white blood cells.[5] Four intragenic RFLP *Dde*I (a 50 bp insertion polymorphism in intron 1), *Xmn*I (in intron 3), *Taq*I (in intron 4) and Malmö polymorphism (in exon 6), and one extragenic RFLP (*Cfo*I/*Hha*I) located 8Kb 3' to the FIX gene were analyzed as described previously.[6-8]

### Mutation screening

To study exon *b* of the FIX gene, DNA was amplified by polymerase chain reaction (PCR) using the primers F9BC1 and F9BR that correspond to the nucleotides 6240 to 6274 and 6533 to 6514, respectively, according to the FIX gene sequence of Yoshitake *et al*.[9] PCR conditions were: a first step at 94°C for 5 min, 30

PCR cycles (45 sec at 94°C, 30 sec at 55°C, and 50 sec at 72°C), and a final single step of extension at 72°C for 7 min. For single-strand conformation polymorphism(SSCP) analysis, 2.0 µL of amplified DNA were mixed with 10.0 µL of loading buffer containing 95% formamide, 10 mM NaOH, 0.05% bromophenol blue, and 0.05% xylene cyanol. The samples were heated at 94°C for 7 min and then cooled in ice. Six microliters of the denatured DNA were applied to 10% polyacrylamide (37.5:1) gel, which was run for 19 h at 700 V and 4°C. The bands were detected by silver staining, and the samples that showed an anomalous pattern were sequenced.

### Sequence analysis

The amplified DNA was purified using microfiltration (Centricon-100), and quantified by electrophoresis vs. ϕX174. Then 30 ng of DNA were sequenced by the fluorescent dideoxy terminator method and analyzed in an ABIPRISM DNA sequencer. The sequence analysis was performed with the GCG program from the Wisconsin Sequence Analysis Package.

### Evaluation of the methylation status

X-inactivation was analyzed on peripheral blood lymphocytes. The X chromosome inactivation pattern (XIP) was determined by PCR analysis of a polymorphic trinucleotide repeat in the first exon of the androgen receptor (AR) gene.[10] For each DNA, two reactions were performed. DNA samples (1.8 ng) were separately digested with two restriction enzymes: *Rsa*I as a control, and *Hpa*II a methylation sensitive endonuclease, which only gives a PCR product from the inactive X chromosome. All the reactions performed were incubated in a total volume of 20 µL at 37°C overnight. The digested samples (2.0 µL) were amplified using the primers reported by Allen *et al.*[10] PCR conditions

were 10 cycles (each comprising 40 sec at 95°C, and 1 min 30 sec at 65°C) and 20 cycles (each comprising 40 sec at 95°C, 1 min 30 sec at 65°C, and 30 sec at 72°C), with an initial denaturation at 94°C for 4 min, and a final extension at 72°C for 7 min. The PCR product was electrophoresed in 10% non-denaturing polyacrylamide (29:1) gels, which were run at 500V for 15h, and stained with silver nitrate. Band intensities were measured by densitometry using the program 1D Manager V2.0 (T.D.I.) after digitalizing the image in a SNAPSCAN 1236 (Agfa). The ratio of intensities of the two alleles after *Hpa*II digestion was compared with that obtained in the control amplification (*Rsa*I) as described elsewhere.[10]

## Results

### Mutation screening

A single base substitution, 6488C→G, corresponding to a missense mutation T38R, at exon *b* of the FIX gene was found in the patient (III:11), her mother (II:7), and her younger sister (III:12) in heterozygous state. To date, no other mutation has been detected in these females. A maternal aunt of the propositus (II:4) does not carry the detected mutation. The propositus has another maternal aunt (II:2), whose DNA was unavailable for testing, although it is unlikely that she is a carrier since she has three healthy sons (Figure 1).

