The factor V (FV) gene ASP79HIS polymorphism modulates FV plasma levels and affects the activated protein C resistance phenotype in the presence of the FV Leiden mutation

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Background and Objectives. In carriers of the factor V (FV) Leiden mutation, different trans-acting gene variants (HR2 haplotype and FV Cambridge mutation) affect activated protein C (APC) sensitivity. Among a series of FV gene variants characterized, the Asp79His polymorphism appeared to be a good candidate for the modulation of FV activity.

Design and Methods. In a group of 150 apparently healthy subjects without the FV Leiden mutation and in 55 apparently healthy subjects with mutation, genotypes of the Asp79His polymorphism and of the HR haplotype were characterized and plasma levels of FV coagulant activity and APC ratios evaluated.

Results. In the group without the FV Leiden mutation, 16 subjects (10.7%) carried the His 79 allele and 15 subjects (10.0%) the HR2 haplotype. Two of them carried both gene variants. As compared to FV activity levels in non-carriers (106.4+18.5%), values were lower in subjects with the His79 allele (95.2+25.2%; p=0.025) and in those with the HR2 haplotype (93.7+16.2%; p =0.007). FV activity levels were further reduced in carriers of both FV gene variants (78.7+3.3%; p =0.009). APC values were similar among individuals carrying different FV genotypes. In the group with the FV Leiden mutation, APC ratios were lower in subjects carrying the His 79 allele (0.63; p =0.008) or the HR2 haplotype (0.63; p =0.026) than in subjects without (0.69), reflecting FV activity values.

Interpretation and Conclusions. Present data suggest that carriership of the His79 allele modulates plasma levels of FV coagulant activity and, in subjects carrying the FV Leiden mutation, affects APC sensitivity.

Key words: FV gene, polymorphism, antigen, activated protein C resistance

Haematologica 2003; 88:286-289
http://www.haematologica.org/2003_03/88286.htm

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Coagulation factor V (FV) is a 330 kDa single-chain pro-cofactor essential for the function of the blood coagulation pathway.[1] After activation by thrombin, the B domain is excised by proteolysis and two chains are formed, one containing the A1 and A2 domains (heavy chain) and the other the A3, C1, and C2 domains (light chain).[2] Activated FV shares a common domain structure with activated coagulation factor VIII, displaying approximately 40% of identity in heavy and light chains.[3]

Genetic factors were found to account for about 45% of the between-individual variance of FV plasma levels.[4]

A series of polymorphisms (HR2 haplotype), strictly related to each other, have been reported within the FV gene. The HR2 haplotype has been consistently associated with reduced FV plasma levels.[5-6] Carriership of the HR2 haplotype has been associated with increased resistance to activated protein C (APC) both in normal subjects and in thrombophilic patients, independently of the FV Leiden mutation.[7] The co-inheritance of HR2 and FV Leiden mutations determines a higher degree of functional resistance to APC than that observed in FV Leiden heterozygotes.[7] Carriers of the HR2 haplotype have an imbalance between two glycosylated and functionally different isoforms of plasma FV.[8,9] The mechanisms by which the HR2 haplotype favors reduced FV plasma levels would, in this case, be due to the imbalance between the two different isoforms of plasma FV.

Recently, a new polymorphism (G409C) leading to an Asp to His substitution at position 79 has been described within exon 3 of the FV gene.[10] This gene variation occurs within the A1 domain of the heavy chain and it is conceivable that it could affect FV phenotypic expression. We investigated whether the Asp79His polymorphism of the FV gene locus contributes to the modulation of FV plasma levels and, in turn, affects the APC phenotype.

Design and Methods

After approval from the local Ethics Committee, the study was carried out according to the Principles of the Declaration of Helsinki; informed consent was obtained from all the subjects.

Subjects. One hundred and fifty apparently healthy subjects (63 men and 87 women; median age 36.0 years, range 22-65) randomly selected from a Southern Italian general population[11] without a history of hemorrhages were investigated. As an inclusion criterion, none of

them carried the FV Leiden mutation. An additional group of 55 apparently healthy subjects (25 men and 30 women) known to carry the FV Leiden mutation was investigated. Acquired conditions known to affect the APC resistance assay, such as oral anticoagulation, factor VIII levels, lupus anticoagulant, and pregnancy were excluded.

