Evaluation of factor V mRNA to define the residual factor V expression levels in severe factor V deficiency

We evaluated FV mRNA in severe factor V deficiency caused by the -12T/A IVS18 mutation, activating a cryptic splice site and leading to premature translation termination. Quantitative evaluation of factor V cDNA from homozygous and heterozygous subjects, and correction for nonsense mediated decay, suggested the presence of 0.1% of normal factor V mRNA.

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Patients with severe factor V (FV) deficiency have been found to be homozygous for FV gene small deletions, nonsense and missense mutations,[1] or substitutions affecting invariant bases in the consensus splicing sequences,[2-4] which indicate that very low FV levels are compatible with life. On the other hand, the absence of extended deletions, which cannot be corrected by ribosomal slippage or somatic reversion events, might indicate that traces of FV activity are also essential in humans. The contribution of maternal FV and early replacement therapy, which could rescue null mutations by permitting development in utero, live birth and survival, should also be evaluated.

Evaluation of FV mRNA in homozygotes for mutations impairing but not abolishing correct RNA maturation would help determine residual FV expression by exploiting very sensitive amplification assays for nucleic acids.

We studied FV mRNA in a chronic patient (FV:C <1%) who suffered from gastrointestinal bleeding at the age of 5 months. He has since experienced easy bruising and gum, joint and muscle bleeding. At 11 years old, intracranial bleeding, required surgical intervention. Fresh frozen plasma or, in the last three years, virus inactivated plasma (Octapharma) has been administered on demand. The presence of FV Leiden, FVH1299R and FII20210G/A, potential phenotype modulators, was excluded. This study has been approved by the Bratislava University Hospital committee on human experimentation and informed consent has been obtained from the patient and parents.

Nucleotide sequencing of FV exons and splicing junctions[5] confirmed the patient to be homozygous for the novel -12T/A transversion in the IVS18 polypyrimidine tract (Figure 1A), a crucial region to define the acceptor splice site (3'ss). The mutation creates a cryptic 3'ss with scores (0.99, Neural Network Prediction Tool; 0.85, SpliceView program) overlapping with those of the downstream canonical 3'ss (0.99; 0.86). RT-PCR and sequencing of the FV mRNA region spanning exons 16-20 clearly confirmed the presence of the altered FV transcript bearing the 10 bp insertion in the patient's leukocytes (Figure 1A-B). The inferred frameshift and premature termination of translation at codon 1883 would completely remove the C1 and C2 FV domains.

To investigate whether the homozygous condition for the -12 T/A mutation in FV IVS18 were compatible with residual normal splicing, thus producing wt mRNA, we used fluorescent labeling of RT-PCR products and denaturing capillary electrophoresis (ABI-3100) for quantitative evaluation. Two FV cDNA fragments (primers 2F-2R, Figure 2A or 1F-2R, Figure 2B) were studied. Study of a

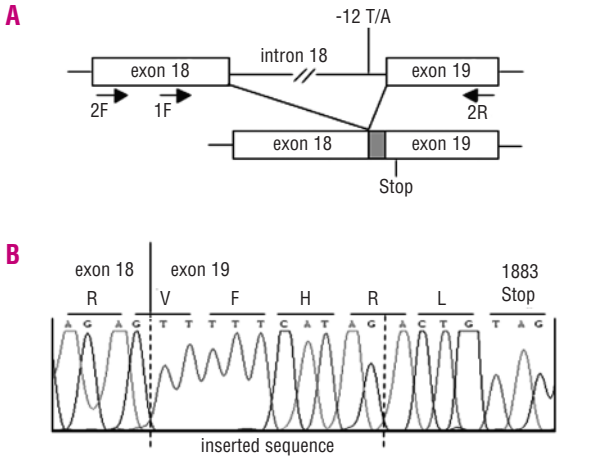

Figure 1. Mutated DNA and cDNA region bearing the -12 T/A transversion in intron 18 (A). The following primers (arrows) were used for RT-PCR: 1F (5'-GAAAACCAGAGAGCAGGG-3'), 2F (5'-TAAAACTCTTGAAATGAAGGCA-3'), 2R (5'-FAM-CTCTGAAGCCTTGATCTGTG-3'). Grey rectangle: 10 bp insertion. Sequencing of the propositus cDNA showing the 10 bp insertion, together with the predicted frameshift and premature stop codon (B).

