Molecular genotyping of the Italian cohort of patients with hemophilia B

Donata Belvini
Roberta Salviato
Paolo Radossi
Federica Pierobon
Piergiorgio Mori
Giuseppe Castaldo
Giuseppe Tagariello
and the AICE HB study group

Background and Objectives. The aim of the study, funded by the Italian Ministry of Health, was to identify the causative mutation in all known patients with hemophilia B in Italy.

Design and Methods. Overall, 269 patients followed by 25 regional centers were considered in the study; after exclusion of the related individuals, 238 unrelated patients were analyzed (153 with severe, 59 with moderate and 26 with mild hemophilia B). Screening of the factor IX gene was performed using conformation sensitive gel electrophoresis (CSGE) followed by denaturing high performance liquid chromatography (dHPLC) or direct sequencing in negative cases, or by dHPLC/sequencing (36 cases).

Results. A mutation was identified in 236 of the 238 patients: 6 had large gene deletions (4 total and 2 partial), 14 small deletions, 1 combined deletion/insertion and 215 single nucleotide substitutions. A correlation was observed between the type of mutation and severity of hemophilia; however, a number of patients with the same genotype had varying severities of the disease. Eight of the 169 patients with severe hemophilia B (4.7%) developed inhibitors: 2 of these had a complete gene deletion, 1 had a large partial deletion (from exon A to part of exon H) while 5 had 3 different nonsense mutations. One patient with a nonsense mutation developed anaphylaxis. We also studied 65 families with hemophilia B involving 144 females (14 obligatory carriers, 85 carriers and 45 non-carriers) and performed 12 antenatal diagnoses.

Interpretation and Conclusions. The data have been used to build the Italian mutation database to provide each family with knowledge of the disease-causing defect for genetic counseling. This Italian study confirms the marked heterogeneity of factor IX mutations in the population and the presence of a degree of genotype/phenotype discordance. The identification of the mutation can also be used to predict risk of inhibitor development.

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

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From the Dipartimento di Laboratorio, Servizio Trasfusionale, Centro Regionale per le Malattie del Sangue e Presidio Regionale Malattie Rare per le Coagulopatie Congenite, Castelfranco Veneto Hospital (TV), ASL 8 Regione Veneto (DB, RS, PR, FP, GT); Laboratorio di Ematologia ed Emofilia, IV Divisione di Pediatria, Istituto Gaslini, Genova (PM); Dipartimento di Biochimica e Biotecnologie Mediche, Università di Napoli "Federico II", CEINGE-Biotecnologie Avanzate, Napoli, and Università del Molise, Isernia, Italy (GC).

Correspondence:
Dr. Giuseppe Tagariello, MD, Castelfranco Veneto Hospital, 31033 Castelfranco Veneto TV, Italy.
E-mail: gtagariello@ulssasolo.ven.it

Mutations in the factor IX gene (*F9*) may result in deficient or defective coagulation factor IX causing the bleeding tendency known as hemophilia B (HB), an X-linked recessive disease that occurs in about 1:30,000 male live births. The *F9* gene maps to Xq27; it spans about 34 kilobases of genomic DNA and contains eight exons encoding a 2.8 Kb mRNA, 1.4 Kb of which are translated.[1] The entire gene, including introns, was cloned in 1982[2] and sequenced in 1985.[3] The protein is a vitamin K-dependent serine protease released into plasma as a single glycoprotein of 415 amino acids, activated by factor VIIa and factor XIa through the proteolytic cleavage of two intramolecular bonds.[1] Hemophilia B is typically expressed in males and diagnosis is confirmed by the low activity of plasma factor IX (FIX). Carrier females are usually asymptomatic and plasma activity of FIX in carriers is variable. Genetic analysis for carriership is currently performed by the direct search for the causative mutation or by the indirect study of intragenic polymorphisms (linkage analysis).[4,5] Mutation analysis based on DNA sequencing is widely used, but as yet this procedure is too expensive to be used for large scale analyses in most laboratories. Thus screening procedures such as denaturing gradient gel electrophoresis (DGGE),[6,7] single strand conformation analysis (SSCP),[8] conformation sensitive gel electrophoresis (CSGE),[9,10] and denaturing high performance liquid chromatography (dHPLC)[11] have been utilized. These studies have confirmed that there is no common mutation pattern in particular ethnic groups. A database of *F9* gene mutations was assembled in 1990 and it is updated annually

Table 1. PCR primers and conditions used for amplification of the factor IX gene: sequence from Hinks *et al.*[13]