Blood collection and coagulation tests. Blood samples were collected into vacuum plastic tubes containing 3.8% trisodium citrate and centrifuged at 2,000 g for 15 min to obtain platelet-poor plasma. A modified APC resistance assay was performed in FV-depleted plasma using Staclot APC-R (Diagnostica Stago, Asnières, France).[12] Results were expressed as a normalized ratio, defined as the APC of the sample to the APC of normal plasma obtained in the same run. FV activity was measured in a one-stage clotting assay using Thromborel S (Behringwerke AG, Marburg, Germany) and a FV-deficient plasma (IL, Milan, Italy).

DNA extraction and analysis. Isolation of DNA and polymerase chain reaction (PCR) analysis were done according to standard procedures.[10] A 220-bp DNA fragment of the factor V gene that includes the nucleotide 1691, was amplified and digested with Mnl I, as previously described,[13] with some modifications.[14] The FV HR2 haplotype was detected using the method described by Bernardi *et al.*[7]

Amplifications of the FV exon 3 containing the Asp79His polymorphic site were achieved using sense and antisense oligonucleotides designed on the basis of known sequences of the FV gene (Genbank accession numbers L32757). The oligonucleotide custom synthesis service was from Life Technologies (Paisley, UK). Primer sequences were 5'-TGACCCTGAATACAGACATAG-3' (sense: 6-26) and the mutated 5'-ATGGATGCTCAAGGGCTGAT-3'(antisense: 136-127). PCR was carried out on 50 μL volume samples, in a 480 Perkin Elmer-Cetus thermal cycler (Perkin-Elmer Cetus, Norwalk, CN, USA). Each sample contained 0.2 μg of genomic DNA, 25 pmoles of each primer, 200 μM of dNTP, 5 mM Tris HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 1 U Taq polymerase. The solution was overlaid with 50 μL of mineral oil and, after an initial denaturation step (3 min at 95°C), it was put through 30 cycles each consisting of 1.5 min at 94°C, 40 sec at 56°C and 2.0 min at 72°C. For the screening of the G-to-C transversion using the mutated antisense primer, which abolishes a Mbo I restricton site, 20 μL of the 131bp PCR products were digested with 3 units of Mbo I (New England Biolabs Inc., Beverly, MA, USA) at 37°C for 4 hours, according to the manufacturer's recommendations. Fragments were separated in a 2.0% agarose gel.

Statistical analysis. All the analyses were performed using the Statistical Package for Social Science (SPSS 6.1 for Macintosh). Means and standard deviations (SD) are presented. The significance of any difference in means was evaluated by non-parametric tests, whereas the significance of any difference in proportions was tested by χ^2 statistics. The allele frequencies were estimated by gene counting, and genotypes were scored. The observed numbers of different Asp79His genotypes and HR haplotypes were compared with those expected for a population in Hardy-Weinberg equilibrium using a χ^2 test. Statistical significance was taken as $p<0.05$.

Results

Asp79His polymorphism in normal subjects

In this general population without the FV Leiden mutation, 16 subjects (10.7%) carried a histidine at position 79 (Table 1). None of the individuals studied was homozygous for the His79 allele. The frequency of the His79 allele was 5.3%, not at variance with that previously reported.[10] The HR2 haplotype was found in 15 subjects (10.0%), all heterozygotes (Table 1). The figures observed were consistent with those predicted by the Hardy-Weinberg equilibrium. Among the subjects investigated, 2 carried both the His79 allele and the HR2 haplotype (Table 1).

The mean plasma levels of FV coagulant activity are reported in Table 1. As indicated, mean FV plasma concentrations were significantly lower both in carriers of the His79 allele (p=0.025, Mann-Whitney exact test) and in those of the HR2 haplotype (p=0.007, Mann-Whitney exact test). FV activity levels were further decreased in carriers of both the His79 allele and the HR2 haplotype (p=0.009, Mann-Whitney exact test).

