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Blood Spotlight

The intron-22–inverted F8 locus permits factor VIII synthesis: explanation for low inhibitor risk and a role for pharmacogenomics

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Intron-22-inversion patients express the entire Factor VIII (FVIII)-amino-acid sequence intracellularly as 2 non-secreted polypeptides and have a positive "intracellular (I)-FVIII-CRM" status. Mutations conferring a positive I-FVIII-CRM status are associated with low inhibitor risk and are pharmacogenetically relevant because inhibitor risk may be affected by the nature of the therapeutic FVIII-protein (tFVIII), the affinity of any tFVIII-derived foreign peptide (tFVIII-fp) for any HLA class-II isomer (HLA-II) comprising individual major histocompatibility complex (MHC) repertoires, and the stability of any tFVIII-fp/HLA-II complex. We hypothesize that mutations conferring a completely or substantially negative I-FVIII-CRM status are pharmacogenetically irrelevant because inhibitor risk is high with any tFVIII and individual MHC repertoire. (*Blood*. 2015;125(2):223-228)

Introduction

The development of neutralizing antibodies ("inhibitors") to therapeutic Factor VIII (FVIII) proteins (tFVIIIs) is a serious complication of hemophilia A (HA) treatment, occurring in approximately 25% of patients with severe HA. Patient-, treatment-, and product-related variables contribute to inhibitor risk. The most salient patient-related determinant is the nature of the HA-causing FVIII gene (*F8*) mutation.[1-3] The highest inhibitor incidence, 68% to 88%, occurs in patients with large *F8* deletions, and the lowest (<10%) occurs in patients with missense mutations. This is consistent with the postulate that an endogenous FVIII polypeptide synthesized during immune system development can, through the induction of central tolerance, confer preexisting immunologic unresponsiveness to infused tFVIIIs. Patients with the intron-22-inversion (I22I) mutation[2] have no circulating endogenously synthesized FVIII; thus, this mutation might be expected to be associated with a high rate of inhibitor development, but in large studies, only ~20% of such subjects had developed inhibitors.[4] We recently discovered a plausible mechanism for this conundrum[5] and, in so doing, defined a new biomarker for the risk of immunogenicity of tFVIIIs, which we refer to as a patient's "intracellular-FVIII-cross-reactive material" (I-FVIII-CRM) status. Other mutation types, obviously including missense mutations, but possibly other mutations, may be associated with a positive I-FVIII-CRM status. This biomarker identifies patients who may have a preexisting tolerance to tFVIIIs, a group of patients for whom pharmacogenomics may be most helpful.

Does the effect of *F8* mutation type on endogenous FVIII synthesis underlie inhibitor risk?

As we alluded to before, the ability or inability of patients to induce and maintain tolerance toward their own (self-) FVIII protein underlies the well-established relationship between risk of developing inhibitors to tFVIIIs and underlying *F8* mutation type, where risk increases roughly with the degree to which the gene is disrupted structurally and/or functionally. The physiologic mechanisms of central and peripheral immune tolerance require endogenous protein synthesis. Individuals first become tolerized to the FVIII protein they express during lymphocyte development.[6] Patients with missense mutations are tolerant to their own endogenously expressed missense mutation–containing full-length FVIII (mm-$FVIII_{FL}$) proteins. These mm-$FVIII_{FL}$ proteins are variably secreted and/or dysfunctional because of the nature and location of their mutant residues, but all may differ from the tFVIIIs by only one amino acid.[7-10] Despite the existence of >980 distinct missense mutations, only ~80 of these mutations have been identified in patients with inhibitors (HADB, http://hadb.org.uk). Overall, inhibitors develop in <10% of all HA patients with missense mutations, although the risk of developing inhibitors can be very high with a specific subset comprising <10 mutations, in so called "hot spots."[11]

Although nonsynonymous single-nucleotide polymorphisms (ns-SNPs) allow the expression of fully functional endogenous FVIII proteins, in contrast to missense mutations, from an immunologic