Exon 1 (bp)	*Primer*	*Primer Sequence (5' to 3')*	*Exon 2 (bp)*	*Primer*	*Primer Sequence (5' to 3')*	*Annealing Temp (°C)*	*$MgCl_2$ (mM)*
P+A (407)	P+Af	CCCATTCTCTTCACTTGTCC	D (245)	Df	CTGCAGGGGGAGGACCGGGCATTCTA	58	2.5
	P+Ar	CCTAGCTAACAAAGAACCAGT		Dr	GAATTCAACTTGTTTCAGAGGGAA		
A (256)	Af	GATGGACATTATTTCCCAGA	G (393)	Gf	AAGCTCACATTTCCAGAAAC	55	2.5
	Ar	GAAGAAGACAGCATCAGATA		Gr	TGGGTTCTGAAATTATGA		
B+C (506)	B+Cf	AGAGATGTAAAATTTTCATGATGTT	E (272)	Ef	CATGAGTCAGTAGTTCCATGTACTTT	58	2.5
	B+Cr	GCAGAGAAAAAACCCACATAAT		Er	TGTAGGTTTGTTAAAATGCTGAAGTT		
F (458)	Ff	TTTAAATACTGATGGGCCTG	–	–	–	55	1.5
	Fr	GTTAGTGCTGAAACTTGCCT		–	–		
H1 (394)	H1f	TAAGAATGAGATCTTTAACA	–	–	–	55	2.5
	H1r	CTAAGGTACTGAAGAACTAA		–	–		
H2 (391)	H2f	AAGATGGGAAAGTGATTAGTTA	Poly A (521)	Poly Af	AAGAGAACCGTTCGTTTGCA	58	1.5
	H2r	GAAGAGTCTTCCACAAAGGG		Poly Ar	AGAACTAAAGGAACTAGCAAG		

CSGE analysis is based on 4 duplex (P+A/D, A/G, B+C/E and H2/PolyA) and 2 single (F and H1) PCR.

(*http://www.kcl.ac.uk/ip/petergreen/haemBdatabase.html.*) In 2001 a national project, funded by the Italian Ministry of Health, was started with the aim of identifying the causative mutation in all known HB patients in Italy. A national database of mutations would permit more effective genetic counseling, providing each family with the knowledge of the disease-causing defect, timely carrier analysis and antenatal diagnosis. Furthermore, the knowledge of *F9* mutations could also be used to identify patients with a high risk of developing an inhibitor.

We enrolled 286 HB patients from 25 Italian hemophilia centers and examined the whole *F9* coding and regulatory regions to establish the molecular epidemiology of FIX mutations in Italy. Of the 286 patients considered, 238 unrelated patients were studied after exclusion of 17 duplicate samples and of 31 related patients.

Design and Methods

Patients

Each patient gave informed consent to be included in the study. A blood sample (5 mL) was collected in EDTA or sodium citrate from each patient. All HB patients currently followed by the 25 Hemophilia Centers in Italy were included in the study. Twenty-three centers sent the blood sample or DNA (250 samples) to our laboratory at Castelfranco Veneto Hospital for molecular analysis; two centers performed their own analyses (36 samples) and sent the results. For each patient clinical and laboratory data (including FIX clotting activity and inhibitor) were recorded. The form used is available on the web site of the Italian Association of Hemophilia Centers (*AICE, available at URL: http://www.aiceonline.it*). Of the 286 samples, 17 were excluded as they were duplicates and 31 were from relatives of other patients. Therefore, data from 269 HB patients were considered: 169 with severe HB (FIX:C<1%), 68 with moderate HB (FIX:C 1-5%) and 32 with mild (FIX:C 5-25%).

Factor IX coagulant activity (FIX:C). FIX:C was determined in a one-stage recalcification system by each laboratory.

Mutation analysis

Genomic DNA was extracted as previously described.[12] For all 238 unrelated samples, the *F9* gene was studied by polymerase chain reaction (PCR) analysis, amplifying all exonic and flanking intronic regions, the promoter and the region spanning the polyadenylation site.

DNA from 209 samples was analyzed by CSGE using the procedure previously described.[13] The patients negative by CSGE analysis were tested again by dHPLC[11] or by direct sequencing. CSGE analysis was based on 4 duplex and 2 single PCR. Each reaction was as follows: PCR ReddyMix Master Mix – ABGene Surrey UK (1.25 U Taq DNA polymerase, 75 mM Tris-HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.001% Tween 20 and 0.2 each of dNTP), 100 ng of each primer, 500 or 1000 ng of DNA in a single or duplex PCR, respectively, in a final volume of 50 µL. The concentration of magnesium chloride for each amplification and the sequence of the primers are detailed in Table 1. Figure 1 shows representative CSGE patterns. CSGE was repeated on a new PCR product for

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Table 2. Type of mutations found in the three subgroups of unrelated patients with severe, moderate and mild hemophilia B.