### Evaluation of the methylation status

The results of X inactivation analysis are shown in Figure 2. The mutated X-chromosome is active in 97% and 15% of the cells in the patient and her healthy sister, respectively. The DNA amplified after digestion with *Rsa*I gave two equally strong bands for the three females studied. However, the lane of the PCR product
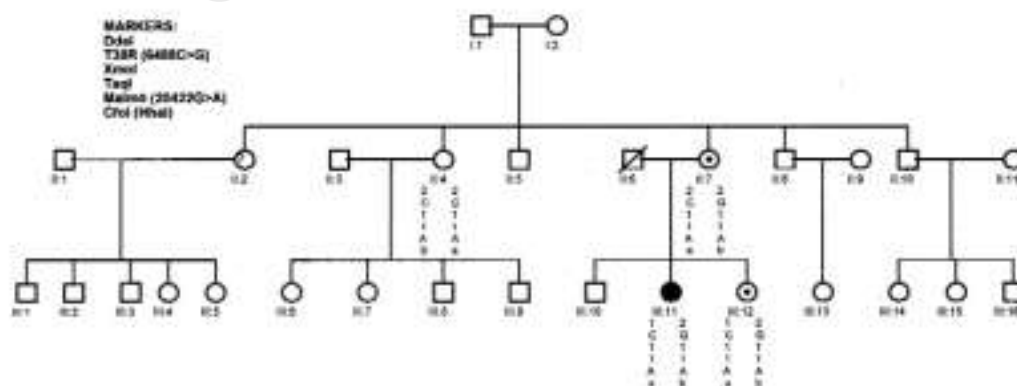


**Figure 1. The patient's family tree. The mutation 38R was identified in II:7, III:11, and III:12, but not in II:4. Since DNA from female II:2 was not available, it was not possible to determine whether she carries the 38R change, although this seems unlikely.**

obtained after digestion with *Hpa*II showed evident differences in the degree of inactivation between both chromosomes, except for the mother, whose DNA presented a random XIP for this digestion. The propositus' sample only showed the band inherited from the healthy father, while in her sister the band inherited from her mother was more intense (methylated). The patient therefore has an extremely skewed XIP towards the paternal X chromosome, while her healthy sister shows preferential inactivation of the maternal chromosome.

### Cytogenetic analysis

Cytogenetic analysis of the propositus showed a normal karyotype (46, XX, 21ps+), with no deletion, inversion, or gross structural abnormality of the X chromosomes. Her karyotype shows a polymorphism that does not have any pathologic implication as it is commonly found in the general population.

## Discussion

Both the propositus and her sister have a defect (T38R) on FIX gene within their maternal X chromosome that is associated with severe cases of HB (*http://www.umds.ac.uk/molgen/haem-Bdatabase*). The patient suffers clinically moderate-to-severe disease, whereas her sister is healthy. To date, no other mutation has been detected. Therefore, the manifestation of the disease in the propositus could be due to extreme bias in favor of inactivity of the normal paternal X chromosome observed in leukocytes. Although the liver was not tested, the low level of factor IX activity suggests a similar inactivation pattern in this organ. Moreover, her sister presents the opposite: a significant inactivation of the mutated chromosome inherited from her mother. This fact agrees with her clinical phenotype, since she has no symptoms of HB (FIX: C= 75 UI/dL). The propositus is the only affected member in her family. Both the family tree and the haplotype analysis strongly suggest that the mutation arose *de novo* in the patient's mother (II:7). Several reports of females with HB have been previously published.[1-4] Preferential inactivation in all of these cases have been explained by different mechanisms: cytogenetic aberrations affecting the X chromosomes, negative selection by mutations affecting the cellular growth, familial skewed X inactivation, or just by chance. A few cases of HB females have been explained because of the presence of cytogenetically detectable aberrations,[11] which can be excluded in our patient, since her karyotype is normal.

The assessment of the XIP in relatives of HB female patients has been studied in few cases. In
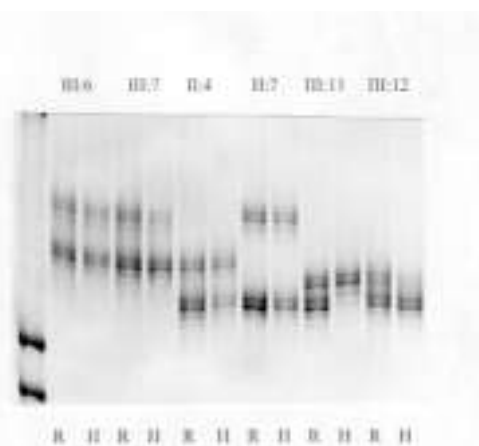


