Disorders of Hemostasis

Coagulation factor V gene analysis in five Indian patients: identification of three novel small deletions

Congenital factor V (FV) deficiency is a rare coagulopathy associated with moderate to severe bleeding symptoms. A total of 34 mutations, all located in the FV gene (*F5*), have been described in patients with severe FV deficiency, only eight of them being of Asian descent. Sequencing of *F5* in five unrelated Indian patients identified three novel small deletions in exon 13, all present in the homozygous state (g.50936-50937delAA or AG and g.51660delA, both occurring in two different patients, and g.52162delC). Besides widening the knowledge on the mutational spectrum of FV deficiency in Asian populations, these data will also be useful for purposes of prenatal diagnosis.

Haematologica 2006; 91:1724-1726
(*http://www.haematologica.org/journal/2006/12/1724.html*)

Coagulation factor V (FV) is a 330-kDa single-chain plasmatic pro-co-factor, with an A1-A2-B-A3-C1-C2 domain structure. FV is converted into its active form (FVa) by α-thrombin or activated factor X (FXa) through proteolytic removal of the large B domain. Once activated, FVa acts as an essential co-factor for the FXa-catalyzed activation of prothrombin, whereas it is rapidly inactivated by activated protein C, thus limiting thrombin generation.[1,2] The FV gene (*F5*) has been mapped to chromosome 1q24.2.[3] Defects in this gene result in either an autosomal dominant form of thrombophilia (activated protein C resistance) or in a hemorrhagic diathesis with an autosomal recessive mode of inheritance [severe or moderately severe FV deficiency; Online Mendelian Inheritance in Man (OMIM)[4] +227400].

The estimated prevalence of severe FV deficiency in the general population is one in one million; the disorder is less rare in populations in which consanguineous marriages are traditionally frequent, such as those from Muslim countries and Southern India.[5,6] FV-deficient patients have a lifelong hemorrhagic diathesis of variable severity, due to the complete absence or extremely low levels of both functional and immunoreactive plasma FV.[5]

Molecular analysis of *F5* in affected individuals has led to the identification of 34 causative mutations.[7-9] Mutations introducing premature termination codons (PTC) account for two thirds of the total; most of them are located in the large exon 13 (six deletions and four nonsense mutations). So far, only ten molecular defects, including the recurrent Tyr1702Cys mutation, have been described in patients of Asian origin (three probands were from China, while one each was from Japan, Korea, Iran, Taiwan, and from a not-specified area of South Asia).[7,9] This sparse information makes it impossible to demonstrate a correlation between genetic defects and the ethnicity or the geographical distribution of the patients, thus hampering the possibility to design a *population-specific* strategy for genetic/prenatal diagnosis of the disorder. To address this issue, a cohort of five unrelated patients with FV deficiency from Northern India was analyzed.

All patients, whose main clinical details are summarized in Table 1, had very low plasma antigen FV levels and did not suffer from any additional concomitant coagulation disorder. No bleeding history was reported in any of the probands' relatives. Genomic DNA of each patient was extracted from blood samples according to standard procedures. All 25 *F5* exons, including exon-intron boundaries and about 300 bp of the promoter region, were amplified by polymerase chain reaction (PCR) under standard conditions from genomic DNA. Sequencing, which was carried out as described elsewhere,[10] revealed three small deletions in exon 13, each present in the homozygous state (Table 1). In particular: (i) in patients A and B, a deletion of two nucleotides was found at genomic positions 50,936-50,937 (g.50936-50937delAA or AG), giving rise to a frameshift (introducing three aberrant residues) that is predicted to encode for a severely truncated polypeptide chain because of the presence of a PTC.

The predicted mutant mature FV molecule would be composed of only 834 amino acids (FS835ter). Since the nucleotide position 50,937 is polymorphic (A or G; rs4524), it is impossible to establish unambiguously whether the deletion involves an AA or an AG dinucleotide; (ii) a single bp deletion, involving the nucleotide at genomic position 51,660 (g.51660delA) was identified in both patients C and D. This frameshift mutation would lead to a premature stop at codon 1142 (FS1142ter), preceded by an abnormal and long stretch of 72 amino acids resulting from the translation of the putative mutant FV mRNA in a different reading frame; (iii) in patient E, a single C deletion (within a double C) was found at nucleotide position 52,162 (g.52162delC). This mutation would predict the synthesis of a truncated protein (FS1259ter), which shows a novel aberrant sequence of 21 residues at the C-terminus as a consequence of the frameshift.

In all cases, these three newly identified molecular defects lead to the introduction of a PTC, making them a

Table 1. Clinical and genetic characteristics of the analyzed FV-deficient Indian patients.