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perspective they are akin in that they also encode proteins with amino acid sequences that differ from the sequences of the tFVIIIs at 1 to 3 residues.[12] Mismatches between the infused and endogenous sequences in an individual expressing one or more permissive HLA class-II (HLA-II) molecules can elicit an alloimmune response.[13] In our initial small study of African-American HA patients, those with the underlying black African-restricted haplotypes H3 and H4 were 3.6 times as likely to have developed inhibitors as were those with the underlying nonracially-restricted haplotypes H1 and H2, which were the same as those of white Europeans and those representing the recombinant-protein drugs currently approved for replacement therapy.[14] Given that 2 subsequent studies,[15,16] each with fewer African-descent subjects than in our initial study, did not confirm our observation, and because a third study is not yet complete, further investigation with a larger sample size is warranted. The third ongoing study has a larger sample of HA patients of African descent and it is hoped will be more definitive.[5]

Theoretically, exposures through blood transfusions and/or pregnancies to wild-type FVIII proteins that are allogeneically mismatched at residues encoded by nonsynonymous polymorphisms can elicit alloimmune responses that may crossreact with an individual's endogenously expressed FVIII and cause the autoimmune bleeding disorder–acquired HA.[17] Fortunately, this occurs very infrequently, presumably because any at-risk recipient or mother must have both a permissive HLA-II repertoire and a substantial exposure to the foreign FVIII molecule(s) in the presence of danger signals and other predisposing determinants.

Null-type *F8* defects that result in the absence of circulating FVIII activity and antigen include multiexonic deletions, which preclude endogenous expression of most (or all) of a FVIII protein, and nonsense mutations, which, depending on their location, can result in decreased cytoplasmic levels of the *F8* mRNA and/or can prevent translation of variable amounts of downstream-coding sequence (DCS). Transcription and/or translation of a full-length FVIII mRNA are prevented when the mutation is a deletion of an *F8* exon or a premature termination codon (PTC): in these instances, establishment of complete immunologic unresponsiveness to any tFVIII is prevented. There are, however, a few moderating considerations. Some large deletions and duplications occur in-frame[18] and the resultant mutant *F8* may direct synthesis of most of an endogenous FVIII protein. Similarly, with "exon skipping," the exons immediately flanking the exon bearing a PTC may be spliced together to generate a shorter but in-frame transcript. Both the deletion of in-frame exons and the exon-skipping mechanism allow for the production of somewhat smaller proteins that, although they are not secreted into plasma and/or are functional in coagulation, are still likely to produce immunologic unresponsiveness to corresponding sequences of the infused tFVIIIs.[19,20]

The *F8* intron-22 inversion

The I22I mutation, which causes ~40% to 45% of all cases of severe HA,[1-3] results in truncation of the wild-type *F8* transcription unit (Figure 1A) and inversion of exons 1 to 22 toward the telomere of the X-chromosome long arm (Figure 1B).[21] Transcription of the I22-inverted *F8* locus followed by primary mRNA processing yields a polyadenylated fusion transcript, $F8_{I22I}$, containing FVIII exons 1 to 22 spliced to 2 cryptic (C) exons,[21] referred to as Exons 23_C and 24_C (Figure 1B).[5] Exon-23_C contains 16 in-frame codons followed by an in-frame stop codon and 38 bp of untranslated sequence. Because this stop codon is situated <50 to 55 nucleotides upstream of the 3′-most exon-exon junction,[21] the fusion transcript is not predicted to trigger nonsense-mediated mRNA decay.[20] We recently demonstrated that $F8_{I22I}$ is translated, and it generates a protein with the 19-residue wild-type FVIII leader-peptide, the first 2124 amino acids of the mature FVIII protein, and 16 C-terminal non-FVIII amino acids encoded by exon-23_C (ie, a fusion protein [$FVIII_{I22I}$] containing 2159 amino acids).[5]