Mean APC ratios were similar among the different subsets (Table 1). Carriers of the His79 allele or the HR2 haplotype, as well as compound heterozygotes for both gene variants, showed normalized APC ratios that were not different from those found in normal subjects without mutations.

Carriers of the FV Leiden mutation

To test the hypothesis that the His79 allele can affect the APC phenotype in subjects carrying the Leiden mutation, as occurs in those with the HR2 haplotype, we investigated a set of 55 apparently healthy subjects carrying the FV Leiden mutation. Of them, 5 (9.1%) carried the His79 allele and 3 (5.5%) the HR2 haplotype (Table 2). None was found to carry both gene variants. The effect of the association between the His79 allele and the Leiden mutation on normalized APC ratios is shown in Table 2. Compound heterozygous carriers for both mutations had lower normalized APC ratios than did subjects with the Leiden mutation only (p<0.05). Mean plasma levels of FV activity were consistent with the reduction of the normalized APC ratios (Table 2), further supporting the hypothesis that the His79 allele can

Table 1. FV coagulant activity levels and APC ratio in subjects with different FV gene variants.

	No mutation	*His79 allele*	*HR2 haplotype*	*Both mutations*	*All subjects*
Numbers	121	14	13	2	150
FV activity %, mean (SD)	106.4 (18.5)	95.2 (25.2)*	93.7 (16.2)°	78.7 (3.3)§	103.9 (19.4)
APC ratio (range)	1.02 (0.87-1.46)	0.96 (0.94-0.99)	0.98 (0.96-1.03)	0.97 (0.96-0.98)	1.00 (0.87-1.46)

**p=0.025 vs. no mutation group (Mann-Whitney exact test). °p=0.007 vs. no mutation group (Mann-Whitney exact test). §p=0.009 vs. no mutation group (Mann-Whitney exact test).*

Table 2. FV coagulant activity levels and APC ratio in subjects with different FV gene variants and the FV Leiden mutation.

		No mutation	*His79 allele*	*HR2 haplotype*
Numbers		47	5	3
FV activity %	Mean (SD)	104.9 (18.6)	93.1 (22.3)	91.4 (15.9)
APC ratio	Mean (range)	0.69 (0.62-0.75)	0.63 (0.61-0.65)°	0.63 (0.62-0.64)*

*°p=0.008 vs. no mutation group (Mann-Whitney exact test); *p=0.026 vs. no mutation group (Mann-Whitney exact test).*

modulate the APC phenotype when co-segregated with the Leiden mutation. As predicted, subjects with the HR2 haplotype and the FV Leiden mutation showed a reduced APC sensitivity that was comparable to that found in carriers of the His79 allele.

Discussion

Activated protein C resistance is a poor anticoagulant response of plasma to APC and is almost always associated with the presence of a mutation in one of the APC cleavage sites of the FV (Arg506). However, FV genetic components, different from the FV Leiden mutation, have been found to contribute to the APC resistance phenotype. The HR2 haplotype[5,6,15] and the FV Cambridge mutation (Arg306→Thr)[12,16] have been reported to be associated with a reduced sensitivity for APC, mainly in subjects carrying the FV Leiden mutation. In addition to polymorphic sites enclosed in those arising the HR haplotypes, different gene variants have been characterized within the FV gene.[10] In exon 3 of FV, a G to C transversion occurring at nucleotide 409 has been identified; this leads to an Asp to His substitution at position 79. This gene variation occurs within the A1 domain of the FV heavy chain and causes the substitution of a small charged amino acid for an aromatic one. Thus, it is conceivable that this missense mutation may affect the correct folding and the stability of the FV molecule and, in turn, impair functionality.

