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

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**ABSTRACT**

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, F8 still challenges mutation characterization 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)\(^2\) and intron 1 inversions (Inv1).\(^3\) 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.

**Protocol for F8-mutation characterization**

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

The Inv22 was investigated by Southern blot\(^2\) and by a novel approach based on inverse-PCR.\(^4\) The Inv1 was analysed by double-PCR.\(^4\)

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

**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 were studied. Informed consent was obtained in all cases.

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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. Novel aminoacid (aa) changes were examined for their conservation in murine, porcine and canine FVIII using the sequence alignment line-up (http://europe- um.esc.mrc.ac.uk/WebPages/Database/Protein/lineups.html) and the conservation in human factor V (CAB16748), Ceruloplasmin (BAU08084) 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.ncbi.nlm.nih.gov/GenoSplicer/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 assisted by Deep View/Swiss-PdbViewer software (ver.3.7).

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). 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. The Inv1 was found in one family, less than 2% of Inv1 carriers (15 of them belong to families with sporadic disease). 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 intron-splicing mutations; and in Table 1B: 11 ins/del in-frame, 1 shift (14%) and 2 ins/del in-frame.

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_EX15del (62.5kb), g.EX10_EX11del (4.4kb), g.EX5_EX26del (→15kb), g.EX23_EX26del (→30kb), g.EX2_EX12del (int1h-1_EX12, →55.6kb), 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. 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.

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).

Annotation of all 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; α1, α2 and α3, minor acidic peptides. The 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 p. numbering is based on F8 cDNA sequence (GenBank entry NM_000132.2), with nucleotide +1 corresponding to A of the ATG translation initiation codon.
Interestingly, we found two novel missense mutations, p.L2297P and p.L2301S that affect 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),

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 affect 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
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.9 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 aa 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.7,13 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; 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

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