Sixteen novel hemophilia A causative mutations in the first Argentinian series of severe molecular defects

Liliana C. Rossetti, Claudia Pamela Radic, Miguel Candela, Raúl Pérez Bianco, Miguel de Tezanos Pinto, Anne Goodeve, Irene B. Larripa, Carlos D. De Brasi

From the Instituto de Investigaciones Hematológicas Mariano R. Castex, Academia Nacional de Medicina de Buenos Aires, Argentina (LCR, CPR, MC, RPB, MdTP, IBL, CDDB); Division of Genomic Medicine, University of Sheffield Medical School, Sheffield, UK (AG).

Acknowledgements: we wish to thank patients, relatives and the Hemophilia care staff; and the scientists Derrick Bowen, Héctor Targovnik and Eduardo Tizzano for their help in different phases of the work.

Fundings: this study was supported by grants from the René Barón Foundation, the Alberto J. Roemmers Foundation, the Academia Nacional de Medicina de Buenos Aires, the National Research Council (CONICET), the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) and the Word Federation of Hemophilia.

Manuscript received November 30, 2006.
Manuscript accepted April 6, 2007.

Correspondence:
Liliana C. Rossetti, Departamento de Genética, Academia Nacional de Medicina. Pacheco de Melo 3081, Cdad. de Buenos Aires (1425), Argentina.
E-mail: rossetti@hematologia.anm.edu.ar

ABSTRACT

Hemophilia A (HA) is caused by heterogeneous mutations in the factor VIII gene (*F8*). This paper reports 16 novel small F8-mutations and rearrangements in a series of 80 Argentinian families with severe-HA. Using an updated scheme for *F8*-analysis, we found 37 *F8*-inversions (46%), 10 large deletions (13%), 13 small ins/del (16%), 7 nonsense (9%) and 8 missense mutations (10%), including 4 new ones (p.T233K, p.W1942R, p.L2297P and p.L2301S). The potential changes leading to severe-HA of these latter mutations were suggested by bioinformatics. The *F8*-mutation was characterised in 76 families (95%). They received genetic counselling and precise information about treatment design.

Key words: *F8*, HEMA, severe phenotype, mutation characterization.

Haematologica 2007; 92:842-845

Hemophilia A (HA), the commonest X-linked coagulopathy, is caused by heterogeneous mutations in the coagulation factor VIII gene (*F8*). Due to its size and complexity,[1] *F8* still challenges mutation characterisation worldwide.

HA can be classified as severe when the residual clotting activity of FVIII is less than 1% and accounts for about 40% of the patients with HA. In approximately one half of severe-HA, the disease can originate from large DNA inversions which truncate *F8*: intron 22 inversions (Inv22)[2,3] and intron 1 inversions (Inv1).[4] Other HA-causative mutations include a spectrum of *F8* defects that are compiled in international databases (HAMSTeRS, *http://europium.csc.mrc.ac.uk*). This paper describes the first integrated severe-HA mutation series from Argentina using an updated scheme for *F8* analysis. Here we report 16 novel *F8* small defects associated with severe-HA and a discussion of potential 3D-structural changes of new missense mutations.

Design and Methods

Studied populations

Eighty families affected by SHA, including 173 individuals (85 probands, 40 mothers, and 48 relatives) and individuals from an Argentinian DNA-sample collection from healthy donors[5] were studied. Informed consent was obtained in all cases.

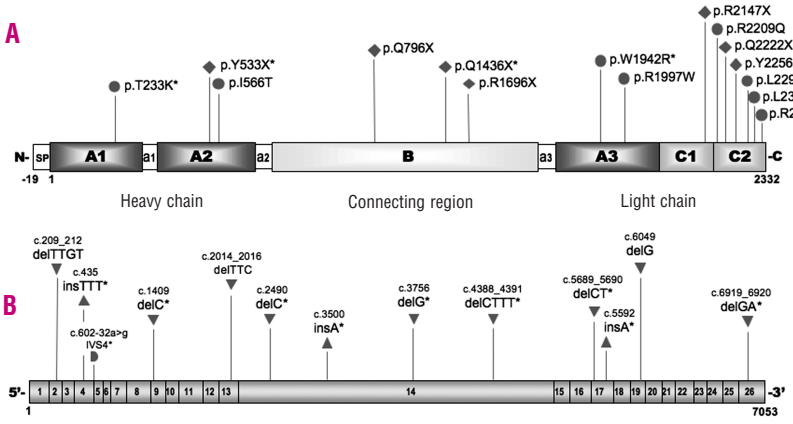
Protocol for F8-mutation characterization

Genomic DNA was prepared from peripheral blood leukocytes using phenol-chlorophorm or salting-out methods.

