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QUARTERLY

Molecular analysis of hemophilia B in Poland: 12 novel mutations of the factor IX gene°

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We examined the molecular basis of factor IX deficiency in 53 unrelated Polish patients with hemophilia B. Heteroduplex analysis and direct sequencing of polymerase chain reaction (PCR) products were applied to identify the gene defect. Forty-three different point mutations were detected in the factor IX gene of 47 patients. There were 29 missense mutations, 9 nonsense mutations, 4 splice site mutations and 1 point mutation in the promoter region. Twelve mutations were novel. The results of this study emphasize a very high degree of heterogeneity of hemophilia B.

Hemophilia B is a recessive X-linked bleeding disorder in which normal clotting factor IX (FIX) is absent, deficient or has been replaced by a functionally abnormal FIX protein. The FIX gene is located on the long arm of chromosome X at the band Xq27.1. It is about 34 kb long and consists of eight exons *(a–h)*. The exons code for a precursor protein of 454 amino acids, which is synthesized in the liver. The mature FIX is present in plasma as a 415 amino-acid glycoprotein and consists of a γ-carboxyglutamic acid region, two epidermal growth factor-like domains, activation domain and catalytic domain (High & Roberts, 1995).

Cloning and sequencing of the FIX gene (Choo *et al.*, 1982; Kurachi & Davie, 1982; Yoshikate *et al.*, 1985) has made possible the investigation of genetic defects in hemophilia patients. To date, over 600 distinct mutations have been identified in 1918 patients and submitted to an international hemophilia B data-

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Abbreviations: FIX , factor IX; APTT, activated partial thromboplastin time; PCR, polymerase chain reaction.

base (Giannelli *et al.*, 1998). Over 90% of the mutations resulting in hemophilia B are single nucleotide substitutions, small insertions (< 30 bp) or small deletions (< 30 bp). Only 3% of total mutations are gross deletions or rearrangements (Lillicrap, 1998).

Hemophilia B is usually quoted as four to eight times less common than hemophilia A (factor VIII deficiency). In Poland, 295 hemophilia B patients from 223 families have been reported to the national registry of congenital blood coagulation disorders. In 160 patients (54%) the disease is severe (FIX < 1 u/dL), in 74 (25%) moderate (FIX 1–5 u/dL), and in 61 (21%) mild (FIX > 5 u/dL). We have undertaken efforts to characterize the mutations of the FIX gene in 53 unrelated patients as part of a nationwide programme to provide an efficient hemophilia B genetic counselling service.

MATERIALS AND METHODS

Patients. Blood samples were obtained from 53 unrelated patients, aged 17 to 54 years, with severe (n = 41), moderate (n = 9) or mild (n = 3) hemophilia B. Consent forms were signed by all patients. All were of Polish origin.

Coagulation studies. Blood samples (4.5 ml) for assays of FIX coagulant activity and FIX antigen were drawn into tubes containing 0.11 M trisodium citrate (0.5 ml) and plasma was separated after centrifugation at 2000 × *g* for 20 min at +4°C. The platelet-poor plasma was frozen and stored at −70°C until assayed. FIX coagulant activity was determined by measuring the ability of the patient's plasma to correct the activated partial thromboplastin time (APTT) of FIX deficient plasma in a standard one-stage assay. The APTT was measured using the reagent Neothromtin (Behring AG, Germany). FIX antigen levels were determined using a commercial ELISA kit (Asserachrom FIX:Ag, Diagnostica Stago, France).

DNA extraction and polymerase chain reaction (PCR). Blood samples (10 ml) for DNA preparation were drawn into EDTA. DNA was isolated from white blood cells by the salting out method of Miller *et al.* (1988). Primer pairs for the FIX gene and PCR conditions were the same as previously described (Wulff *et al.* 1995; 1997b).