Disease severity	Large deletion	Small deletion	Missense	Nonsense	Splice site	Promoter	Total
Severe	6	15	89	32	9	1	152
Moderate	–	–	53	1	4	1	59
Mild	–	–	22	1	1	1	25
All	6	15	164	34	14	3	236

regions displaying an abnormal migration pattern prior to sequencing. A NucleoSpin Extraction kit (Macherey-Nagel) was used for PCR product purification. The sequence reaction was performed using a BigDye Terminator cycle sequencing kit (Applied Biosystems) and electrophoresis was performed on an automated capillary sequencer (Applied Biosystems ABI PRISM 3700 DNA Analyzer). Each patient's sequence was compared to the wild type sequence using BLAST software available at the National Centre for Biotechnology Information website (*NCBI; www.ncbi.nlm.nih.gov*). The remaining 29 samples were analyzed by dHPLC or direct sequencing.

Analysis of polymorphic markers

Five *F9* intragenic polymorphisms were investigated using PCR and restriction enzyme digestion: Dde I, Taq I and Xmn I according to Bowen *et al.*;[14] Mnl I and Hha I according to Graham *et al.*[15] and Winship *et al.*,[16] respectively.

Results

We studied 153 unrelated patients with severe HB, 59 with moderate HB and 26 with mild disease. A mutation was identified in 236 of the 238 (99%) patients. In two cases (1 severe and 1 mild) our study failed to identify *F9* gene variants. Table 2 shows the type of mutations found in the three subgroups of HB patients with varying degrees of disease severity.

Molecular analysis revealed 137 different mutations in the 236 HB patients (Table 3). Six patients had large deletions indirectly revealed by failure of PCR amplification of the lost *F9* exons, while a control PCR product obtained for the *F8* gene locus on the X chromosome (Xq28) provided a positive result. The absence of FIX PCR products in 6 DNA samples led to the conclusion that they had large deletions: 4 patients had a complete gene deletion (one has already been reported by Bernardi *et al.*)[18] and 2 large partial deletions: the first from the beginning of the gene to the 3'end of exon H (previously reported by Hassan *et al.*)[19] and the second from exon G to part of exon H. The international database reports 41 total gene deletions and 37 partial gene deletions. Except for the two already mentioned, we cannot tell whether the large deletions identified in this cohort have already been reported as the deletion size or the breakpoints have not been established. However, the breakpoints presumably differ in each patient and the deletions could be considered as new reports.

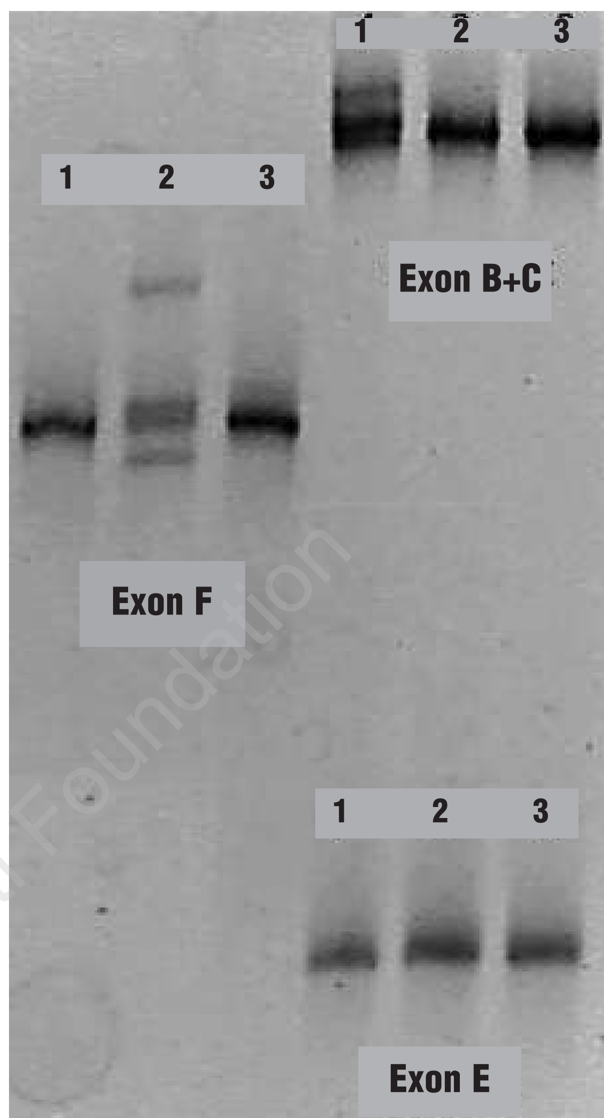

Figure 1. Conformation sensitive gel electrophoresis of PCR amplified *F9* exons: Exon F single PCR and Exon B+C plus E duplex PCR. Lane 1: patient #23 (mutation 6442T→C in exon B); lane 2: patient #75 (mutation 20562G→A in exon F); lane 3: negative control.