*Patient**	*A*	*B*	*C*	*D*	*E*
Sex	M	M	M	M	M
Present age (y)	28	30	n.a.	30	16
Consanguinity	No	No	No	Yes°	No
Main symptoms	Gum, nose, and joint bleeds	n.a.	Gum and nose bleeds	Hematomas	Gum and nose bleeds
FV:Ag (%)#	2.4	2.4	2.9	2.9	<1
Mutation†					
Status Genomic††	Homozygous g.50936-50937 delAA or AG	Homozygous g.50936-50937 delAA or AG	Homozygous g.51660delA	Homozygous g.51660delA	Homozygous g.52162delC
cDNA†††	c.2662-2663 delAA or AG	c.2662-2663 delAA or AG	c.3386delA	c.3386delA	c.3888delC
Mature protein	FS835ter	FS835ter	FS1142ter	FS1142ter	FS1259ter

** Informed consent was obtained from all examined patients; the study was approved by the Institutional Review Board of the University of Milan. °Unknown degree of consanguinity. #FV antigen levels (FV:Ag) were determined using an in-house developed sandwich enzyme immunoassay (EIA), based on a sheep anti-human polyclonal antibody (Affinity Biologicals, Hamilton, ON, Canada). FV levels were expressed as percentage of control plasma pooled from 40 normal individuals, set as 100% (normal range: 64-139%; sensitivity of the test:1%). † Primer pairs for mutation detection were designed from flanking intronic sequences (GenBank, accession number Z99572) and their sequences are available on request. ††Numbering according to GenBank, accession number Z99572, inverted and complemented. †††Numbering according to GenBank accession number M16967. n.a.: not available; g.: genomic sequence; c.: cDNA sequence; FS: frameshift.*