Because the I22I arises through a homologous recombination event that restores completely the sequence within which it occurs—which includes the promoter and first exon of the nested $F8_B$ gene—transcription and translation of $F8_B$ is expected to be unaffected (ie, wild type).[5,22] The $F8_B$ locus encodes a widely expressed, moderately abundant 2.6-kb transcript with exons 23 to 26 of *F8* spliced in-frame to an unrelated first exon that has a Kozak's consensus initiation codon.[22] The $F8_B$ mRNA is predicted to encode a 216-amino-acid protein, $FVIII_B$, corresponding to exons 23 to 26 in *F8*. We have now identified the $FVIII_{I22I}$ and $FVIII_B$ proteins in peripheral blood mononuclear cells (PBMCs) and liver sections from patients with I22I.[5] Between them, these polypeptides contain the entire coding sequence for a full-length FVIII protein.

The last base of exon 22 corresponds to the third nucleotide of translated codon 2143, which encodes residue 2124 of the mature protein. Moreover, the first base of exon 23 corresponds to the first nucleotide of adjacent codon 2144, which encodes residue 2125 of the mature protein (Figure 2). Thus, with the I22I, truncation of *F8* after exon 22 does not split a codon and every FVIII amino acid residue should be expressed.[5]

Results from quantitative real-time reverse transcriptase polymerase chain reaction–based assays showed that the cytoplasmic levels of the $F8_{I22I}$ mRNA are similar to those of the full-length wild-type *F8* mRNA.[5] The $F8_B$ transcript in I22I patients was also found to be present at levels similar to those of the $F8_B$ mRNA in persons without hemophilia.[5] Because the $FVIII_{I22I}$ and $FVIII_B$ proteins are found to be expressed in liver samples and PBMCs from HA patients with the I22I at similar levels to the $FVIII_{FL}$ and $FVIII_B$ proteins in normal persons,[5] it is reasonable to postulate that these polypeptides provide central tolerance even though expression of these antigens in thymic tissue has not yet been demonstrated. There is a promiscuous expression of tissue-restricted self-antigens by medullary thymic epithelial cells. Thus, antigens expressed in the thymus mirror those of all tissues in the body.[23,24] Recent transgenic mouse studies indicate that endothelial cells may be the only cell type capable of secreting their intracellularly-expressed FVIII protein. However, secretion of proteins may not be necessary to begin the process of tolerance induction. There are at least 2 documented mechanisms by which endogenously expressed intracellular polypeptides can be presented by the major histocompatibility complex (MHC)-II system, the type of antigen presentation necessary for inducing tolerance in $CD4^+$ T cells. In 1 mechanism, peptides from endogenously expressed proteins can be displayed on antigen-presenting cell (APC) surfaces, bound to MHC-II molecules, through the cross-presentation process.[25] In the other mechanism, the intracellularly expressed polypeptides are delivered to the MHC-II loading compartments of APCs in the form of apoptotic bodies that arise as a function of cellular senescence or targeted killing by cytolytic cells.[26]

However, peptides that are encoded by the nucleotide sequence spanning the exon 22/exon 23 junction (Met2124-Val2125) cannot be generated by patients with the I22I mutation (Figure 2). Fourteen 15-mer overlapping peptides could potentially be recognized as "foreign" if derived from the infused wild-type FVIII protein and presented by a patient's repertoire of HLA-II receptors. However, not

