Disorders of Hemostasis

Rescue of activated protein C-resistance phenotype by *cis*-acting factor V Gly2032Asp mutation

A 60-year-old Italian woman presenting with factor V_{Leiden} mutation but a normal activated protein C (APC) resistance, low functional and antigenic factor (FV) plasma levels, was found to have a novel heterozygous Gly2032Asp substitution located on the same allele. In transfected cells, the Gly2032Asp mutation caused an approximately 2-fold reduction of the intracellular FV protein and a 9-fold reduction of the secreted protein, suggesting that the Gly2032Asp substitution acts *in cis* on the allele carrying the FV_{Leiden} mutation and rescues the APC-resistance phenotype.

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A common mutation (Arg506Gln) within the factor V (FV) gene (FV_{Leiden} mutation) is the most frequent cause of inherited thrombophilia. Several mutations within the FV gene have been reported to be associated with FV deficiency, a rare bleeding disorder that follows an autosomal recessive inheritance.[1,2] The *in trans* association of mutations causing FV deficiency with the FV_{Leiden} mutation leads to *pseudohomozygous APC-resistance*, a condition characterized by an APC-resistance phenotype similar to that of FV_{Leiden} homozygotes.[3-5] We reported the case of a 60-year old Italian woman with a recent retinal vein thrombosis and diabetes mellitus in whom the FV_{Leiden} mutation was not associated with an altered response to the APC-resistance test. APC-resistance assays performed using Staclot APC-R (Diagnostica Stago, Asnières, France) and both undiluted and diluted test plasma in FV-depleted plasma gave similar results. No further thrombophilic risk factor was found. There was no family history of thrombotic episodes.

FV activity was measured in a one-stage clotting assay (Thromborel S, Behringwerke AG, Marburg, Germany) and a FV-deficient plasma (International Laboratories, Milan, Italy). FV antigen was measured by an enzyme immunoassay (EIA) using a polyclonal antibody (Affinity Biologicals Inc, Hamilton, Canada) and a normal plasma pool as standard. Reduced levels, expressed as percentage of values measured in pooled normal plasma, of both FV activity (normal range: 70-130%) and FV antigen (normal range: 60-140%) were detected only in the patient and in relatives carrying the FV_{Leiden}mutation (Figure 1).

Direct DNA sequencing of the entire coding region of the FV gene showed a heterozygous G⟶A transition in exon 22 at cDNA position 6269 (numbered according to GenBank accession number M16967), leading to a Gly to Asp (GGT⟶GAT) substitution at amino acid position 2032 (numbering omits the signal peptide) (Figure 1). The same mutation was found in the heterozygous state in both relatives (II-6 and III-1) who had reduced FV levels, but not in 57 control subjects. No gene variants contributing to the APC-resistance phenotype, such as the HR2 haplotype and the FV Cambridge mutation (Arg306Thr), were found.

Haplotypes constructed using informative polymorphisms suggested that the FV_{Leiden} and the Gly2032Asp mutations are inherited in the same haplotype together with an adenine at cDNA position 327 in exon 2 and a cytosine at cDNA position 3943 in exon 13 (Figure 1). Site-directed mutagenesis of pMT2/FV plasmid using mutagenic primers (5'-GATTGGAACTGCAAGATTGTGAGGTAA-

