The spectrum of mutations and molecular pathogenesis of hemophilia A in 181 Portuguese patients

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Disease-causing alterations within the *F8* gene were identified in 177 hemophilia A families of Portuguese origin. The spectrum of non-inversion *F8* mutations in 101 families included 67 different alterations, namely: 36 missense, 8 nonsense and 4 splice site mutations, as well as 19 insertions/deletions. Thirty-four of these mutations are novel. Molecular modeling allowed prediction of the conformational changes introduced by selected amino acid substitutions and their correlation with the patients' phenotypes. The relatively frequent, population-specific, missense mutations together with *de novo* alterations can lead to significant differences in the spectrum of *F8* mutations among different populations.

Key words: hemophilia A, molecular modeling, spectrum of mutations, splicing mutations, vWF.

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Factor VIII (FVIII), a plasma glycoprotein, is a crucial co-factor required by activated factor IX (FIXa) to activate factor X (FX) in blood coagulation. Deficiency of this co-factor results in the bleeding disorder hemophilia A (OMIM #306700), a typical X-chromosome linked recessive disorder that results in a bleeding diathesis.[1] Clinically, although the severity of bleeding correlates well with residual FVIII coagulant activity (FVIII:C), phenotypic variation can be observed between patients with identical FVIII:C levels or even between patients with the same genetic defect. However to date, F5 Leiden has been the only consistently reported genetic thrombotic risk factor to moderate the severity of hemophilia A.[2] With the exception of recurrent intron 22 (IVS22) and intron 1 (IVS1) inversions (resulting in severe disease) and frameshift mutations in the "6-9 consecutive As" hotspot sequence motifs in exon 14, the molecular basis of hemophilia A is characterized by a large number of heterogeneous family-specific mutations. Based on the residual circulating coagulant activity (FVIII:C) and antigen (FVIII:Ag) level, hemophilia A patients are classified into two main phenotypic groups termed as cross-reacting material-negative (CRM) and CRM-positive (CRM^+), and a subgroup of the latter denominated CRM-reduced (CRM^r).[1] Generally, CRM^- indicates that disease is due to a lack of circulating FVIII protein, while CRM^+ results from normal circulation of a dysfunctional protein. Within a hemophiliac population there is an equilibrium between the elimination of life-threatening mutations and a constant input of *de novo* alterations. Currently, hemophilia A is no longer regarded as a life-threatening disorder: therefore, elimination of mutations leading to severe hemophilia A may be expected to slow down. This may not be the case with certain FVIII alterations leading to inhibitory antibody formation, which is still a major life-threatening complication of hemophilia A. During the last two decades a large number of *F8* mutations have been reported and these are summarized in the HAMSTeRS *F8* mutation database (*http://europium.csc.mrc.ac.uk/*). Despite the large number of laboratories reporting *F8* genotypic data, identification of novel mutations, as is the case here, continues to add significant information regarding the structural-functional, genotype-phenotype correlations and contributes to a better understanding of the molecular pathology of hemophilia A.

Design and Methods

Blood samples were obtained from hemophilia A patients of Portuguese origin and their relatives following informed consent. FVIII:C was measured by the one-stage clotting assay, while FVIII:Ag was determined by ELISA (ASSERACHROM, Diagnostica Stago, Asnières, France). FVIII inhibitors were determined by the standard Bethesda method. Genomic DNA was extracted with the Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA). A total of 14 kb of the *F8* gene, including the entire coding sequence, exon-intron junctions and part of the 5' and 3' untranslated regions, were amplified by polymerase chain reaction (PCR). Most of the primers and amplification conditions have been previously reported;[3] primer sequences, annealing temperatures and the

size of PCR fragments are available on request. Single-strand conformation polymorphism analysis (SSCP) was performed essentially as described previously.[3] Presently, fragments are sequenced using the BigDye sequencing kit (Applied Biosystems, Foster City, CA, USA) and reaction products are separated on Applied Biosystems 377 PRISM automated sequencer according to the manufacturer's instructions. Screening for the *F8* IVS22 inversion is presently carried out by the long-distance sub-cycling-PCR method[4] while the recently reported *F8* IVS1 inversion was screened for by multiplex-PCR.[5] Isolation of Poly A^+ RNA and reverse transcript RT-PCR was performed as previously described.[6]

Whenever possible, family studies were performed in all available relatives by two independent methodologies (SSCP analysis, direct sequencing or restriction enzyme cleavage). One hundred and fifty healthy unrelated male individuals were used as controls. Haplotype analysis for three intragenic *F8* polymorphisms, the biallelic *Bcl*I within IVS18 and the two dinucleotide repeats: IVS13-(CA)16-24 and IVS22-(GT)n(AG)n, of the *F8* gene, was performed. The families were also screened for the *F5* Leiden mutation (R506Q) by a multiplex PCR-SSCP. Molecular modeling was carried out using the five-domain FVIII model[7] and the porcine FIXa model,[8] using InsightII software (Accelrys, Cambridge, UK) mounted on a Silicon Graphics Indigo graphics workstation (Reading, UK).