The Inv22 was investigated by Southern blot[2] and by a novel approach based on inverse-PCR.[6] The Inv1 was analysed by double-PCR.[4]

All the relevant sequences of *F8* were represented in 35 amplimers (260-547bp) that were designed for mutation screening by conformation sensitive gel electrophoresis (CSGE) (*Supplementary Table 1*). The identity of these amplimers was analyzed by agarose gel electrophoresis.

Large deletions were defined as a consistent absence of PCR amplification products from a group of *F8*-exon sequences. Long-distance PCR amplifications (LD-PCR) analyses were designed case by case using vicinal exon-specific primers. These LD-PCR amplifications were performed using *Taq/Pwo* DNA-polymerase mixture (Roche).[5] Small mutation screening was performed using CSGE.[7] High-resolution CSGE was achieved throughout the entire *F8* in all patients, by long runs (41 cm), and when possible was also resolved by intermediate (17 cm) or short runs (7 cm). Selected purified-PCR products were subjected to DNA-sequencing. To simplify and accelerate mutation diagnosis in a particular family we used intermediate-low resolution CSGE, mainly useful for frameshifts, or restriction analysis.



A. Location of 8 missense changes (circles) and 7 nonsense (diamonds) in the FVIII domains. SP, indicates signal peptide; A1, A2, A3, B, C1 and C2, major FVIII domains; a1, a2 and a3, minor acidic peptides. **B.** Location of 12 small Ins/Del mutations in the *F8* cDNA, 3 small insertions (upward triangles), and 9 small deletions (downward triangles) and 1 potential splice site mutation (half-circle). The *p.* numbering corresponds to mature secreted FVIII protein, aminoacid +1 indicates codon +19 downstream from the initiation codon ATG (excluding the signal peptide). The *c.* mutation numbering is based on *F8* cDNA sequence (Genbank entry NM_000132.2), with nucleotide +1 corresponding to A of the ATG translation initiation codon.

Figure 1. The distribution of small HA causative mutations in human *F8* including all 16 newly described*. *F8* encodes a signal peptide and a mature glycoprotein of 2,332 amino acids, composed of a heavy-chain (domains A1-A2-B) and a light-chain (domains A3-C1-C2).[14]

Bioinformatics

Annotations, mappings and alignments were performed using EditSeq, MapDraw and MegAlign software (LaserGene, DNA Star). Genomic *F8* sequences were obtained from GenBank AY769950. *F8* mutation nomenclature followed the recommendations of the HGVS.[8] Novel aminoacid (aa) changes were examined for their conservation in murine, porcine and canine FVIII using the sequence alignment line-up (*http://europium.csc.mrc.ac.uk/WebPages/Database/Protein/lineups.html*) and the conservation in human factor V (CAB16748), Ceruloplasmin (BAA08084) and Hephaestin (CAC35365) by MegAlign-based alignments. Analysis of ESEs (Exonic Splicing Enhancers) was performed using ESE-Finder software on-line (*http://exon.cshl.edu/ESE/*). The GeneSplicer software was used to seek and score potential splicing sites on-line (*http://www.tigr.org/tdb/GeneSplicer/gene_spl.html*).