The study revealed 11 small deletions (1 in a splice site, 1 in combination with an insertion, 8 frameshift and 1 in frame), and 120 single nucleotide substitutions (86 missense, 19 nonsense, 12 in splice sites and 3 in the promoter). Fifty of these 131 small changes (small deletions and single nucleotide substitutions)

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Table 3. Summary of the 137 different factor IX gene mutations found in 236 Italian patients with hemophilia B.

ID number	Nucleotide change[1]	CpG[2]	Amino acid change	Type of mutation	Severity of disease[3]	Number[4]
1	g→t -26	no	promoter	–	severe	1
2	t→c -20	no	promoter	–	mild	1
3	a→t -5	no	promoter	–	moderate	1
4	88T→C	no	Leu-27Ser	missense	severe	1
5	107-110delTGAA*	–	Ala-21 fs Stop-7	4 bp deletion	severe	1
6	111T→C	no	Cys-19Arg	missense	mild	1
7	112G→A	no	Cys-19Tyr	missense	severe	4
8	116A→G	no	Thr-18Thr	splice site	severe	1
9	118-121delgttt	–	Splice site	4 bp deletion	severe	4
10	122 g→a	no	IVS A +5 g→a	splice site	mild	1
11	122g→c	no	IVS A +5 g→c	splice site	severe	1
12	6320t→g	no	IVSA -6 t→g	splice site	severe	1
13	6347C→A*	no	Ala-10Asp	missense	severe	1
14	6364C→T	yes	Arg-4Trp°	missense	moderate/severe	2
15	6365G→A	yes	Arg-4Gln	missense	severe	3
16	6372G→C*	no	Lys-2Asn	missense	severe	1
17	6392T→C*	no	Leu6Ser	missense	severe	1
18	6395A→G*	no	Glu7Gly	missense	moderate	1
19	6400T→A	no	Phe9Ile	missense	mild	2
20	6410G→A	no	Gly12Glu	missense	mild	1
21	6410G→C	no	Gly12Ala	missense	moderate	1
22	6422-5delGAGA	–	Arg16 fs Stop56	4 bp deletion	severe	1
23	6442T→C	no	Cys23Arg	missense	severe	1
24	6449T→G*	no	Phe25Cys	missense	mild	1
25	6460C→T	yes	Arg29Stop	nonsense	severe	2
26	6461G→A	yes	Arg29Gln	missense	mild	1
27	6463G→C*	no	Glu30Gln	missense	moderate	1
28	6472G→A*	no	Glu33Lys	missense	severe	2
29	6472G→C*	no	Glu33Gln	missense	severe	1
30	6488C→T	no	Thr38Ile	missense	mild	1
31	6494 g→a	no	IVS B +5 g→a	splice site	severe	2
32	6494g→t*	no	IVS B +5 g→t	splice site	severe	1
33	6494g→c*	no	IVS B +5 g→c	splice site	moderate	1
34	6685T→G*	no	Phe41Cys°	missense	moderate/severe	5
35	6706A→G	no	IVSC +4 a→g	splice site	moderate	1
36	10380c→g*	no	IVSC -12 c→g	splice site	severe	1
37	10396-7delAG*	–	Gly48 fs Stop51	2 bp deletion	severe	1
38	10400C→T	no	Gln50Stop	nonsense	severe	1
39	10422T→A*	no	Leu57Stop	nonsense	severe	1
40	10425-6delAT*	–	Asn58 fs Stop64	2 bp deletion	severe	1
41	10430G→A	yes	Gly60Ser	missense	mild	4
42	10437G→T*	no	Cys62Phe	missense	severe	1
43	10438C→G*	no	Cys62Trp	missense	severe	1
44	10464G→A	no	Cys71Tyr	missense	severe	1
45	10497G→C*	no	Cys82Ser	missense	severe	1
46	10506g→t*	no	IVS D +1 g→t	splice site	severe	2
47	17677T→C	no	Cys88Arg	missense	severe	1
48	17678G→C	no	Cys88Ser	missense	severe	1
49	17689A→C	no	Asn92His°	missense	mild/moderate/severe	5
50	17692G→A	no	Gly93Ser	missense	severe	1
51	17698T→C	no	Cys95Arg	missense	severe	1
52	17700C→A	no	Cys95Stop	nonsense	severe	1
53	17701G→T*	no	Glu96Stop	nonsense	severe	1
54	17704C→A*	no	Gln97Lys	missense	severe	1
55	17704C→G	no	Gln97Glu	missense	moderate	1
56	17734G→A*	no	Val107Met	missense	mild	1
57	17741G→C	no	Cys109Ser	missense	severe	1
58	17748T→A*	no	Cys111Stop	nonsense	severe	1
59	17755G→T*	no	Gly114Stop	nonsense	severe	1
60	17760delT*	–	Tyr115 fs Stop156	1 bp deletion	severe	1
61	17761C→T	yes	Arg116Stop	nonsense	severe	1
62	17764C→T*	no	Leu117Phe	missense	severe	1
63	17785T→C	no	Cys124Arg	missense	severe	1
64	17786G→A	no	Cys124Tyr	missense	severe	1
65	17810 a→g	no	IVS E +13 a→g	splice site	moderate	1
66	20375G→A	no	Cys132Tyr	missense	severe	1
67	20413C→T	yes	Arg145Cys	missense	moderate	7
68	20414G→A	yes	Arg145His°	missense	mild/moderate	10
69	20414G→T	no	Arg145Leu	missense	moderate	1
70	20464G→T	no	Glu162Stop	nonsense	severe	1
71	20518C→T	yes	Arg180Trp	missense	severe	3
72	20519G→A	yes	Arg180Gln	missense	severe	5
73	20530G→A	no	Gly184Arg	missense	severe	1
74	20549G→A*	no	Gly190Asp°	missense	moderate/severe	17
75	20562G→A	no	Trp194Stop	nonsense	severe	2 (2)

