Hemophilia A: different phenotypes may be explained by multiple and variable effects of the causative mutation in the *F8* gene

Giancarlo Castaman

Center for Bleeding Disorders, Department of Oncology, Careggi University Hospital, Florence, Italy

E-mail: giancarlo.castaman@unifi.it

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In this issue of *Haematologica*, Donadon *et al.*[1] investigated at molecular level the effect of a mutation in the frequent *F8* gene (p.R2016W) in determining the circulating Factor VIII (FVIII) level in patients with hemophilia A carrying this missense mutation. The p.R2016W change was expressed and found to impair both FVIII secretion and activity. Furthermore, the nucleotide change (c.6046C>T) associated with the mutation also decreased the correct splicing at mRNA level, contributing to a further lowering of the already reduced FVIII level expected on the basis of impaired secretion and activity. Interestingly, other mutations clustered in the same area of p.R2016W displayed a variable proportion of these mechanisms, providing a good explanation for the genotype-phenotype relationships in patients with hemophilia A carrying these mutations.

Mutations in coding regions are usually thought to produce an altered biosynthesis or dysfunction of proteins. But in hemophilia this is usually suggested on the basis of phenotype characterization: using immunological assays in plasma to measure the concentration of the protein, or to measure protein activity by coagulation or chromogenic assays. Thus, it is generally assumed that null mutations (e.g. intron 22 inversion in hemophilia A, large gene deletions, stop codons, etc.) reduce or abolish the synthesis and/or the release of the protein while mutations altering the amino acid composition (missense mutations) may affect in particular the activity of the protein in addition to reducing biosynthesis. However, the combination of different mechanisms potentially associated with a given mutation has only very rarely been investigated at molecular level in hemophilia. There has been previous evidence to suggest that, indeed, missense mutations may have pleiotropic effects impairing not only biosynthesis and activity, but also mRNA properties, at least for some coagulation factors.[2,3] Donadon *et al.*[1] were able to quantitatively evaluate the pleiotropic effects of a nucleotide change at the RNA and protein (codon) levels. To do so, they chose the p.R2016W F8 mutation which is very frequent in Italy[4] and has been putatively suggested to induce also mRNA splicing impairment.[5] Very few hemophilia A missense mutations have so far been characterized because of the very low secretion efficiency of recombinant full-length FVIII. Donadon *et al.*[1] used a lentiviral-mediated delivery of expression cassette consisting of the codon-optimized FVIII cDNA lacking the B domain. By this means, they showed that the p.R2016W mutation impairs both FVIII secretion and function. Furthermore, they investigated the *F8* mRNA splicing pattern by using mRNA from patients' leukocytes. The mutated c.6046C>T associated with the mutation was found to alter splicing and to decrease the proportion of correct transcript to approximately 75% of wild type by inducing a variable degree of skipping of exon 19. Surprisingly, they found that several other missense changes in the same exon 19 (p.G2013R, p.E2018G, and p.N2038S) are responsible for variable splicing alterations, and that only the combination of altered RNA processing and abnormal protein biology produced a clinically relevant defect (see Figure 4 in Donadon *et al.*[1]). This is probably a general feature of several other hematologic disorders frequently associated with mutations that are defined simply as "missense" without the interplay among several molecular mechanisms producing the disease being known. However, the results obtained by Donadon *et al.*[1] suggest slightly milder hemophilia phenotypes than those observed in patients by clotting assays (see Table 1 in Donadon *et al.*[1]). It is tempting to speculate that other components could have a role in influencing the phenotype in addition to the mechanisms highlighted by Donadon *et al.*[1] For example, studies evaluating the half life of different missense mutations are very rare and yet these are needed in order to assess the impact of clearance mechanisms on these mutant proteins.[6] Thus, other studies should be carried out with other *F8* mutations to confirm that the experimental conditions used in this study are reliably reproducible and to compare the effects of the different pleiotropic mechanisms with other mutants located in different domains of the FVIII protein. Unfortunately, demonstrating the combination of different mechanisms can be very complicated, because expression studies are needed both at the recombinant protein and at RNA levels. Very few missense mutations among the thousands causing hemophilia A have been characterized for FVIII protein expression,[7-11] and this study makes a significant contribution to current knowledge in showing that it is possible to dissect the different molecular mechanisms influencing the phenotype, especially in mild and moderate hemophilia A. However, this is a demanding approach, particularly with FVIII mutants with very reduced synthesis and activity. Donadon *et al.*[1] exploited the technology for B-domainless FVIII, used to magnify FVIII expression for substitutive therapy of hemophilia A, to obtain a reliable estimate of the residual level of altered FVIII protein and function, to be combined with the partial negative effects of the RNA splicing defect. Thus, the combination of differentially altered mRNA processing and FVIII biosynthesis and co-factor activity has been shown to make a substantial contribution to variable significant FVIII deficiency caused by clustered variants generated by missense mutations in exon 19 of the *F8* gene.

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