Heteroduplex analysis. The PCR products of the FIX gene were screened for mutations by heteroduplex analysis. The heteroduplex method (White *et al.*, 1992) was modified as follows: PCR products of a test sample were mixed with an aliqout of PCR product from a male control (wild type) and denatured for 5 min at 95°C. After reannealing for 2 h at 37°C the reaction was stopped (Wulff *et al.*, 1997a). Small mutations (< 30 bp) in exons *b, c, e, f* and *g* were identified by the presence of shifted extra bands. Mutations in exons *a* and *h* did not always produce the extra bands. If these were not visible, the PCR products of exons *a* and *h* were subjected to direct sequencing.

Sequencing. Double-stranded PCR products were purified and concentrated in a Microcon 100 concentrator (Amicon GmbH, ?). Sequence analysis was performed by cycle sequencing using the Taq Dye Deoxyterminator FS sequencing kit (PE/Applied Biosystems, U.S.A.) and an automatic sequencer type 373 A (PE/Applied Biosystems) as previously described (Wulff *et al.*, 1995).

Haplotype analysis. Data were obtained by examining the following restriction endonuclease polymorphisms in the FIX gene: *Dde*I in intron A, *Xmn*I in intron C, *Tag*I in intron D and *Hha*I/8 kb to exon *h* (Peake, 1995; Wulff *et al.*, 1995).

RESULTS

Forty-three different mutations of the FIX gene were characterized in 47 of the 53 (89%) examined patients with hemophilia B. No mutation could be found in 6 patients despite sequence analysis of the regions of likely func-

Table 1. Coagulation data and FIX gene mutations in 47 unrelated patients with hemophilia B

Patient	FIX:C u/dL	FIX:Ag u/dL	Amino acid change	Nucleotide change	CpG site	Exon
052 (PK)	<1	<1	None	G→A (-26)	no	Promoter
043 (GB)	4	4	Cys (-19)>Arg	T→C (111)		a
055 (MK)	<1	<1	Splice donor	G→A (122)	no	-
031 (KL)	<1	48	Arg (-4) → Gln	G→A (6365)	yes	b
048 (DS)	<1	52	Arg (-4) → Gln	G→A (6365)	yes	b
014 (AD)	2	65	Arg (-1) → Ser	G→T (6375)	no	b
003 (SG)	<1	n.d.	Glu 7 → Val	A→T (6395)	no	b
044 (RD)	<1	100	*Glu 7 → Asp	G→C (6399)	no	b
001 (WP)	<1	<1	Splice donor	G→A (6702)	no	-
053 (SJ)	2	2	Splice donor	*G→A (6707)	no	-
006 (TM)	<1	24	Cys 88 → Arg	T→C (17677)	no	e
033 (SS)	<1	4	Cys 109 → Phe	G→T (17741)	no	e
062 (LK)	<1	<1	Ser 110 → Pro	T→C (17743)	no	e
064 (JC)	12	73	Arg 145 → His	G→A (20414)	yes	f
034 (BK)	<1	77	Arg 180 → Trp	C→T (20518)	yes	f
060 (WW)	<1	90	Arg 180 → Pro	G→C (20519)	no	f
047 (SC)	<1	117	Val 181 → Phe	G→T (20521)	no	f
049 (BO)	3	17	*Pro 193 → Ser	*C→T (20557)	no	f
058 (AK)	<1	20	Gly 207 → Glu	G→A (30073)	no	g
057 (MB)	<1	<1	*Splice donor	*G→A (30154)	no	-
017 (PM)	<1	<1	Arg 248 → stop	C→vT (30863)	yes	h
056 (RR)	<1	<1	Arg 248 → stop	C→T (30863)	yes	h
019 (AL)	<1	<1	Arg 248 → Gln	G→A (30864)	yes	h
054 (SM)	<1	<1	Arg 252 → stop	C→T (30875)	no	h
051 (PP)	<1	<1	*Tyr 266 → stop	*C→A (30919)	no	h
010 (KS)	<1	<1	Asp 269 → Val	A→T (30927)	no	h
040 (PB)	<1	<1	*Tyr 295 → stop	*C→G (31006)	no	h
027 (JG)	6	9	Thr 296 → Met	C→T (31008)	yes	h
061 (ZW)	<1	44	*Trp 310 → Arg	*T→A (31049)	no	h
005 (RM)	<1	118	Gly 311 → Arg	G→A (31052)	no	h
050 (JK)	<1	<1	Gly 311 → stop	G→T (31052)	no	h
004 (RM)	<1	5	Gln 324 → Pro	A→C (31092)	no	h
026 (TP)	<1	<1	Tyr 325 → stop	C→A (31096)	no	h
042 (MW)	<1	<1	Arg 333 → stop	C→T (31118)	yes	h
025 (RW)	<1	51	Cys 336 → Arg	T→C (31127)	no	h
016 (MK)	<1	<1	Arg 338 → stop	C→T (31133)	yes	h
037 (LM)	4	15	*Ser 339 → Pro	*C→T (31136)	no	h
045 (MM)	<1	<1	*Cys 350 → Arg	*T→C (31169)	no	h
041 (ZA)	<1	3	Gly 352 → Asp	G→A (31176)	no	h
009 (AP)	<1	25	*Cys 361 → Gly	*T→G (31202)	no	h
035 (MD)	<1	102	*Pro 368 → His	*C→A (31224)	no	h
059 (LM)	5	22	*Ser 384 → Cys	*A→T (31271)	no	h
036 (AC)	<1	74	*Glu 387 → Lys	*G→A (31280)	no	h
063 (KJ)	<1	63	*Glu 387 → Lys	*G→A (31280)	no	h
039 (MS)	<1	5	Cys 389 → Tyr	G→A (31287)	no	h
022 (JT)	<1	<1	Trp 407 → stop	G→A (31342)	no	h
028 (DM)	<1	<1	Trp 407 → stop	G→A (31342)	no	h