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ID number	Nucleotide change[1]	CpG[2]	Amino acid change	Type of mutation	Severity of disease[3]	Number[4]
76	20563C→T	no	Gln195Stop	nonsense	severe	1
77	30076G→T*	no	Gly208Val	missense	severe	1
78	30099A→T*	no	Ile216Phe	missense	severe	1
79	30111G→A*	no	Ala220Thr	missense	severe	1
80	30114delC	–	His221 fs Stop250	1 bp deletion	severe	1
81	30117T→C	no	Cys222Arg	missense	severe	1
82	30144G→T*	no	Val231Phe	missense	severe	1
83	30150G→A	yes	Ala233Thr°	missense	moderate/severe	2
84	30153G→A*	no	Gly234Ser	missense	severe	1
85	30153G→C	no	Gly234Arg	missense	severe	1
86	30852delC*	–	Thr244 fs Stop250	1 bp deletion	severe	2
87	30854G→A	no	Glu245Lys	missense	severe	1
88	30855A→G	no	Glu245Gly°	missense	mild/moderate	2
89	30863C→T	yes	Arg248Stop	nonsense	severe	4 (2)
90	30864G→A	yes	Arg248Gln°	missense	moderate/severe	11
91	30870T→A*	no	Val250Glu	missense	severe	1
92	30875 C→T	yes	Arg252Stop	nonsense	severe	4 (1)
93	30897A→G	no	Tyr259Cys	missense	severe	1
94	30919C→G	no	Tyr266Stop	nonsense	severe	1
95	30973C→A	yes	Tyr284Stop	nonsense	severe	1
96	30987G→A*	no	Cys289Tyr	missense	severe	1
97	31007A→G	no	Thr296Ala	missense	mild	1
98	31008C→T	yes	Thr296Met°	missense	moderate/severe	2
99	31023A→T*	no	Lys301Ile	missense	mild	1
100	31028G→T*	no	Gly303Stop	nonsense	mild	1
101	31035G→T*	no	Gly305Val	missense	severe	1
102	31049T→G*	no	Trp310Gly	missense	severe	2
103	31053G→A	no	Gly311Glu	missense	severe	2
104	31055A→G*	no	Arg312Gly	missense	mild	1
105	31071-9 delGGAGATCAGinsA*	no	Gly317 fs Stop324	del-ins	severe	1
106	31080C→G*	no	Ala320Gly	missense	mild	1
107	31088C→T*	no	Leu323Phe	missense	moderate	1
108	31091C→T	no	Gln324Stop	nonsense	severe	2
109	31103G→T	no	Val328Phe	missense	severe	1
110	31118C→G	no	Arg333Gly	missense	moderate	1
111	31118C→T	yes	Arg333Stop°	nonsense	moderate/severe	5
112	31119G→A	yes	Arg333Gln°	missense	mild/moderate/severe	6
113	31119G→C*	no	Arg333Pro	missense	moderate	1
114	31128G→A	no	Cys336Tyr	missense	severe	1
115	31133C→T	yes	Arg338Stop	nonsense	severe	3
116	31152T→A*	no	Ile344Asn	missense	severe	1
117	31161-3del ACA	/	del Asn 347	3 bp deletion	severe	1
118	31164T→G*	no	Met348Arg	missense	severe	1
119	31203G→A	no	Cys361Tyr	missense	severe	1
120	31213T→G*	no	Asp364Glu	missense	severe	1
121	31220G→A	no	Gly367Arg	missense	moderate	1
122	31223C→A	no	Pro368Trp	missense	severe	3
123	31224C→A	no	Pro368His	missense	severe	1
124	31260C→T	no	Thr380Ile	missense	severe	2
125	31262G→A	no	Gly381Arg	missense	severe	1
126	31263G→A*	no	Gly381Glu	missense	severe	1
127	31280G→A	no	Glu387Lys	missense	moderate	1
128	31290C→T	no	Ala390Val	missense	severe	1
129	31344T→A*	no	Ile408Asn	missense	severe	1
130	31355delA	–	Thr412 fs Stop436	1 bp deletion	severe	1
131	32528A→G	no	–	new splice site	moderate	1
132	–	–	–	del from ex G to H	severe	1
133	–	–	–	del from ex A to H	severe	1 (1)
134	–	–	–	complete gene del.	severe	1
135	–	–	–	complete gene del.	severe	1
136	–	–	–	complete gene del.	severe	1 (1)
137	–	–	–	complete gene del.	severe	1 (1)