Hypotheses about the structural effects caused by new missense mutations were provided using the atomic 3D-coordinates of an integrated template of the FVIII, Stoilova-McPhie *et al*. model[9] assisted by Deep View/Swiss-PdbViewer software (ver.3.7).[10]

Results and Discussion

Large DNA rearrangements

Former Inv22 analysis identified 19 cases with the distal pattern (76%) and 6 with proximal (24%). These agree with those reported in literature (81% and 17% respectively).[11] Inverse-PCR analysis led to molecular diagnosis of 11 additional families with the Inv22. Therefore, the Inv22 has been identified in 36 out of 80 families with severe-HA (45%). Among the 83 individuals from Inv22 positive families that received genetic counselling, all 21 mothers resulted carriers (15 of them belong to families with sporadic disease). This finding agrees with the meiotic origin of Inv22 in men.[12] The Inv1 was found in one family, less than 2% in our series. Primary *F8*-scanning enabled detection of 10 large deletions (13%): g.EX4_EX10del (30.9kb), g.EX10_EX18del (62.5kb), g.EX10_EX11del (4.4kb), g.EX3_EX26del (→158kb), g.EX23_EX26del (→30kb), g.EX2_EX12del (int1h-1_EX12, →53.6kb), g.EX2_EX3del (int1h-1_EX3del, →22kb), g.EX1del (→0.5kb), g.EX1del (*F8* promoter_*int1h*-1del, >18kb) and g.EX26del (→0.5kb). Further characterisation of these deletions led to precise molecular diagnosis (hemi-heterozygous diagnosis) in a specific family and analysis of the deletion breakpoints. Thirteen percent of large deletions in severe-HA represents a higher frequency than other published series.[13] This can be explained by the correlation *F8*-deletions and inhibitors. Families with large deletions are more involved in the hemophilia care system and tend to seek genetic counselling. This, therefore, introduces a bias in our series.

Small mutations

We characterized 29 small mutations (Figure 1) in 33 severe-HA affected families without large rearrangements. Thus the efficacy of CSGE screening was 88%. Molecular diagnosis was provided in probands and 31 female relatives. Full details of small mutations are displayed in Table 1A: 8 missense (10%), 7 nonsense (9%) and 1 intronic/splicing mutations; and in Table 1B: 11 ins/del frameshifts (14%) and 2 ins/del in-frame. p.T233K is a non-conservative missense change, polar neutral to positively charged residue. T233 is only partially exposed to the protein surface (fractional accessibility, FA, 0.036) and its electrostatic potential results modified upon p.T233K. In the 3D-model of FVIII, the hydroxyl in the T233-residue is normally hydrogen-bound (hb) to A200 providing a specific interaction between β-strands (S10-S9) in A1, which is lost upon the p.T233K (*Supplementary Figure S1*). At this location, p.T233I is a reported mutation associated with mild/moderate phenotype in HAMSTeRS. Although p.T233I is non-conservative and abolishes the above

Table 1A. Single nucleotide substitutions.

Case	Mutation Description	Exon/Domain	Reports	FVIII Inhibitor	Origin	Observations
Missense 8/80 (10%)						
1	c.5881T→A p.1942W→R	18/A3	NR	–(HIV)	F	Transversion; A3 β-S49 disturbance; huF5→L.
2	c.6683G→A p.2209R→Q	24/C2	25	–	S	CpG
3	c.6959T→C p.2301L→S	26/C2	NR	–	F	Loop/β-S2301-14; VWF/PL binding site; huF5→I
4	c.6947T→C p.2297L→P	26/C2	NR	–	S	β-S2293-7/loop/β-S2301-14; VWF/PL binding site; huF5→F
5	c.755C→A p.233T→K	6/A1	NR	–	F	Transversion; A1 β-S10-S9 interactions; huCe→S; huHe→A
6	c.6967C→T p.2304R→C	26/C2	8	–	S	CpG
7	c.6046C→T p.1997R→W	19/A3	30	–	F	CpG
8	c.1754T→C p.566I→T	12/A2	3	–	F	
Nonsense 7/80 (9%)						
9	c.6825T→A p.2256Y→X	25/C2	NR	–	S	Transversion
10	c.6496C→T p.2147R→X	23/C1	18	+ (HIV)	F	CpG
11	c.2443C→T p.796Q→X	14/B	1	T,–	S	
12	c.1656C→A p.533Y→X	11/A2	NR	T,–	F	Transversion
13	c.6721C→T p.2222Q→X	24/C2	2	–(HIV)	S	
14	c.5143C→T p.1696R→X	14/B	3	–	S	CpG
15	c.4363C→T p.1436Q→X	14/B	NR	–	S	
Intronic single nucleotide mutation 1/80						
16	c.602-32A→G	IVS4/A1	NR	–	S	In-phase with SHA in the family. Causative?