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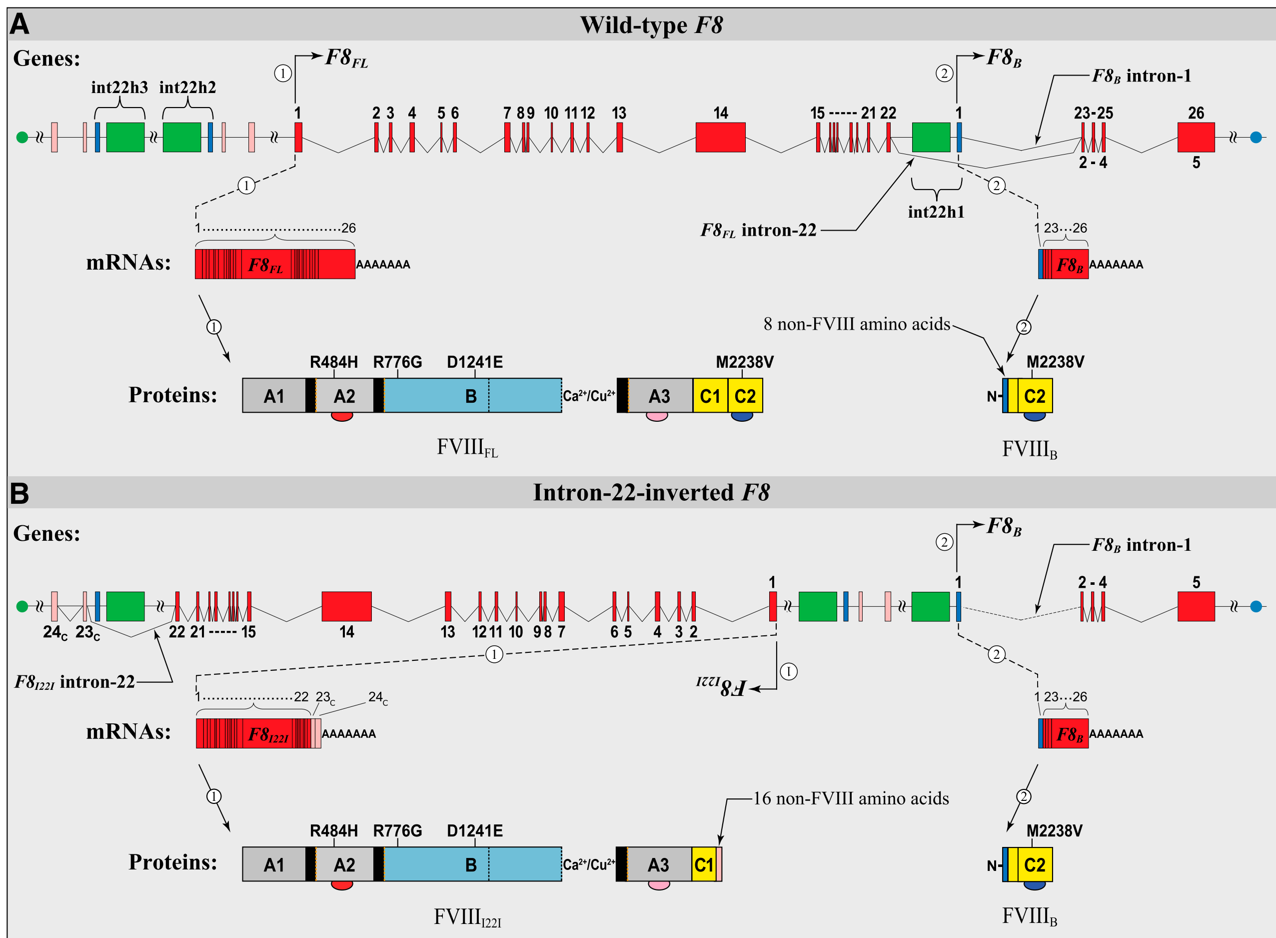