*[1]DNA numbering system from Yoshitake et al.[3] The reference sequence is genomic DNA (Genbank accession number K02402). The mutation nomenclature is that described by den Dunnen JT et al. *novel changes, not previously reported in the International Hemophilia B database. °Mutations found in more than one patient with phenotypically different disease severity. Range of severity in patients is shown in the "severity of disease" column. [2]C to T or G to A transitions due to deamination of 5-methylcytosine to thymine in CG dinucleotides. [3]Severity of disease in the patient or in the group of patients, based on factor IX coagulation activity (see text). [4]Number of patients with the same mutation and in brackets, the number of patients with an inhibitor.*

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Table 4. Characteristics of patients with an inhibitor.

Patient	*Mutation*	*Type of treatment*	*Exposure days*	*Peak titer (BU)*
CF 26	Trp 194 Stop	pd/od	15	25
FI 1071	Trp 194 Stop	r/od	45	36
VI 880	Arg 248 Stop	pd/od	30	2.6
VR 1357*	Arg 248 Stop	r/od	9	0.8
NA 4	Arg 252 Stop	pd/od	14	117
BA 1111°	Del. from ex A to H	pd/od	nk	25
FI 718°	Complete gene del.	pd/od	48	4.8
TO 1386	Complete gene del.	r/od	31	6.4

*Pd, plasma derived; od, on demand; r, recombinant; nk, not known *patient with anaphylaxis; °previously studied patients: BA 1111 reported by Hassan et al.;[19] FI 718 reported as Pisa 1 by Bernardi et al.[18]*

had not yet been reported in the international hemophilia B database. Seventy-six (35%) of 216 single nucleotide substitutions identified in our 236 patients involve a CpG dinucleotide, which confirms the figure (40%) reported in the international database.[20] CpG dinucleotides have been shown to be a hot-spot for mutations and account for many of the identical mutations found in unrelated families.[21] In fact, more than 30% of all single nucleotide substitutions detected in many inherited disorders are C to T or G to A transitions due to the spontaneous deamination of 5'-methylcytosine to thymidine in the CG doublet.[22]

Among the 137 different mutations identified in the 236 patients, 33 mutations (31 single base substitutions and 2 small deletions) were present in more than 1 patient (see the *number* column in Table 3) accounting for mutations in 132 patients. Six of these 33 mutations have not been previously reported, and 16 out of 31 single base substitutions (72 unrelated patients) were at CpG dinucleotides. To examine whether a founder effect contributed to the presence of these mutations in our cohort, haplotype analysis was performed in 104 of 132 HB patients with the same mutations. Different haplotypes were found in 38 patients, indicating that their mutations were independent events. In particular, the Gly190Asp substitution was detected in 17 apparently unrelated patients displaying 2 different haplotypes. A common ancestor could be considered in 8 patients of this subgroup who originated in the same region. This novel mutation does not involve a CpG dinucleotide, and it has been reported only in our cohort of Italian patients. In this subgroup of patients, 5 are severely affected and 12 have moderate disease.