Table 1B. Small insertions and deletions.

Case	Mutation Description	Exon/Domain	Reports	FVIII Inhibitor	Origin	Observations
Frameshifts Ins/Del (1-4 bp) 11/80 (14%)						
17	c.3500insA p.N1148fsX8	14/B	NR	+	S	Within an A-run
18	c.5592insA p.K1845fsX16	17/A3	NR	– (HIV)	S	Within an A-run
19,20	c.209_212delTTGT p.L68fsX1	2 / A1	6	–; –	S; S	2 unrelated families; {case 19: [- ; C; - ; -] ≈haplotypes: {case 20: [+; A; +; +]
21	c.6049delG p.R1997fsX13	19 / A3	1	+	S	
22	c.3756delG p.T1232fsX4	14 / B	NR	T (HIV)	S	
23	c.6919_6920delGA p.Q2287fsX77	26 / C2	NR	–	S	Mutation X77 > wtFVIII X46 !
24	c.5689_5690delCT p.A1877fsX3	17 / A3	NR	–	S	H[+], M[–/–] Neomutation*
25	c.1409delC p.G450fsX12	9 / A2	NR	+, –	F	
26	c.2490delC p.E810fsX15	14 / B	NR	+, –	F	
27	c.4388_4391delCTTT p.L1443fsX1	14 / B	NR	–	S	
In-frame Ins/Del (3 bp) 2/80						
28	c.435insTTT p.D126_K127insF	4 / A1	NR	– (HIV)	F	
29	c.2014_2016delTTC p.F659del	13 / A2	1	–	F	

*Origin, S: sporadic, F: familiar. NR: non-reported. +: Inhibitor present. -: Undetectable inhibitor. T: Transient antibody that disappears over a period of 6 months. HIV: Human Immunodeficiency Virus. VWF: Von Willebrand Factor. PL: phospholipids; H: hemophilic; M: mother. Only aminoacidic residue sequence differences are indicated in novel missense mutations. Human FVIII was aligned and compared with 6 homologous proteins (exact residue conservation is assumed by default): orthologous porcine; murine; canine FVIII; human factor V, huF5; human ceruloplasmin, huCe and human hephestin, huHe. Spurious stop codon (X) created by frameshifts mutations are predicted by sequence analysis. E.g., p.E810fsX15 indicate that a spurious stop codon is created 15 codons downstream E810 that is the site affected by the frameshift. Clearly out of the norm, c.6919_6920delGA predicts an extension of 77 codons from the frameshift at Q2287 that pass 31 aa through the normal stop codon. Wild type factor VIII, wtFVIII. Haplotype analysis was performed using 3 restriction fragment length polymorphisms (RFLP) [5] and 1 exonic single nucleotide polymorphism [16] in F8: [IVS18-BclI-RFLP(+/-); c.3864(A/C); IVS22-XbaI A-RFLP(+/-); IVS22-MspI A-RFLP(+/-)]. *Among 5 cases of isolated HA not caused by inversions (2 large deletions, 1 nonsense and 2 frameshifts), only case #24 showed the mutation in the proband but not in his mothers' peripheral leukocytes. This indicates a neomutation or a germinal mosaicism.*