Results and Discussion

F8 IVS22 and IVS1 inversions

Of 135 unrelated severe hemophilia A patients (47% with sporadic hemophilia A), 73 (54%) had IVS22 inversions and two (1.5%) had an IVS1 inversion. Sixteen percent of the patients with an inversion developed inhibitory antibodies against FVIII, thus supporting the conclusion that *F8* inversions are not a major predisposing factor for this complication.

Missense and nonsense mutations

Forty-four different point (missense and nonsense) mutations were identified in 70 Portuguese families and are summarized in *Supplementary Table 1 and Figure 1, online version*. Eighteen of these are novel mutations and are discussed below. The C153F substitution reported here confirms the expected CRM^- status of alterations involving this structurally important residue. Replacement leads to disruption of a conserved disulphide bridge (C153-C179) in the A1 domain. The D163A substitution, identified in a patient with moderate hemophilia A, lies at the A1/A3 interface with H-bonds predicted to H2007. Replacement with the smaller Ala sidechain can be tolerated in the models but with loss of interdomain H-bonds. Although FVIII:Ag data from our patient are unavailable, we predict a CRM^- phenotype for this variant. K166 is a core residue close to the A1/A3 interface, and replacement with the Glu sidechain, in the K166E variant associated with mild hemophilia A, is sterically difficult due to clashes with S170 and/or K206. In the absence of a FVIII:Ag value we suggest this variant is likely to be CRM^-. The H281P substitution is an alteration identified in a patient with severe hemophilia A. The His residue is conserved in all FVIII and FV A1 domains, but not in A2, A3 or in CP: in the homologous region A3 has a short insertion sequence. H281 is placed at one end of the pseudo-threefold A1/A2/A3 axis at the A1/A2 interface and very close to A3. A single H-bond is predicted from the His sidechain to that of S524, forming another A1-A2 link adjacent to that predicted for R282-D525, already implicated in the stability of activated FVIII.[9] Replacement with the Pro sidechain can be sterically tolerated but the H-bond is lost. The Y431C substitution is associated with moderate to mild CRM^- hemophilia A. The Tyr residue is conserved in domain A2 of all species of FVIII, and related proteins FV and CP. Replacement with Cys is sterically allowed but illegitimate disulphide linkage is likely. This alteration, in family F35, was found in association with the recently reported polymorphic von Willebrand factor (vWF) R924Q variant.[10]

The novel Y511C substitution, found in eight apparently unrelated families, is one of the most frequently identified missense mutations in Portuguese patients with mild hemophilia A, having a clear CRM^- phenotype. *F8* haplotype analysis in these families indicates that this alteration was introduced in the Portuguese population by an ancestral founder effect. Y511 is an A2 core residue, packed closely by I463, F465, N474, I475, R484 and W513 (*Supplementary Figure 2, online version*). Replacement with Cys likely gives rise to steric clashes with I463 or W513 and there is also the possibility of illegitimate disulphide bond formation resulting in a low circulating FVIII:Ag level, although the protein that does circulate appears functionally normal.

The R698Q substitution leads to a phenotype similar to that of two different variants of the same residue reported in the database (FVIII:C/FVIII:Ag 44%/63%). R698 is conserved in the A2 domains of all FVIII species at the A2/A3 interface and mutation results in a mild functional defect and enhanced A2 domain dissociation.[11]

The R1696P and D1769G substitutions can be treated together since both affect residues which are predicted to interact through a H-bond, holding together two beta-strands on the surface of the A3 domain;[9] each might therefore lead to a similar hemophilia A phenotype. R1696P was identified in a patient with moderate hemophilia A but no FVIII:Ag value is available, while D1769G was identified in a moderately affected CRM^- patient. Replacement of R1696 with proline is predicted to abolish the H-bond, and introduce a steric clash with D1769 leading to misfolding and intracellular retention or instability. Variant A1701D was found in a patient with CRM^- severe hemophilia A. The A1701 sidechain lies in a small pocket near the A3 domain surface, enclosed by six A3 residues. The larger Asp sidechain cannot be accommodated due to clashes with Q1736, F1743, R1776 or Q1778 predicted to result in severe misfolding or poor stability of the FVIII variant.