CSGE detected an abnormal migration pattern in 179 of 201 unrelated individuals (89%). For 24 patients CSGE failed to identify the mutation: 7 mutations were found by the subsequent dHPLC analysis, while DNA sequencing identified the change in 15 additional patients. We failed to identify the mutation in 2 HB patients (1 with severe disease, the other with mild HB). The defect might be located outside the fragments amplified for the analysis or might not involve the *F9* gene.

Eight HB patients from our study carried two different *F9* gene variants. Besides the candidate mutation the second change appeared to be a polymorphism: the g.10512A→G in intron D (1 patient) and the insertion g.30802 insA in intron G (2 patients) had been previously reported as polymorphic variants, while the g.6550G→C in intron B (1 patient) and the g.32847T→C 3' to the usual poly A addition site (4 patients) has not previously been described. The A allele of the known polymorphism g.20422 G→A (Ala148Thr) had a frequency of 72% in our cohort and these results are in agreement with previous reports in which the frequency of the A allele was 67%.[4,5]

Sporadic cases of hemophilia account for approximately 30-50% of the total hemophilic population.[5,23] In our cohort, 148 of 238 (62%) families have only one known case of HB. Even when considering only the families with severe or moderate hemophilia to avoid bias from possible undiagnosed patients with mild disease, the percentage did not change, probably because of the low number of patients with mild HB in this cohort. This high figure could be partially explained by the failure to recognize multiple cases in the same family since large families could be dispersed to more than one hemophilia center.

Eight of the 169 patients with severe HB developed inhibitors: 2 patients had a complete gene deletion, 1 had a partial deletion (from exon A to part of exon H) while the other 5 had 3 different nonsense mutations (Table 4). In three patients (NA 4, BA 1111 and FI 714) the disease was familial, but only patient NA 4 had a cousin with an inhibitor. Patient VR 1357 developed an anaphylactic reaction during the ninth exposure to treatment. None of the patients underwent immunotollerance treatment. Table 4 reports the details of mutations and clinical information on this group of patients.

The data recorded in the Italian *F9* gene mutation database provided the basis for the study of 65 families with HB involving 144 females (14 obligatory carriers, 85 diagnosed carriers and 45 non-carriers) and to the performance of 12 antenatal diagnoses.[24]

Discussion

Genetic counseling is an important part of the care of hemophiliacs and their families. A mutation database would provide each family with the knowledge of the disease-causing defect, permitting effective genetic

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counseling and rapid carrier and antenatal diagnosis within the family. Identification of the mutation provides many advantages compared to linkage analysis, leading to precise carrier and antenatal diagnosis. In this study we used CSGE with a detection rate of 89%. The subsequent application of dHPLC and of *F9* gene sequencing increased the mutation detection rate to 99%. These data compare with previously reported detection rates of 91% for DGGE[6] to 100% for amplification and mismatch detection,[25] CSGE,[13] direct sequencing[26] and dHPLC.[11] The variable detection rates reported for the different techniques could depend on the different sensitivity of each procedure; alternatively, the spectrum of mutations in the different ethnic groups analyzed may be slightly different. In any case, our data suggest that scanning procedures such as CSGE or dHPLC may contribute to efficient mutation detection in HB patients. Direct sequencing can be used as a second level procedure in patients negative by scanning procedures, identifying virtually all mutations in HB patients.

Our study revealed 137 different mutations considered as unique molecular events. In the group of 131 with small changes, the most prevalent gene defects were missense mutations (65%), followed by nonsense mutations (15%), splice site mutations (9%) and small deletions (9%) and finally by defects in the promoter (2%). Similar frequencies are reported in the international hemophilia B mutations database: missense mutations (68%), nonsense mutations (14%), splice site mutations (6%), small deletions and insertions (6%) and finally defects in the promoter (3%).

Among the small changes, 50 were novel mutations, 34 of which lie within codons in which mutations have been already reported. Among the 16 mutations involving codons in which no mutation had been previously described, 6 were small deletions with frameshift, 1 was a splice site mutation, 3 were nonsense and 6 missense changes, involving conserved amino acids; thus, all these mutations are strong candidates for being disease-causing.