FIX:C, factor IX coagulant activity; FIX:Ag, factor IX antigen; n.d., not determined; * Novel mutation

tional significance. In 2 of these subjects hemophilia was severe, in 3 moderate, and in 1 mild.

The coagulation and molecular data from the 47 patients are shown in Table 1. FIX coagulant activity ranged from < 1 u/dL to 12 u/dL, and FIX antigen levels ranged from < 1 u/dL to 118 u/dL. No FIX inhibitors have yet been detected in any of the subjects examined. All mutations were single base substitutions: 1 in the promoter region, 1 in exon *a*, 4 in exon *b*, 3 in exon *e*, 5 in exon *f*, 1 in exon *g*, 24 in exon *h*, and 4 in splice sites of the FIX gene. Of the 43 different mutations, 8 were C → T or G → A transitions at CpG dinucleotides resulting in a termination codon or amino acid substitution. Twelve novel mutations, hitherto not reported in the database (Giannelli *et al.*, 1998), were identified. None of these lesions were located within a CpG dinucleotide.

At nucleotide positions 6365, 30863, 31280 and 31342 more than one patient had the same mutation. Extensive family pedigree analysis indicated that these patients were not related. Haplotype analysis of patients 031 and 048 with the frequent mutation 6365G →A (Arg-4 to Gln substitution) in the CpG dinucleotide (45 cases of this mutation have been submitted to the database) showed different polymorphic fragments in these two subjects (Table 2). Thus, these patients were unrelated and the two identical lesions were of different origin. On the other hand, patients 036 and 063 with the novel mutation 31280G →A (Glu-387 to Lys substitution) had the same haplotype. The lesions in these two patients, not located within a CpG dinucleotide, could have the same origin. In patients with the same mutations at nucleotide positions 30863 and 31342 the haplotype analysis was not performed. The distribution of different types of mutations and their relation to FIX antigen levels are shown in Table 3.