T233-A200 hb (*data not shown*), the dissimilar phenotype may be explained by the disturbances introduced by a positively-charged residue versus a smaller-neutral one. p.W1942R is non-conservative, a practically inaccessible (FA, 0.009) large non-polar residue (W1942) part of S49 β-pleated sheet is replaced by a positively charged aa. Structural modelling of p.W1942R indicates a significant disturbance in the network of both hydrophobic and hb interactions (e.g., the formation of a new hb between M1988 and the most stable rotamer of mutated R1942) (*Supplementary Figure S1*). Interestingly, we found two novel missense mutations, p.L2297P and p.L2301S that afect L residues, located on nearby N- and C- ends of two neighboring β-strands within C1 (i.e., β-strands 2293-2297 and 2301-2314, *Supplementary Figure S1*). Although semi-conservative, p.L2297P (Pro is known to defuse secondary structure) disturbs the integrity of β-strand 2293-2297 and the set of hbs that L2297 normally keep up with D2298 and T2241 (*Supplementary Figure S1*). p.L2301S is non-conservative (non-polar>neutral-polar) and it may influence the interactions with spatial neighbors. By altering the structure of this region of the C2 domain, these two latter defects are thought to destabilize part of a proposed von Willebrand factor (VWF) and phospholipids surface (PLS) binding sites in FVIII (residues 2303-2332),[13] therefore, affecting FVIII/VWF and FVIII/PLS interactions. All recurrent missense and nonsense mutations in our series are associated with nucleotide transitions and most of them affected the hypermutable dinucleotide CpG. Notably, no nucleotide transversions leading to missense or nonsense defects were reported (Table 1A). c.209_212delTTGT was observed in two unrelated families and there were, therefore, linked to different F8-haplotypes (Table 1B). Remarkably, c.209_212delTTGT is repeatedly reported in

HAMSTeRS (6 entries). This recurrence may reveal the acquisition of a precise molecular mechanism. c.209_212delTTGT originated within the exonic sequence c.206_212TGTTTGT. The presence of micro direct repeats (GT…GT) and micro inverted repeats (TCT…AGA) flanking the target site of this mutation suggested the mechanism of slipped-mispairing for the generation of short deletions at the replication fork.[15] Software-based analysis indicated that the intronic transition c.602-32a→g decreases the score of the wild-type IVS4-acceptor splicing site and would create a competitor acceptor splicing site 32 bases upstream. Further *in silico* analysis showed that c.602-32a→g alters the binding pattern for splicing enhancer proteins of the SR family. Nevertheless the causal role of this mutation remains unclear. Regardless of its severe-HA causative status, investigation of c.602-32a→g allowed us to exclude the allele at-risk in the proband's sister and therefore diagnose her as non-carrier by indirect analysis. The absence of the 6 novel mutations affecting single aas and the potential IVS4-splicing mutation in a set of 100 unrelated X-chromosomes from our general population confirmed they were not common DNA polymorphisms. All new missense changes involve highly conserved residues in orthologous FVIII and moderately conserved in paralogous proteins (Table 1A).

FVIII inhibitors

A total of 17.6% of severe-HA patients (16/91) developed FVIII-antibody inhibitors. Large deletions involving more than 1 exon showed 71% of inhibitor risk (5/7); light-chain nonsense mutations, 1 out of 3; frameshifts, 31% (4/13); deletions of 1 exon, 1 out of 4; and Inv22, 12% (5/42). No inhibitors have been observed in 6 patients with heavy-chain nonsense mutations, 8 missense, 2 in-frame ins/del, 1 splicing mutation, 1 Inv1 and 4 uncharacterized small mutations. These data agree with most international reports.[17,18] Transient inhibitory antibodies (antibodies that disappeared over 6 months) were found in 11 patients (1 large-deletion, 1 frameshift, 2 nonsense and 7 Inv22). Also, we detected 4 out of 10 families with hemophiliacs presenting inhibitor status discordance (2 Inv22 and 2 frameshifts).

This represents the first comprehensive molecular series of severe-HA affected families in Argentina. A set of 16 novel *F8* gene small-defects were characterised using a cost-effective analysis scheme that improves the provision of secure information for genetic counseling. The early molecular *F8* diagnosis can offer the physician a prediction of probable side effects of replacement therapy, such as the risk of inhibitor formation.

Furthermore, the characterization of new HA causative mutations helps further out understanding of the relationship between genotype and the hemophilic phenotype.