R1721M is an alteration that was found in a patient with severe CRM^- hemophilia A. R1721 is a FVIII A3-specific residue which probably lies in an accessible surface loop close to the A1/A3 interface but distant from other predicted interactions, and is reported to be a FXa cleavage site (*Supplementary Figure 3, online version*).[12] The

replacement Met sidechain can be sterically accommodated so no reason for a CRM⁻ phenotype is apparent. This substitution, affecting the last amino acid of exon 14, also reduces the donor splice site consensus value (CV) from 0.83 to 0.70, most likely reducing *F8* pre-mRNA processing efficiency.

The D1740N alteration leads to moderate CRM⁺ hemophilia A with a dysfunctional FVIII variant circulating at mildly reduced protein levels. D1740 lies in an A3 surface loop on the FVIII model and the sidechain is likely to be solvent-exposed. Replacement with Asp creates an N-glycosylation signal (N-G-S) and it is therefore likely to be glycosylated. On inspection of a model of the membrane-bound FVIII/FIXa complex,[7,8] we predict that the functional defect results from interference with the FVIII-FIXa interaction on the membrane, since the glycosylated N1740 residue lies close to the predicted position of FIXa (*Supplementary Figure 4, online version*). H1755R was identified in a severely affected CRM⁻ hemophilia A patient. H1755 is completely conserved in the A3 domains of FVIII, FV and CP but not in A1 or A2. The residue lies in a cleft on the A3 surface distant from A1/A2 and FIXa but fairly close to the predicted position of C1.[7] Replacement with the larger Arg sidechain appears sterically forbidden due to clashes with L1752, E1754 and V1873.

The W2062C substitution in the C1 domain was identified in a patient with severe CRM⁻ hemophilia A. In the C1 homology model[7] the large Trp sidechain is completely buried, packed by S2040, A2047, P2153, S2160, T2064 and S2069. The smaller Cys sidechain can hypothetically be accommodated into the pocket, so the CRM⁻ phenotype probably results from illegitimate disulphide bonding leading to poor secretion or stability. We found two novel CRM⁻ substitutions in the C2 domain, Q2246K and G2285R. Q2246 is buried near the surface of the C2 domain and distant from the putative phospholipid interface, enclosed by L2210, H2211, F2290, P2292 and R2320 and H-bonded to main chain atoms. Replacement with the larger Lys sidechain may lead to a steric clash but this might be mitigated by local refolding in the region of F2290. This mutation is associated with a moderate rather than a severe secretion/stability defect.

G2285R is the third most frequent missense mutation in the Portuguese population. This alteration was also introduced by a founder effect. This residue is in a β-sheet environment near the domain surface, distant from putative interactions with other domains or the phospholipid interaction surface. The large Arg sidechain cannot be accommodated leading to severe steric clashes with T2245, V2257, K2258, P2260 and L2319, presumably resulting in misfolding and a fairly severe secretion/stability defect.

Eight nonsense mutations (novel K992X), were also identified in patients with severe CRM⁻ hemophilia A patients (*Supplementary Table 1, online version*). R583X, identified in a patient with sporadic hemophilia A, was the only case in which somatic mosaicism was observed in the patient's mother.

Insertions and deletions

Nineteen different insertions or deletions (14 of which are novel) were identified in 28 hemophilia A families of Portuguese origin (*Supplementary Table 2, online version*). The model of slipped mispairing during DNA replication can explain most of these insertions/deletions. The insertion:deletion ratio in runs of consecutive 6-9 As in *F8* exon 14, reported in the HAMSTeRS database (3:0 at codons 961-963 and 1080-1081; 19:5 at codons 1439-1441; and 12:33 at codons 1191-1194) suggests, in accordance with our data, that shorter runs are more insertion-prone while longer runs are more deletion-prone.

Unusually, three frameshift mutations were associated with a phenotype of only moderate severity. In two families, F1207 and F2614, the 1-nt deletion in the A^{9} sequence was associated with moderate hemophilia A.The third patient (F674) had an insertion in the A_{6} sequence at codons 961-964 and was also heterozygous for the *F5* Leiden mutation. The *F5* Leiden mutation may contribute to the moderate phenotype observed.