Figure 1. Structure and function of the wild-type and I22-inverted *F8* loci: DNA, mRNA, and protein. (A) The wild-type *F8* locus is comprised of 2 separately regulated transcription units that direct synthesis of 2 *F8*-exonic-sequence–containing polyadenylated transcript variants, 1 and 2, which are designated here as $F8_{FL}$ and $F8_B$, respectively. $F8_{FL}$ contains all 9030 bases found in *F8* exons 1 to 26 and encodes the full-length FVIII protein, $FVIII_{FL}$, whose mature circulating form, containing 2332 amino acid residues, is essential for normal blood coagulation. $F8_B$ contains 2598 bases —including 169 bases from its first exon, which is not found in $F8_{FL}$, and 2429 bases from its last 4 exons (exons 2-5), which are identical to exons 23 to 26 of $F8_{FL}$—and encodes wild-type $FVIII_B$, a recently identified intracellular protein comprised of 216 amino acids whose function, if any, remains unknown. Homologous recombination between *int22h1* and *int22h3* (depicted as green and dark blue rectangles, respectively) incompletely inverts $F8_{FL}$. (B) The I22-inverted *F8* locus is also comprised of 2 separately regulated transcription units that direct synthesis of 2 *F8*-exonic-sequence–containing polyadenylated transcript variants, 1 and 2, which are designated here as $F8_{I22I}$ and $F8_B$, respectively. Because homologous recombination restores the genomic DNA sequence within which it occurs, it has long been suspected,[2] but only recently proven experimentally,[5] that the $F8_B$ gene and transcript, and hence the $FVIII_B$ protein, are identical to that in healthy persons without HA. $F8_{I22I}$ contains the 6756 bases found in *F8* exons 1 to 22 and, together with the 48 additional DCS bases in exon 23_C, encodes the $FVIII_{I22I}$ protein, which contains 2159 translated amino acid residues and is trapped intracellularly.

all foreign peptides elicit an immune response. A critical determinant for T cell–dependent immunization is the affinity with which any foreign peptide(s) derived from the infused protein binds to the distinct HLA-II repertoire on the surface of the patient's APCs.[29] Figure 2C illustrates the population-level "promiscuity scores," a synthesis of (1) the frequencies of common (prevalence ≥0.5%) HLA-II variants in the specified populations, and (2) the predicted binding affinities of all overlapping FVIII peptides between positions 2090 and 2165 to those HLA-II variants. The set of overlapping peptides encoded by the nucleotide sequence spanning the exon 22/exon 23 junction (15 amino acids upstream and downstream of the green dotted line, Figure 2C) do not bind with high affinity to most of these HLA-II alleles. This analysis is supported by the clinical observation that although 15 individual missense mutations have been described in the region of the exon 22/exon 23 junction, none of these HA patients developed inhibitors (HADB, http://hadb.org.uk). The promiscuity scores also show that, farther upstream and downstream of the junction, there are regions where peptides bind with high affinity to many of these common HLA-II variants (Figure 2C). Note that these regions include amino acid positions 2105 and 2150, which correspond to sites of the highly recurrent mild HA-causing missense mutations Y2105C and R2150H, associated with a high inhibitor prevalence of 50% and 16%, respectively.[11,27]

We and others have wondered why the inhibitor rate in patients with the I22I is as high as ~20% if almost all of the entire normal FVIII protein is present intracellularly. Although at the population level, the peptides encoded by the exon 22/exon 23 junction do not bind strongly (Kd <50 nM) to the most common HLA-II variants, the individual HLA-II repertoire of some patients binds with intermediate affinity (Kd <500 nM). Because all I22I patients had severe HA and were probably transfused heavily, in contrast to patients with missense mutations, the vast majority of whom have mild HA, the intermediate affinity of the junction peptides could be responsible for the immune response observed in some patients.

