Molecular analyses in hemophilia B families: identification of six new mutations in factor IX gene

Twenty-two Spanish families with hemophilia B have been studied in order to characterize the mutation responsible for the disorder and to carry out family studies. All the essential regions of the factor IX gene were sequenced and the pathologic mutation was identified in every case. Twenty different molecular changes were identified, six of them undescribed elsewhere.

haematologica 2003; 88:235-236
(*http://www.haematologica.org/2003_02/88235.htm*)

Hemophilia B (HB) is an X-linked bleeding disorder caused by mutations in the factor IX gene.[1] To date, a wide range of mutations, showing large molecular heterogeneity, has been described in HB patients.[2] The aim of the present study was to characterize the mutation responsible for this disorder in order to carry out family studies in 22 Spanish families with severe (16), moderate (3) or mild (3) hemophilia B, who had given their consent to be included in this study. A family history was not found in 11 of the families studied: nine were sporadic cases with one affected male, one had a hemophiliac female, and the last had two possible carriers on account of low FIX levels.

Parallel analyses of mutation searching (SSCP and heteroduplex) and direct sequencing were performed in these families, most of whom had already undergone segregation studies. Using both methods, all functional regions of the factor IX gene (exons, intron/exon splice junctions, and the 5' and 3' untranslated regions) were analyzed in at least one propositus from every family. Primers used in both techniques were the same, taking into account that exon 2 was sequenced together with exon 3, and exon 8 was considered as a single fragment (Table 1). Sequencing of a second independent polymerase chain reaction (PCR) product from the propositus, and/or other hemophiliacs or carrier females belonging to the same family was always performed in order to validate mutations. Lastly, when a mutation created or removed the digestion site of a restriction endonuclease (Table 2), the PCR product was digested with the required enzyme to corroborate the nucleotide change and also to perform familial restriction analyses.

Table 2 shows the results obtained, indicating with which method (SSCP/HD or direct sequencing) the molecular defect was detected. The accuracy and efficiency presented by SSCP/HD analyses in our screening were only around 60%. The pathologic mutation was identified in the 22 cases analyzed and no double mutants were found in any case. In total, 20 different molecular defects were detected. Two previously reported nucleotide changes (31008C→T and 31118C→T) were both found in two unrelated families, although a common origin might be dismissed in these cases. On the other hand, two of the families studied are striking: in family 15, carrier status was confirmed in two women prior to the appearance of a hemophiliac male in their family,[3] and in family 18, a female with HB is the only hemophiliac whilst her mother and her sister are carriers.[4]

Six of these mutations have not previously been described. Three of them are frameshift mutations leading to the creation of a premature stop codon: 31035-36insT (327X) detected in a patient with family history who showed a functional and antigenic deficiency (family 1); 31057-58delAG (327X), characterized in a sporadic case who was clinically diagnosed at birth and for whom basal functional data are not available (family 8); and 31222-31223insA (372X) which was identified in a patient who is the first case in his family (family 21). The other three new mutations are missense substitutions. The transversion 31013A→T (I298L) was characterized in two hemophilic relatives (family 14), who suffer from a functional deficiency, with low levels of FIX: C and normal values of FIX: Ag (Table 2). Mutation F349L is reported in the HB database, but due to a 31166T→C transition, while the case included here presents 31168C→G transversion (family 13). Since it was a sporadic case and the hemophilic patient has

Table 1. Primers numbered according to the FIX gene sequence reported by Yoshitake *et al*.[1]

Exon	Forward primer	Reverse primer	PCR size (bp)	
1	-42 - -64	198-175	240	
2	6239-6263	6533-6514	295	507
3	6514-6533	6746-6722	233	
4	10300-10321	10550-10528	251	
5	17588-17609	17897-17878	310	
6	20311-20335	20612-20593	302	
7	29956-29975	30273-30254	318	
8 (I)	30730-30749	31107-31088	378	
8 (II)	31015-31036	31222-31205	208	670
8 (III)	31079-31098	31399-31380	321	

Table 2. Mutations characterized in our population.

F	DS	FIX: C (%)	FIX: Ag (%)	Mutation	Exon (E) Intron (I)	RA	Method
20	M	2.7	(d)	6364C→T, R-4RW	E2	*Hinf*I	(a)
16	S	(d)	(d)	6460C→T, R29X	E2	*Nla*III	SSCP
18	(c)	4	33	6488C→T, T38R	E2	*Ear*I	SSCP
2	S	0.65	(d)	10487G→A, G79R	E4	––	SEQ
12	S	0.2	<1.6	10488G→T, G79V	E4	––	SEQ
17	S	<1	(d)	17786G→T, C124F	E5	––	SSCP
11	S	0.8	0.9	17796A→G, A127A	E5	*Aci*I	SSCP
6	S	1	1.5	20360T→G, acceptor splice	I5	*Taq*I	SEQ
7	Md	8	(d)	30888A→G, H256R	E8	*Hph*I	SSCP
22	M	4.9	14	31008C→T, T296M	E8	*Nla*III	(a)
3	M	2.1	7.3	31008C→T, T296M	E8	*Nla*III	SEQ
14	Md	7.2	81	31013A→T, I298L (b)	E8	––	SEQ
1	S	<1	<1	31035-31056insT, 327X (b)	E8	––	HD
8	S	(d)	(d)	31057-31058delAG, 327X (b)	E8	*Hinf*I	SSCP
15	(c)	16	13	31118C→T, R333X	E8	*Dde*I	SSCP
4	S	<1	<1	31118C→T, R333X	E8	*Dde*I	SSCP
13	S	(d)	(d)	31168C→G, F349L (b)	E8	––	SSCP
21	S	<1	(d)	31222-31223insA, 372X (b)	E8	*Nla*IV/*Bsm*FI	(a)
9	S	1.3	72	31223C→A, P368T	E8	*Ava*II	SEQ
5	S	(d)	(d)	31287G→T, C389Y	E8	––	SEQ
19	S	(d)	(d)	31308G→A, G396E (b)	E8	––	SEQ
10	Md	40	94	31328C→T, R403W	E8	*Msp*I	SSCP