**Figure 2. X chromosome inactivation analysis at the androgen receptor locus. R= *Rsa*I digested DNA; H= *Hha*II digested DNA. The female members of this family show a random XIP, except III:11 and III:12 who showed evident differences in the degree of the activation for both chromosomes for *Hha*II digested DNA. III:11 presents complete inactivation of the paternal band, and III:12 shows a preferential inactivation of the maternal chromosome.**

all these instances the inactivation bias is a feature that is segregated in the family. They may correspond to familial skewed X as described by Naumova *et al.*[12] without being associated with any disease. In the family studied, the patient's mother and aunt showed a balanced inactivation pattern. Since both sisters (III: I1 and III: I2) presented dissimilar XIP, this trait cannot have been inherited from their father. We can, therefore, conclude that the inactivation bias in this patient is not a familial transmission feature. A skewed inactivation may also be due to a chance occurrence. But the extreme lyonization observed in our propositus is rarely found in the general population, so it is unlikely that in this case X inactivation is due to chance. The event of a second mutation in the patient's paternal chromosome favoring the use of the maternal X chromosome is likewise improbable. There is no report of other genetic diseases or multiple abortions in the family. Therefore, we conclude that XIP in the present patient is probably due to a *de novo* event affecting the X inactivation process itself as previously reported,[13,14] or other gene/s that play an essential role in cell viability.[15] As far as we know, this is the first case of a HB female patient with a *de novo* skewed inactivation that has been described. HB females are rarely reported, and when they are, non-random XIP is given as an explanation for most of these cases. However, it is not usual to study the remaining

females from these families to assess the possibility of a familial XIP. This kind of study should be performed in order to predict new affected females.

### Disclosures

*Conflict of interest: none.*
*Redundant publications: no substantial overlapping with previous papers.*

## References

1. Kling S, Coffey AJ, Ljung R, et al. Moderate haemophilia B in a female carrier caused by preferential inactivation of the paternal X chromosome. Eur J Haematol 1991; 47:257-61.
2. Schröder W, Wulff K, Wolling K, Herrmann FH. Haemophilia B in female twins caused by a point mutation in one factor IX gene and non-random inactivation patterns of the X-chromosomes. Thromb Haemost 1997; 78:1347-51.
3. Chan V, Chan VW, Yip B, Chin CS, Chan TK. Hemophilia B in a female carrier due to skewed inactivation of the normal X-chromosome. Am J Hematol 1998; 58:72-6.
4. Orstavik KH, Orstavik RE, Schwartz M. Skewed X chromosome inactivation in a female with haemophilia B and in her non-carrier daughter: a genetic influence on X chromosome inactivation? J Med Genet 1999; 36:865-6.
5. Sambrook J, Fritsch E.F, Maniatis T. Molecular cloning, Cold Spring Harbor Laboratory Press. New York. pp. 9.61-9.22.
6. Graham JB, Kunkel GR, Tennyson GS, Lord ST, Fowlkes DM. The Malmö polymorphism of factor IX: establishing the genotypes by rapid analysis of DNA. Blood 1989; 73:2104-7.
7. Winship PR, Rees DJG, Alkan M. Detection of polymorphisms at cytosine phosphoguanadine dinucleotides and diagnosis of haemophilia B carriers. Lancet 1989; 25:631-4.
8. Reiss J, Neufeldt U, Wieland K, Zoll B. Diagnosis of haemophilia B using the polymerase reaction. Blut 1999; 60: 31-6.
9. Yoshitake S, Shach BG, Foster DC, Davie EW, Kurachi K. Nucleotide sequence of the gene for human factor IX (antihemophiliac factor B). Biochemistry 1985; 24: 3736-50.
10. Allen RC, Zoghbi HY, Moseley AB, Rosenblatt HM, Belmont JW. Methylation of HpaII and HhaI sites near the polymorphic CAG repeat in the human androgen-receptor gene correlates with X chromosome inactivation. Am J Hum Gen 1992; 51:1229-39.
11. Schröder W, Poetsch M, Gazda H, et al. A de novo translocation 46,X,t (X;15) causing haemophilia B in a girl: a case report. Br J Haematol 1998; 100:750-7.
12. Naumova AK, Plenge RM, Bird LM, et al. Heritability of X chromosome inactivation phenotype in a large family. Am J Hum Genet 1996; 58:1111-9.
13. Plenge RM, Hendrich BD, Schwartz C, et al. A promoter mutation in the XIST gene in two unrelated families with skewed X-chromosome inactivation. Nat Gen 1997; 17:353-6.
14. Pereira LV, Zatz M. Screening of the C43G mutation in the promoter region of the XIST gene in females with highly skewed X-chromosome inactivation. Am J Med Genet 1999; 87:86-7.
15. Belmont JW. Genetic control of X inactivation and processes leading to X-inactivation skewing. Am J Hum Genet 1996; 58:1101-8.