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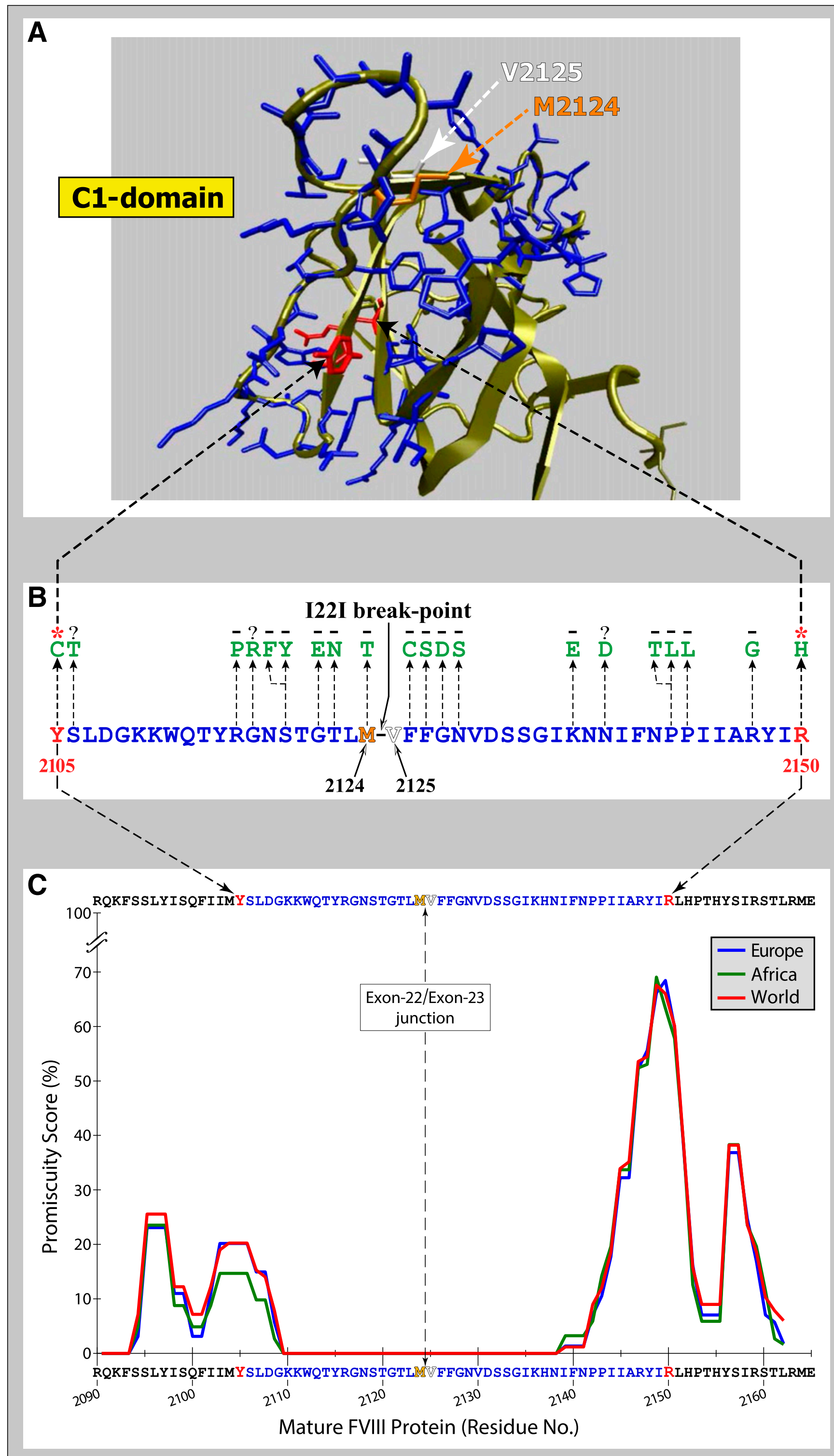

Figure 2. Peptides from the Exon 22/Exon 23 junction and their affinity for common MHC-II variants. (A) Ribbon diagram of the structure of the C1 domain of FVIII highlighted to emphasize the I22I break point encoded by the 3′- and 5′-ends of exons 22 and 23, respectively. Y2105 and R2150 (red) represent the positions of recurrent mild HA-causing missense mutations that are strongly associated with inhibitor development. Residues 2106 to 2123 and 2126 to 2149 (blue) are 2 segments of C1 on either side of the inversion break point. M2124 and V2125 (orange and white, respectively) are the residues flanking the break point. (B) Forty-six amino acids comprising most of the C-terminal one-third of the C1 domain are shown together with the location of known HA-causing missense mutations; the presence (red asterisk) or absence (black minus sign) of inhibitor development in patients found to have one of these abnormalities with these is also indicated. Note that Y2105C and R2150H have been found in many unrelated alloimmunized HA patients and represent the N- and C-terminal missense mutations, respectively, closest to the exon 22/exon 23 junction, which have been identified in inhibitor patients. Although 18 additional missense mutations (green) have been identified more proximal than Y2105C and R2150H to the I22I break point, none of these patients has yet been reported to have developed inhibitors to date. (C) The immunogenicity potential of wild-type tFVIII-derived peptides encoded by the mRNA sequence spanning the exon 22/exon 23 junction is depicted as promiscuity scores.[5,27] The binding affinities of commonly-occurring HLA-II proteins for peptides derived from the C1 domain region corresponding to the exon 22/exon 23 junction were predicted using the NetMHCIIpan-3.0 method (http://www.cbs.dtu.dk/services/NetMHCIIpan/[28]). The method predicts the binding affinity (in nM) for each 15-mer peptide–HLA-II complex. The population-level promiscuity scores for each 15-mer FVIII peptide is then defined as the (normalized) cumulative prevalence of common HLA-II proteins that bind it with high affinity (≤50 nM) in 3 populations: white European (blue), black African (green), and Global (red).