(a) Families studied by automated sequencing only. (b) New mutations. (c) In these families, an affected male has not yet been described: Functional data of family 15 belong to one carrier, and of family 18 to a hemophilic woman. (d) Data not available.
F: identification of family; DS: degree of severity; S: severe; M: moderate; Md: mild; RA: restriction analyses; SEQ: automated sequencing.

died, the mutation was detected in an obligate carrier who showed levels of FIX: C= 32 IU/dL and FIX: Ag= 30 IU/dL, hence there are not discrepancies between both levels. This case was classified as severe HB, agreeing with the clinical history. Finally, the G396E (31308G→A) mutation was identified in obligate carriers, since the hemophiliacs have died (family 19). Other severe HB patients with the same mutated codon, listed in the worldwide mutation database, show extremely low levels of coagulant activity, and normal levels of antigen. All of the six newly described mutations cause severe HB, except the I298L change which produces a mild phenotype.

Finally, segregation studies, together with sequence analyses, indicate that approximately 2/3 of *de novo* mutations occur in germinal cells from the maternal grandfather. Moreover, the pathologic mutation was detected in all patients' mothers except in one case. In this kind of sporadic cases, a possible maternal somatic/germinal mosaicism may be considered, mainly in order to carry out accurate genetic counselling.[5,6]

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Key words: hemophilia B, factor IX gene, mutations.

Funding: this work was partially supported by a grant FIS 99/0633.

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Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Mario Cazzola, Editor-in-Chief. The final decision to accept this paper for publication was taken jointly by Professor Cazzola and the Editors. Manuscript received July 24, 2002; accepted January 2, 2003.

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Evaluation of the factor V HR2 haplotype as a risk factor for ischemic cerebrovascular disease

The HR2 haplotype of the factor V (FV) gene has been identified as a cause of resistance to activated protein C, specially in the presence of FV Leiden. We studied the prevalence of the HR2 haplotype in 115 patients with a first episode of ischemic cerebrovascular disease (CVD) and 115 age- and sex-matched healthy controls. Our results show that the HR2 haplotype is not associated with an increased risk of CVD.

haematologica 2003; 88:236-237
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Activated protein C resistance (APCR), is a biological condition related with venous and to a lesser extent arterial thrombosis. A single point mutation in the factor V gene, G1691A, known as FV Leiden, is the most frequent cause of APCR in the Caucasian population. APCR has been associated with an increased risk of CVD, independently of FV Leiden mutation.[1] On the other hand, the contribution of FV Leiden to the risk of CVD seems to be very low, if any.[2] Recently, a specific factor V gene haplotype known as HR2, which includes at least six polymorphisms in exons 13 and 16, has been described and associated with APCR, particularly in compound heterozygous FV Leiden/HR2 carriers.[3,4] Carriers of the HR2 haplotype have an increased ratio of a hyperglycosylated FV isoform in plasma. This variant could be more resistant to degradation by activated protein C than is the FV in HR2-free controls.[5]

We studied 115 consecutive patients, 56 males and 59 females, with a first episode of CVD (ischemic stroke or transient ischemic attack) before the age of 65 years (mean age 47 years, range 19-64) and without a previous history of venous or arterial thrombosis. Patients were recruited at two Spanish university hospitals, located in Pamplona and Salamanca. CVD was diagnosed by clinical evaluation and computed tomography or magnetic resonance imaging of the brain. Controls (n = 115; mean age 47 years, range 24 – 64) were selected from among hospital staff and people admitted to hospital without a history of venous or arterial thrombosis. Controls were 1:1 matched with patients for age (± 5 years), sex and recruitment area. Genomic DNA was obtained by standard procedures. FV Leiden and the HR2 haplotype (determined by the A4070G polymorphism, which identifies with certainty the entire haplotype) were analyzed by polymerase chain reaction (PCR) amplification and digestion of the PCR product with restriction enzymes MnlI and RsaI respectively, as previously described.[6,7]

Statistical analysis was performed by applying the McNemar 1:1 matched patients-controls χ^2 test, using odds ratios (OR) with corresponding 95% confidence interval (CI) as the measure of association. Results are summarized in Table 1. Briefly, the HR2 alelle was identified in fourteen patients (12.17%) and twenty (17.39%) controls, one of whom was homozygous (OR: 0.57 [95% CI: 0.22–1.45] $p = 0.29$). In our population FV Leiden did not significantly increase the risk of CVD (OR: 4.00 [95% CI: 0.79–27.33] $p = 0.11$). Separate evaluation of patients with ischemic stroke and with transient ischemic attack did not reveal differences between the two groups. The association between FV Leiden and HR2 haplotype could not be assessed as no compound heterozygotes were found in the control group. Regarding gene-enviroment interactions, no clear association between the HR2 alelle and acquired risk factors such as hypertension, hypercholesterolemia and smoking habit was found.

The role of the HR2 haplotype as a risk factor for venous