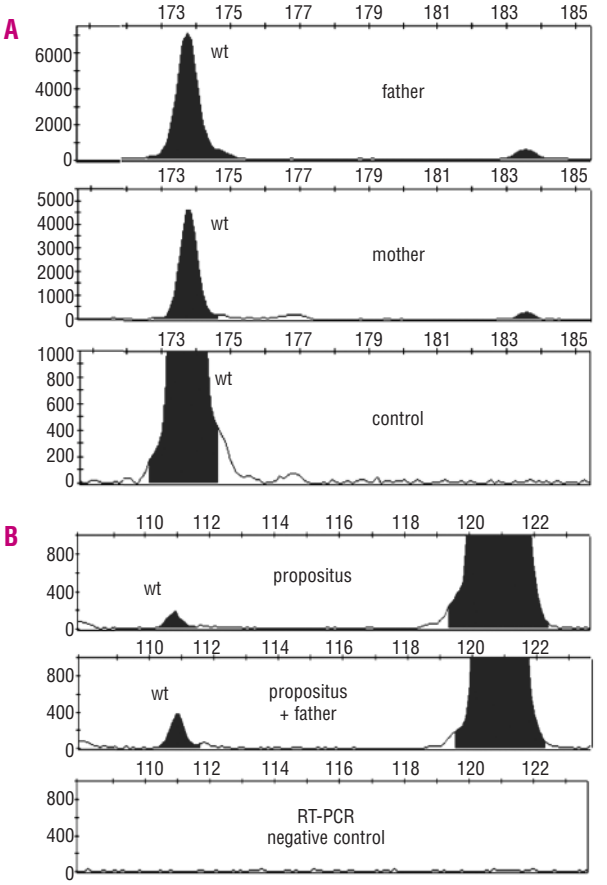

Figure 2 Analysis of the relative abundance of normal (wt) and aberrant cDNAs in a normal control and in -12T/A IVS18 carriers (A), and in the propositus (B). The fluorescence peaks were obtained with primers 2F-2R (A) or primers 1F-2R (B). 1µl of 1:10 diluted (A) or 5µl (B, upper and lower panels) RT-PCR products were loaded. Patient cDNA was also spiked (B, middle panel) with a small amount (~1000 relative fluorescence units, RFU) of RT-PCR products from his father. The size of RT-PCR fragments (X-axis, nucleotides) and fluorescence intensity (Y-axis, RFU) are reported.

control subject (Figure 2A) showed the correct mRNA form only, indicating that the alternative splice site is not used in the absence of the -12 T/A mutation. The heterozygous condition for the -12T/A transversion, detected in both of the propositus' parents, was used to establish the relative amount of normal and altered mRNA forms. In 5 independent assays, the ratio between normal and altered cDNA was 10.7±2.7 in the father and 14.2±1.1 in the mother, suggesting selective degradation of the aberrant transcript through nonsense mediated mRNA decay (NMD) caused by the insertion of a premature nonsense codon.[6] Consequently, the amount of altered mRNA is relatively decreased by one order of magnitude, assuming similar transcription rates of normal and mutated alleles.

The patient's -12T/A homozygous condition allowed us to study the use of the normal 3'ss. The peak compatible with the normal cDNA (114bp) could only be seen in the patient (Figure 2B) after electrophoresis of an increased concentration (20-50X) of the RT-PCR products. The presence of normal cDNA was further supported by the overlapping migration of peaks from the propositus and his father. In particular, mixing experiments confirmed a single peak at the wt position (Figure 2B).

The estimated ratio between the normal and altered cDNA forms was 0.010±0.001 and 0.013±0.001 for the 1F-2R (Figure 2B) and 2F-2R (*not shown*) fragments respectively.

This indicated that the canonical 3'ss was not used efficiently by the spliceosome machinery, leading to barely detectable amounts of normal cDNA. The preferential use of the newly created 3'ss, located upstream to the canonical one and possessing a comparable consensus score, might underlie a mechanism of oriented scanning[7] also in the recognition of the 3'ss.

Evaluation of the patient's normal splicing activity was made easier by the fact that since experiments conducted in both parents indicate that NMD decreases the altered FV mRNA amount by one order of magnitude, the initial ratio between normal and altered mRNA levels should be approximately 0.001 (0.01/10). This indirect estimate would suggest a residual FV expression around 0.1% of normal. This is consistent with simulations in plasma,[8] suggesting that 0.1% (0.02 nM) FV would produce detectable levels of thrombin, and along with observations in transgenic mice, indicating that FV levels <0.1% (<0.09 nM) would be sufficient to support postnatal survival.[9]

This is the first study to date demonstrating residual levels of FV expression in the presence of a homozygous splicing mutation. Although other FV splicing mutations have been characterized,[2-4] the levels of wt FV expression and their association with the degree of FV deficiency have not been reported.

We exploited the presence of a novel FV gene mutation, able to impair splicing while leaving the normal splice site intact, and the combined data from the homozygous and heterozygous conditions, to estimate the relative amounts of normal and altered cDNA forms in leukocytes. Information was obtained, first, about NMD affecting the altered mRNA, and second, about the residual capability of the splicing machinery to produce normal mRNA from the altered gene transcript. Although we did not prove that the estimated mRNA levels give rise to proportional FV activity levels, in patient plasma barely detectable by functional assays, we believe that our findings contribute to quantitative evaluation of residual FV expression in severe FV deficiency.

Barbara Lunghi,[1] Mirko Pinotti,[1] Iva Maestri,[2] Angelika Batorova,[3] and Francesco Bernardi[1]

[1]Department of Biochemistry and Molecular Biology, Ferrara University, Italy; [2]Department of Experimental and Diagnostic Medicine, Section of Anatomic Pathology, Ferrara University, Italy; [3]The National Hemophilia Center, Institute of Hematology and Blood Transfusion, University Hospital, Bratislava, Slovakia

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Correspondence: Francesco Bernardi, via Fossato di Mortara 74, I-44100, Ferrara, Italy. Phone: international +39.0532.974425. Fax: international +39.0532 974484. E-mail: ber@unife.it

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