Authors' Contributions

LCR: designed and performed research, and wrote the paper; CPR: performed research, wrote the paper, and final approval of the manuscript; MC: designed research, performed clinical evaluation of patients and final approval of the manuscript; RPB: performed research, performed clinical evaluation of patients and final approval of the manuscript; MTP: performed research, performed clinical evaluation of patients and final approval of the manuscript; AG: designed research, analyse data and wrote the paper; IBL: designed research, performed research and final approval of the manuscript; CDB: designed and performed research, analyse data and wrote the paper.

Conflict of Interest

The authors reported no potential conflicts of interest.

References

1. Gitschier J, Wood WI, Goralka TM, Wion KL, Chen EY, Eaton DH, et al. Characterisation of the human factor VIII gene. Nature 1984;312:326-30.
2. Lakich D, Kazazian HH, Antonarakis SE, Gitschier J. Inversions disrupting the factor VIII gene are common cause of severe. Haemophilia A. Nature Genet 1993; 5:236-41.
3. Naylor J, Brinke A, Hassock S, Green PM, Giannelli F. Characteristic mRNA abnormality found in half the patients with severe Haemophilia A is due to large inversions. Hum Molec Genet 1993;2:1773-8.
4. Bagnall RD, Waseem N, Green PM, Giannelli F. Recurrent inversion breaking intron 1 of the factor VIII gene is a frequent cause of severe Hemophilia A. Blood 2002;99:168-74.
5. De Brasi CD, Rossetti LC, Larripa IB. Rapid genotyping of XbaI and MspI DNA polymorphisms of the human factor VIII gene: estimation of their combined heterozygosity in the Argentinean population. Haematologica 2003;88:232-4.
6. Rossetti LC, Radic CP, Larripa IB, De Brasi CD. Genotyping the Hemophilia Inversion Hotspot by use of Inverse PCR. Clin Chem 2005;51:1154-8.
7. Williams IJ, Abuzenadah A, Winship PR, Preston FE, Dolan G, Wright J, Peake IR, Goodeve AC. Precise carrier diagnosis in families with Haemophilia A: Use of conformation sensitive gel electrophoresis for mutation screening and polymorphism analysis. Thromb Haemost 1998;79:723-6.
8. Den Dunnen JE, Antonarakis SE. Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. Hum Mutat 2000; 15:7-12.
9. Stoilova-McPhie S, Villoutreix BO, Mertens K, Kemball-Cook G, Holzenburg A. 3-Dimensional structure of membrane-bound coagulation factor VIII: modeling of the factor VIII heterodimer within a 3-dimensional density map derived by electron crystallography. Blood 2002;99:1215-23.
10. Guex N, Peitsch MC. SWISS-MODEL and the Swiss-PdbViewer: An environment for comparative protein modelling. Electrophoresis 1997;18:2714-23.
11. Antonarakis SE and a consortium of more than 50 international authors. FVIII gene inversions in severe haemophilia A: results of an international consortium study. Blood 1995; 86:2206-12.
12. Rossiter JP, Young M, Kimberland L, Hutter P, Ketterling RP, Gitschier J, et al. Factor VIII gene inversions causing severe hemophilia A originate almost exclusively in male germ cells. Hum Molec Genet 1994;3: 1035-9.
13. Graw J, Brackmann HH, Oldenburg J, Schneppenheim R, Spannagl M, Schwaab R. Haemophilia A: from mutation analysis to new therapies. Nat Rev Genet 2005;6:488-501.
14. Vehar GA, Keyt B, Eaton D, Rodriguez H, O'Brien DP, Rotblat F, et al. Structure of human factor VIII. Nature 1984; 312: 337-42.
15. Cooper N, Krawczack M. 1993. Human Gene Mutation. Bio Scientific Publishers.
16. Kemball-Cook G, Tuddenham EG, Wacey AI. The factor VIII structure and mutation resource site: HAMSTeRS version 4. Nucleic Acids Res 1998;1: 26:216-9.
17. Goodeve AC, Peake IR. The molecular basis of hemophilia A: genotype-phenotype relationships and inhibitor development. Semin Thromb Hemost 2003;29:23-30.
18. Oldenburg J, Pavlova A. Genetic Risk factors for Inhibitor to factors VIII and IX. Haemophilia 2006; 12:15-22.