Our study confirms the well known correlation between the type of mutation and the severity of HB: all the deletions were identified in patients bearing a severe phenotype; missense mutations were more frequent in moderate (90%) and in mild disease (88%) than in severe HB (59%). Nonsense mutations are expected to produce truncated unstable proteins and to result in a severe disease.[4] However the international database reports several nonsense mutations with different values of FIX:C (from <1 to 6%). We report 2 nonsense mutations associated with a non-severe disease: Gly303Stop, not previously reported in the international database, was found in a patient with mild HB (6%); Arg333Stop, reported in the international database in many patients with severe HB and also in 2 cases with moderate HB (2% and 4%), was found in 1 patient with moderate HB (1.5%) and in 4 patients with severe HB (Table 3). Cutler[27] and Taylor[28] proposed an explanation for the mitigation of phenotype (higher than expected FIX:C) in 2 cases of HB with a well documented presence of somatic mosaicism for a mutant allele. Other still unrecognized genes may play a role in the balance of coagulation. Analysis of the factor V Leiden and P20210 variants was performed in the 2 patients and the normal G/G genotype was found in each case. Among the 3 substitutions in the promoter, 2 of them (-5A→T and –20T→C) were previously reported as responsible for the Leiden phenotype[29] with increasing FIX clotting activity after puberty. Several HB patients from our study bearing the same *F9* mutation (10 missense and 1 nonsense mutation – Table 3) express variably severe phenotypes. Ten out of the 11 mutations have been previously described in the database with different FIX clotting activities. Such discordance might be due to gene variants in other proteins of the coagulation pathway.[20]

The type of mutation is a distinct risk factor predisposing to inhibitor development.[23] Two types of mutations have been documented in association with inhibitor development: point mutations resulting in frameshifts and premature stop codons or gross gene deletions and rearrangements. In our series, 8 of the 169 patients (4.7%) with severe HB developed inhibitors while no patients with moderate or mild disease did so. None of the 102 patients with severe HB and missense mutations developed inhibitors while 8 of 56 (14.3%) of the patients with severe HB and large deletions (6 patients), nonsense (33 patients) or frameshift (17 patients) mutations did so. In our cohort the Trp194Stop mutation was found in 2 unrelated patients with severe HB who both developed inhibitors; while Arg248Stop was found in 4 unrelated patients with severe HB, of whom two developed inhibitors. One of these inhibitor patients developed an anaphylactic reaction to FIX replacement therapy, as observed in a previous subgroup of HB patients.[30] The same mutation was reported by Thorland in association with anaphylaxis.[31] Arg252Stop was found in 4 unrelated patients with severe HB but only 1 developed an inhibitor. This latest family had 10 affected members but only 2 developed an inhibitor.

In conclusion, a database of *F9* mutations in a well-defined population can be useful in helping genetic counseling and antenatal diagnosis. Furthermore, we confirm the genetic heterogeneity of *F9* mutations and some phenotypic discordance within patients bearing the same mutation. Finally, the type of *F9* mutation may help to predict the risk of developing a FIX inhibitor and anaphylaxis.

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Appendix

Centro emofilia di Bari Policlinico I, Dr. A. Scaraggi; Centro emofilia di Bari Policlinico II, Dr. N. Ciavarella; Centro emofilia di Bologna, Dr.ssa G. Rodorigo; Centro emofilia di Castelfranco Veneto, Dr. G. Tagariello; Centro emofilia di Catanzaro, Dr. G. Muleo; Centro emofilia di Firenze, Dr. M. Morfini; Centro emofilia di Genova, Prof. PG. Mori; Centro emofilia di Milano Policlinico, Prof. P.M. Mannucci, Dr.ssa E. Santagostino; Centro emofilia di Milano Niguarda, Dr. F. Baudo; Centro Emofilia di Napoli, Dr.ssa A. Rocino; Centro emofilia di Padova, Dr. E. Zanon; Centro emofilia di Palermo "G. di Cristina", Prof. G. Mancuso; Centro emofilia di Parma, Dr.ssa A. Tagliaferri; Centro emofilia di Pavia, Prof.ssa G. Gamba; Centro emofilia di Perugia, Dr. A. Iorio; Centro emofilia di Pescara, Dr. A. Dragani; Centro emofilia di Reggio Calabria, Dr. V. Trapani Lombardo; Centro emofilia di Roma, Prof.ssa G. Mazzucconi; Centro emofilia di Torino "Policlinico pediatrico", Dr.ssa L. Perugini, Dr.ssa M Messina; Centro emofilia di Torino "Le Molinette", Dr. G. Tamponi, Dr.ssa P. Schinco; Centro emofilia di Trento, Dr.ssa G. Rossetti; Centro emofilia di Udine, Dr. G. Barillari; U.O. Oncoematologia pediatrica di Verona, Dr. P. Marradi; Centro emofilia di Vicenza, Dr. G. Castaman.

GT, GC and PM: design of the study, interpretation of data, drafting and final version; DB, RS, PR and FP: analysis and interpretation of data, critical revision and approval of final version.

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