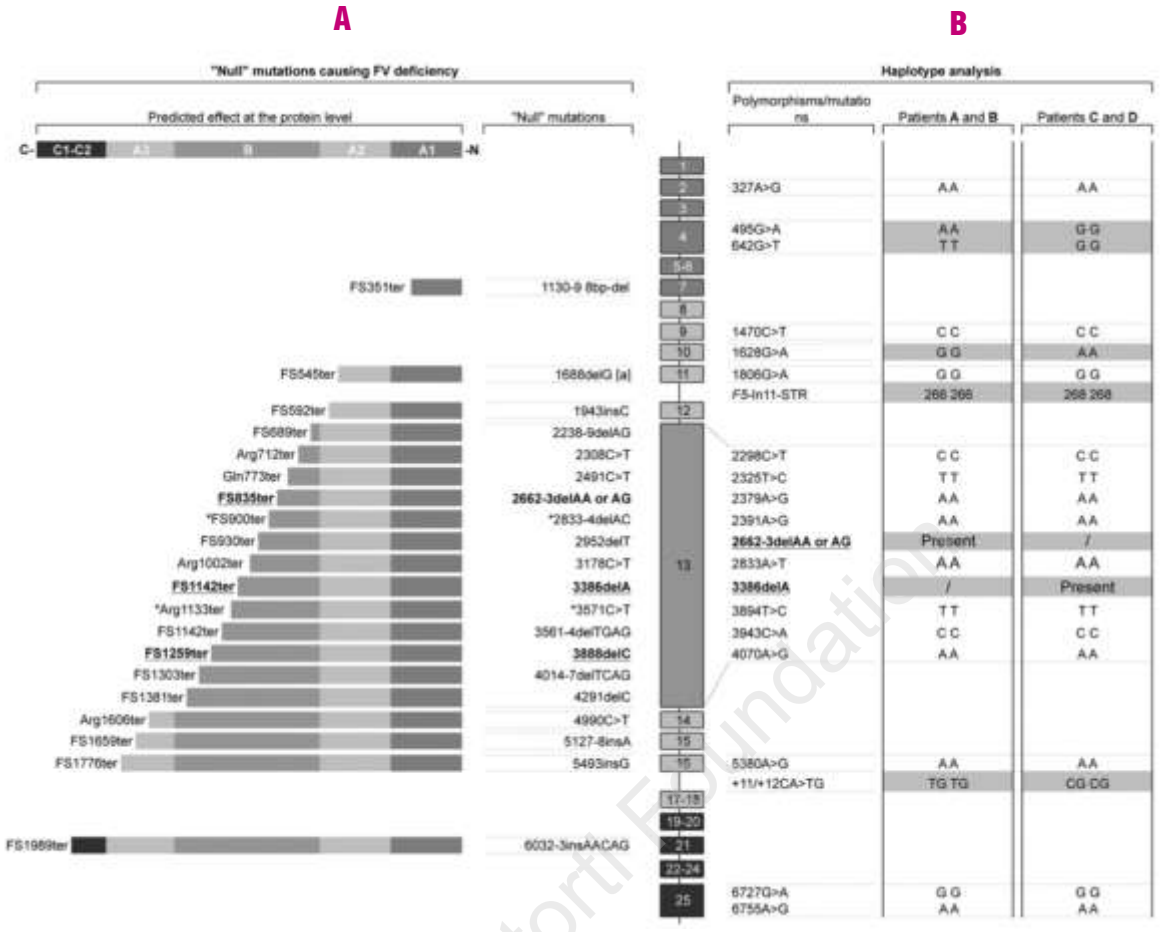

Figure 1. *Null* mutations in severe FV deficiency and haplotype analysis of patients A, B, C, and D. A. *Null* mutations leading to severe FV deficiency are listed according to both their position within *F5* and their predicted effect at the protein level. FV domain structure is indicated in the upper left part of the figure: each domain is represent by a gray box, and is drawn approximately to scale. The structure of the *F5* gene is indicated on the right: exons and introns are represented by boxes and lines, respectively, and are not drawn to scale. Mutations are named on the basis of their predicted effect at the protein level (numbering omitting the signal peptide), and also on the basis of cDNA position (numbering according to GenBank accession number M16967). Only fully-published ins/del and nonsense mutations are reported. Although often leading to PTC, splicing mutations were omitted due to the lack of studies confirming their predicted effects at the protein level. Mutations described in this paper are in bold and underlined; mutations demonstrated to be associated with the degradation of the corresponding mRNA are marked with an asterisk.[7] [a] This nucleotide deletion is indeed located in exon 10 but the resulting frameshift produce a PTC at residue 545, in exon 11. B. Polymorphisms used in haplotype construction, as well as mutations identified in patients A, B, C, and D, are listed on the left according to their position within *F5*. Genotypes are indicated on the right. Gray shading indicates genotypes marking differences between the two identified haplotypes. Biallelic marker genotypes were obtained during the mutational screening performed by sequencing; PCR amplification of the microsatellite located in intron 11[12] was performed using the primer couple *F5*-In11-STR-F-5'-CCAGTCCAAAGACTTGGTGATG-3', and *F5*-In11-STR-R-5'-TTGCCTTCTTCTGACCTAGCC-3' (labeled with Hex), under standard conditions. Allele sizing was accomplished by using an ABI-3100 Genetic Analyzer and Genescan 3.1 software (Applied Biosystems, Foster City, CA, USA). Alleles were labeled according to the genotype calls of the software.

possible trigger for the nonsense-mediated mRNA decay pathway (NMD). Indeed, when investigated at the mRNA level, PTC-causing mutations in *F5* were always demonstrated to induce the selective degradation of the corresponding transcript;[7] should the three PTC-carrying transcripts escape the NMD surveillance system, the encoded proteins would lack part of the B domain and the complete light chain (corresponding to the A3-C1-C2 domains) (Figure 1A). In order to check for the existence of a common ancestor, haplotype analysis for polymorphic markers covering the whole *F5* (18 biallelic[10,11] and one microsatellite in intron 11)[12] was performed in patients carrying the same mutation (g.50936-50937delAA or AG in patients A and B; g.51660delA in patients C and D). All patients were homozygous for each marker (Figure 1B).

In particular, patients A and B shared the same haplotype, suggesting that they had a common ancestor. The same conclusions could be drawn for patients C and D, who both carry the same haplotype, differing by five markers (spread all over *F5*, from exon 2 to intron 16) from the previous one. However, these results are also compatible with the presence of a heterozygous large deletion, leading to the PCR amplification of only one *F5* allele. Unfortunately, the most straightforward approach to test the patients for hemizygosity (i.e. by genotyping their parents) could not be adopted, due to the unavailability of DNA samples from the patients' relatives. Knowledge on the existence of founder effects for specific mutations can be useful to lower costs and speed up procedures for the molecular diagnosis of the disease and, whenever morally warranted by the severity of symptoms, for its prevention through prenatal diagnosis. This is especially true for those countries in which the

disorder is highly prevalent (high level of inbreeding) and resources (facilities and budget) are not abundant.

In this frame, considering that FV deficiency is characterized by a high level of allelic heterogeneity,[7] the identification of two homozygous mutations in four Indian families with FV deficiency, might suggest the presence of prevalent mutations in this population. Nonetheless, the identification of founder effects requires the genetic characterization of large cohorts of patients, an important task for future genetic studies in Asian populations.

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Funding: R.A. is a recipient of a Bayer Hemophilia Early Career Investigator Award 2005. The financial support of Telethon – Italy (Grant n. GGP030261) and of PRIN (Programmi di Ricerca Scientifica di Rilevante Interesse Nazionale, Grant n. 2005058307_002) is gratefully acknowledged.

Acknowledgments: The authors thank Paolo Motta and Roberta Marotta for excellent technical assistance.

Key words: coagulation factor V, factor V deficiency, gene analysis, mutation.

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