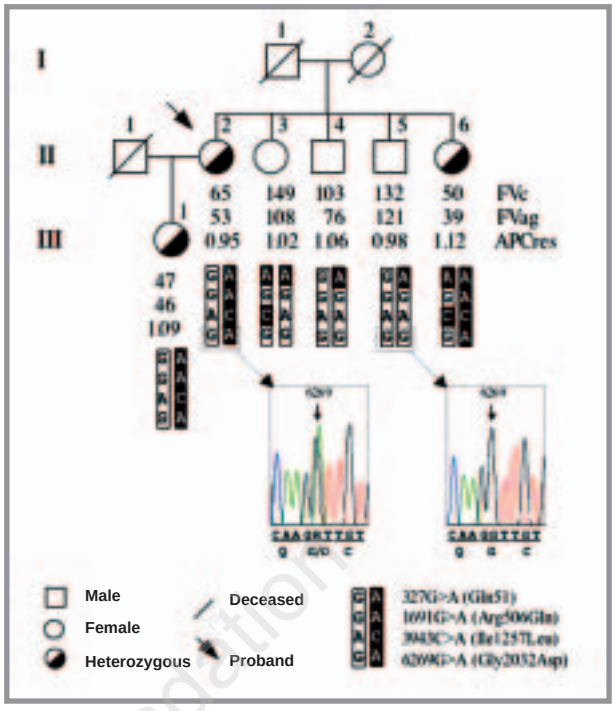

Figure 1. Pedigree of the FV-deficient family, FV coagulant, FV antigen, and APCres values, haplotype analysis and electropherograms showing the identified missense mutation. Haplotypes of the proband and of her relatives constructed with four FV nucleotide variations are shown below each symbol (the first and the third refer to previously reported polymorphisms, the second corresponds to the Leiden mutation, while the fourth represents the newly identified missense mutation described here). Sequence electropherograms of the region surrounding the mutation (whose position is indicated by an arrow) are reported for individuals II-2 (heterozygous for the 6269G>A transition) and II-5 (wild type); R = G or A; the predicted translations of the nucleotide sequences are also reported (one letter code).

ATGG-3' and 5'-CCATTTACCTCACAATCTTGCAGTTCCAATC-3' nucleotide positions 6254-6284 according to GenBank accession number M16967), were performed to replace Gly2032 with an Asp codon (QuickChange Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA, USA). COS-1 cells were cultured and transfected using the Lipofectamine 2000 reagent (Invitrogen). FV antigen levels were measured 72 hours after transfection in conditioned media and cell lysates by EIA.

The level of FV antigen in media conditioned by COS-1 cells expressing the wild-type construct ranged from 520 ng/mL to 665 ng/mL. An approximately 9-fold reduction in FV antigen concentrations was recorded in conditioned media of cells transfected with pMT2/FV-Gly2032Asp plasmid (Figure 2). Co-transfection of wild-type and mutant FV cDNAs gave rise to extracellular FV antigen levels of about 50% of the wild-type (Figure 2). In lysates of cells expressing the mutant allele, FV antigen levels were reduced to approximately 50% of those measured in cells expressing the wild-type allele (Figure 2), whereas in cells co-transfected with both constructs a partial reduction of about 25% was observed.

At variance with previous reports,[6,7] the present work describes the first *in cis* association of the FV_{Leiden} mutation with a missense mutation. The Gly2032 residue is located

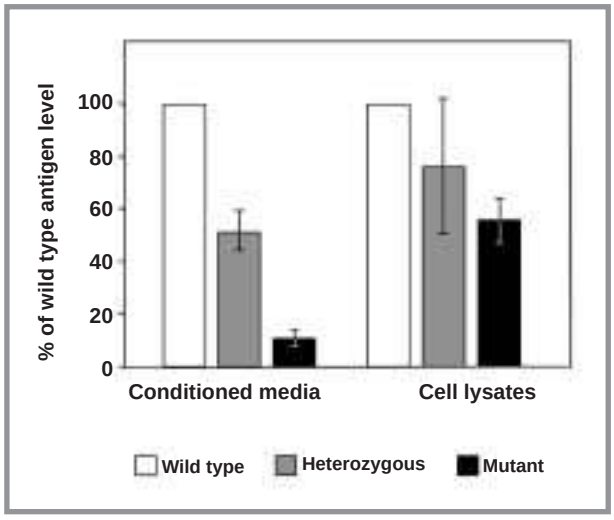

Figure 2. Transient expression of wild-type and mutant FV in COS-1 cells. pMT2/FV (wild-type), pMT2/FV-Gly2032Asp (mutant) or equimolar amounts of both plasmids (heterozygous condition) were transiently transfected in COS-1 cells. Antigen levels of recombinant FV were measured in both conditioned media and in the corresponding cell lysates by an EIA assay, 72 hours after transfection. Bars represent means ± standard deviation of two independent experiments, each performed in duplicate. The mean value of wild-type FV is set as 100%.