We report two novel in-frame 3-bp deletions (R1696del and E1970del) predicting the removal of single amino acids from the A3 domain of FVIII:C, both of which were unusually associated with circulating protein. Modeling in the vicinity of R1696 is tentative, however, this residue is located on a surface strand which could be shortened (*Supplementary Figure 5, online version*) suggesting that folding and secretion might be possible. The phenotype is CRMʳ with an inhibitor, indicating defective secretion of an inactive protein. The phenotype of E1970del indicates a very mild defect in secretion of a functionally normal protein. Recurrent prolonged bleeding after dental extraction was the only manifestation of the FVIII deficiency. E1970 is conserved in FVIII A3, probably at the A1/A3 interface in a short tripeptide insertion loop in comparison with the A2 and A3 domains, which suggests that the single residue could be deleted without significant structural consequences.

A novel large deletion of 4044bp, including the intron 13/exon 14 acceptor splice site was also identified (F1853) in a patient with CRM⁻ severe hemophilia A.

Splice site mutations

Four splice site mutations have also been identified (*Supplementary Table 3 and Figures 1 and 6, online version*), at IVS 6 and 11 donor splice sites (both novel) and IVS 4 and 24 acceptor splice sites. RT-PCR analysis of ectopically transcribed *F8* mRNA of the patient with the IVS24-1G→A splice site mutation (F2672) revealed skipping of exon 25 alone or, in approximately 30% of the processed *F8* mRNA, of exons 19 and 25 (*Supplementary Figure 6D*), predicting in-frame removal of the corresponding polypeptides. Alternative splicing of exon 19 in ectopic *F8* mRNA has been reported in normal control samples as well as in patients with undetected *F8* mutations.[13] The donor splice site mutation in IVS6 +1G→A results in skipping of surrounding exons 4-7 (*Supplementary Figure 6B, online version*) while the IVS11+1G>A, results in excision of exons 10 and 11 (*Supplementary Figure 6C, online version*).

Hemophilia A patients without an obvious causative F8 mutation

No obvious disease-causing *F8* mutation was found in three hemophilia A patients (approximately 1.5% of the

patient group) even after sequencing of the known functionally important *F8* gene regions (*Supplementary Table 4, online version*). This is in accordance with previously reported data[13,14] regarding the proportion of patients with undetectable mutations in the *F8* coding sequences.

The variant V1982V in exon 19 (F2832; *Supplementary Table 4*) was the only *F8* mutation identified in two daughters of a patient diagnosed with mild hemophilia A (FVIII:C= 6%). This *silent* (T→C) mutation at position +5 of exon 19 was not identified in either the male control group or in the group of hemophiliacs or relatives analyzed by SSCP. The CV of the IVS18 acceptor splice site is one of the lowest observed in the *F8* gene (0.72 vs. 0.87, the mean of the CV of all acceptor splice sites in the *F8* gene). A cryptic splice site is present at position +11 in exon 19 with a CV of 0.73, and the apparently silent mutation is located between these two sites. Taking together these data we cannot assume or exclude that this is a causative mutation. In this family the known von Willebrand's disease type 2N R816W substitution was also identified in the heterozygous state (*data not shown*). In this case, the identified vWF mutation alone is unlikely to explain the reduced FVIII:C level of 6% observed in the patient. Family 1377 included the only case of severe hemophilia A in which a disease-causing FVIII mutation was not identified.

Concluding remarks

In 177 of 181 families the relationship between the identified mutation and the FVIII deficiency was clearly demonstrated. Several missense mutations associated with mild to moderate hemophilia A (Y511C, Q2189E and G2285R) were introduced into the Portuguese population by an ancestral founder effect, and presently represent the molecular basis of hemophilia A in a considerable number of patients. These relatively frequent, population-specific, missense mutations together with the *de novo* alterations can lead to significant differences in the spectrum of *F8* mutations among different populations. The analytical sensitivity of the SSCP screening method in this study was near to 100%.

The heterogeneity observed at the mutational level is greatly simplified at phenotypic level. The large majority of mutations are associated with CRM^- hemophilia A, mostly due to misfolding and secretion/stability defects.

DD planned, developed and supervised the experimental work, and was the principal contributor to the collection, organization and analysis of the data and writing the manuscript. CV was the major contributor to the experimental work. IM conducted the screening of the F8 inversion mutations. MJD, MA, AT, FA, SM, MC were the major contributors to providing blood samples and phenotypic data from hemophiliacs. JL contributed to the initiation of the study and reviewed the manuscript. GK-C performed molecular modeling and contributed to writing the manuscript.

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