DISCUSSION

Hemophilia B may be caused by a large number of different mutations throughout the FIX gene. In this study the molecular basis of hemophilia B could be established in 47 of the 53 (89%) unrelated patients. The mutations identified represent 43 different types of a single base substitution in the promoter region, exons or splice sites of the FIX gene. Twelve mutations are novel. These include 9 missense mutations (1 in exon *b*, 1 in exon *f* and 7 in exon *h*), 2 nonsense mutations (both in exon *h*) and 1 splice site mutation. Thus, our findings confirm and expand the heterogenity of hemophilia B found in other studies (Thompson *et al.*, 1992; Wulff *et al.*, 1995; Van de Water *et al.*, 1996; Wulff *et al.*, 1997b; Weinmann *et al.*, 1998; Wulff *et al.*, 1999).

Of the mutations that appear more than once in the database, many are at CpG dinucleotides and involve a CG to TG or CA change (Giannelli *et al.*, 1998). There is ample evidence that CpG doublets are "hot spots" for mutation, relating to spontaneous deamination of 5-methylcytosine. It is also clear that many of the mutations at CpG sites, especially those resulting in mild phenotypes (e.g. 87 cases of the 31008 C → T mutation in the database), are due to a founder effect (Giannelli *et*

Table 2. Haplotype analysis in hemophilia B patients with identical mutations in the FIX gene

Mutation	Patient	*Dde*I/intron A	*Xmn*I/intron C	*Taq*I/intron D	*Hha*I/8kb to exon *h*
Arg (-4) → Gln	031	+	-	-	-
Arg (-4) → Gln	048	+	+	+	+
Glu 387 → Lys	036	-	-	-	+
Glu 387 → Lys	063	-	-	-	+

Table 3. Factor IX antigen levels in different mutation types of the factor IX gene

Mutation type	Number of mutants	Number of mutants with FIX:Ag		
		<1 u/dL	2-25 u/dL	44-118 u/dL
Missense	29* (67%)	4	12	12
Nonsense	9 (21%)	9	-	-
Splice site	4 (9%)	3	1**	-
Promoter	1 (2%)	1	-	-
Total	43 (100%)	17	13	12

*In one patient the antigen was not determined; **Reduced to 2 u/dL

al., 1998; Lillicrap, 1998). In our study this frequent mutation 31008C → T was identified only in patient 027.

Defects within the FIX promoter are less frequent than those in the coding region and represent 3% of total mutations (Lillicrap, 1998). The mutation at position -25 found in patient 052 was previously identified also in other studies (the database, Gianelli *et al.*, 1998) and caused severe hemophilia B. Position -25 represents the binding site for hepatocyte nuclear factor 4 (HNF-4), a protein facilitating FIX transcription (High & Roberts, 1995).

Splice site mutations were found in 4 of our patients. Three had severe disease and undetectable FIX antigen (< 1 u/dL), and in 1 the disease was moderate with the antigen markedly reduced (2 u/dL).

Nonsense mutations were detected in 8 different codons in 9 patients. All the patients with this type of mutation had severe disease and undetectable FIX antigen. Their phenotypes can be described as cross-reactive material negative (CRM-negative).

Missense mutations represented 67% of all distinct mutants found in our study and caused different phenotypes. Twenty-two patients with this mutation type suffered from severe hemophilia B and had FIX antigen levels normal (11 cases), reduced (7 cases) or undetectable (4 cases), while in 5 patients the disease was moderate with the antigen levels normal (1 case) or reduced (4 cases) and in the remaining 2 the disease was mild with the antigen levels normal or reduced.

The determination of mutations within the FIX gene in hemophilia B has generated a wealth of information about the structure and function of the molecule. The identification of the molecular defect in a family greatly facilitates carrier detection and prenatal diagnosis of hemophilia B, both of which are necessary for controlling this inherited disease.

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