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Intracellular FVIII cross-reacting material

I-FVIII-CRM appears to be a major determinant of immunologic tolerance to tFVIIIs in HA patients. We propose grouping *F8* mutations into either “pharmacogenetically relevant” or “pharmacogenetically irrelevant” categories to guide inhibitor prediction and mitigation. Pharmacogenetics is the study of differences in individual responses to the same drug as a result of genetic variations in the patient population. Patients with pharmacogenetically relevant *F8* mutations would thus be presumed to have immune tolerance to most FVIII proteins, and their risk of developing inhibitors may be related to such variables as the degree to which infused FVIII matches their endogenous FVIII, immune system differences that are highly patient-specific, and patterns of exposures to tFVIII.

Patients with pharmacogenetically irrelevant mutations would be presumed to have little or no tolerance to FVIII such that all FVIII replacement proteins would be risky in the setting of a permissive immune system and the necessary exposure level.

Currently available recombinant FVIII products represent only 2 of the 8 or more naturally occurring haplotypes of the human FVIII protein that result from combinations of several *F8* ns-SNPs.[12,14] We hypothesize that for HA patients with pharmacogenetically relevant mutations, a FVIII replacement product whose amino acid sequence is identical to that of the patient's premutation haplotype may have lower immunogenicity potential than a mismatched FVIII product.

Patients with pharmacogenetically relevant mutations express most or all of the FVIII protein, whether secreted into the plasma or trapped in intracellular compartments. Because HLA-II variants vary greatly in their affinity for a given peptide, the probability of an alloimmune response developing in a patient with a specific pharmacogenetically relevant mutation will depend in part on whether any of the mismatched peptides derived from the infused FVIII protein can bind to the HLA-II isomers presented by the patient.[27]

Future directions

There has been significant improvement in the quality of FVIII replacement products over the last 2 decades, and numerous bioengineered coagulation factors are in the drug development pipeline.[30,31] Currently, FVIII replacement products are dispensed interchangeably with the premise that each is equally suitable for all recipients. Until recently, there has been little deliberation on the effects of engineered differences in the amino acid sequence of the FVIII product on inhibitor risk. We wonder whether a B domain–deleted tFVIII would be safer for a patient with an in-frame multiexonic deletion involving exon 14. Results from ongoing studies may foster the development of recombinant FVIII products with varied primary sequences that have an improved safety profile for specific populations.

A personalized strategy is not only important for the choice of a FVIII treatment protein but also for the choice of a specific *F8* for gene therapy.

Promiscuity scores help us understand, on a population level, the predicted inhibitor burden of a specific tFVIII product. For the care of the individual patient, however, specific combination of *F8* mutation, FVIII haplotype, HLA-II repertoire, and possibly other immune system variants will have to be weighed.

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Authorship

Contribution: Z.E.S., C.Y., and T.E.H. conducted the research leading to these concepts; Z.E.S., C.K.K., and T.E.H. wrote the manuscript; and J.N.L., C.Y., and T.N. edited the manuscript and contributed critical commentary.

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