in the last β strand (b8) of the C1 domain,[8] a highly-conserved region of this domain and is conserved in the corresponding positions of human factor V (position 2192) and factor VIII (position 2325) C2 domains. A mutation of factor VIII residue Gly2325 to Cys or Ser was previously reported in patients with moderate to severe hemophilia.[9,10] It is conceivable that the substitution of a tiny non-polar amino acid with a charged one may induce a change in the structure of the C1 domain and, in turn, affect the correct folding of the whole FV molecule leading to intracellular degradation of the mutant protein.

The co-existence of both the Gly2032Asp and the Leiden mutations on the same chromosome has the effect of *cis*-acting rescue of the APC-resistance phenotype. Since the missense mutation *in-cis* with the Leiden mutation gives a normal APC resistance, the proband's retinal vein thrombosis cannot be explained by the FV_{Leiden} mutation. This condition, which is the exact counterpart of the *pseudo-homozygous* APC-resistance phenotype, is another possible cause of discrepancy between phenotype and genotype for the FV_{Leiden}.

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References

1. Lane DA, Mannucci PM, Bauer KA, Bertina RM, Bochkov NP, Boulyjenkov V, et al. Inherited thrombophilia. Part 1. Thromb Haemost 1996;76:651-62.
2. Peyvandi F, Duga S, Akhavan S, Mannucci PM. Rare coagulation deficiencies. Haemophilia 2002;8:308-21.
3. Zehnder JL, Jain M. Recurrent thrombosis due to compound heterozygosity for factor V Leiden and factor V deficiency. Blood Coagul Fibrinolysis 1996;7:361-2.
4. Castaman G, Lunghi B, Missiaglia E, Bernardi F, Rodeghiero F. Phenotypic homozygous activated protein C resistance associated with compound heterozygosity for Arg506Gln (factor V Leiden) and His1299Arg substitutions in factor V. Br J Haematol 1997;99:257-61.
5. Guasch JF, Lensen RP, Bertina RM. Molecular characterization of a type I quantitative factor V deficiency in a thrombosis patient that is "pseudo homozygous" for activated protein C resistance. Thromb Haemost 1997;77:252-7.
6. van Wijk R, Montefusco MC, Duga S, Asselta R, van Solinge W, Malcovati M, et al. Coexistence of a novel homozygous nonsense mutation in exon 13 of the factor V gene with the homozygous Leiden mutation in two unrelated patients with severe factor V deficiency. Br J Haematol 2001;114:871-4.
7. Dargaud Y, Trzeciak MC, Meunier S, Angei C, Pellechia D, Negrier C, Vinciguerra C. Two novel factor V null mutations associated with activated protein C resistance phenotype/genotype discrepancy. Br J Haematol 2003;123:342-5.
8. Pellequer JL, Gale AJ, Griffin JH, Getzoff ED. Homology models of the C domains of blood coagulation factors V and VIII: a proposed membrane binding mode for FV and FVIII C2 domains. Blood Cells Mol Dis 1998;24:448-61.
9. Becker J, Schwaab R, Moller-Taube A, Schwaab U, Schmidt W, Brackmann HH, et al. Characterization of the factor VIII defect in 147 patients with sporadic hemophilia A: family studies indicate a mutation type-dependent sex ratio of mutation frequencies. Am J Hum Genet 1996;58:657-70.
10. Akkarapatumwong V, Oranwiroon S, Pung-amritt P, Treesucon A, Thanootarakul P, Veerakul G, et al. Mutations of the factor VIII gene in Thai hemophilia A patients. Hum Mutat 2000;15:117-8.