Among subjects without previous thromboembolism, we found that the His79 gene variant is associated with reduced levels of FV coagulant activity. The reduction observed was similar to that found in the group of subjects carrying the HR2 haplotype. Moreover, subjects presenting with both the HR2 haplotype and the His79 mutation showed a further reduction of FV coagulant activity. Low plasma levels of FV have been demonstrated in subjects with the HR2 haplotype.[5,6] This reduction was not associated with a reduced sensitivity for APC in non-carriers of the FV Leiden mutation.[5,6,15] In the present study, we confirm these data; subjects with the HR2 haplotype showed APC sensitivities comparable to those measured in subjects without the haplotype. Similarly, the His79 mutation was not associated with a reduced sensitivity for APC in non-carriers of the FV Leiden mutation.

On the other hand, the HR2 haplotype was associated with a reduced sensitivity for APC in carriers of the FV Leiden mutation. These findings were consistent with those obtained in previous studies.[5,6] It has been suggested that the HR2 haplotype is associated with an imbalance between two functionally different isoforms of FV.[8,9] Recently, FV mutants carrying different gene variations concurring to the HR2 haplotype have been demonstrated to impair the secretion of the protein.[17] Both these mechanisms can affect APC sensitivity and explain the reduced values in carriers of both mutations. In keeping with this, we reasoned that a reduction of FV plasma levels, such as that observed in carriers of the His79 mutation, would allow for a modulation of the APC sensitivity in subjects presenting with the FV Leiden mutation. In fact, subjects with both the His79 and the Leiden mutation showed still lower APC sensitivity than that found in carriers of the FV Leiden mutation.

The effect of the His79 allele, as well as of the HR2 haplotype, on APC sensitivity in subjects carrying the FV Leiden mutation can be explained by the fact that the APC resistance assay, among other things, depends on the ratio between plasma levels of the wildtype FV molecule and those of the FV Leiden molecule. Since the His79 allele is associated with reduced plasma levels of FV, the relative concentration of the FV Leiden molecule increases, leading to a more pronounced impairment of the APC sensitivity.

The simplest explanation is that the Asp79His polymorphism itself is responsible for the modulation of FV plasma levels. Another possible expla-

nation is that this gene variation is in linkage disequilibrium with a polymorphism in different regions (e.g. promoter) that may cause a reduced expression of the FV gene.

Regardless of the mechanism responsible for the modulation of FV plasma levels, the findings of this study further suggest that trans-acting genetic causes within the FV locus can affect the phenotype resulting from the carriership of the FV Leiden mutation.

References

1. Haemostasis and Thrombosis. 3rd edition. Forbes CD, Thomas DP, Tuddenham EGD, editors. Edinburgh: Churchill Livingstone; 1994. p. 465-76.
2. Molecular Biology of Thrombosis and Hemostasis. Ortel TL, Keller FG, Kane WH. Factor V, in High KA, Roberts HR, editors. New York, NY, Deker; 1995. p. 119
3. Kane WH, Davie EW. Blood coagulation factors V and VIII: structural and functional similarities and their relationship to hemorrhagic and thrombotic disorders. Blood 1988;71:539-55
4. Souto JC, Almasy L, Borrell M, Garì M, Martinez E, Mateo J, et al. Genetic determinants of hemostasis phenotypes in Spanish families. Circulation 2000;101:1546-51
5. Alhenc-Gelas M, Nicaud V, Gandrille S, van Dreded P, Amiral J, Aubry ML, et al. The factor V gene A4070G mutation and the risk of venous thrombosis. Thromb Haemost 1999;81:193-7.
6. De Visser MC, Guash JF, Kamphuisen PW, Vos HL, Rosendaal FR, Bertina RM. The HR2 haplotype of factor V: effects on factor V levels, normalized activated protein C sensitivity ratios and the risk of venous thrombosis. Thromb Haemost 2000;83:577-82.
7. Bernardi F, Faioni EM, Castoldi E, Lunghi B, Castaman G, Sacchi E, et al. A factor V genetic component differing from factor V R506Q contributes to the activated protein C resistance phenotype. Blood 1997:90:1552-7.
8. Castoldi E, Rosing J, Girelli D, Hoekma L, Lunghi B, Mingozzi F, et al. Mutations in the R2 FV gene affect the ratio between the two FV isoforms in plasma. Thromb Haemost 2000;83:362-5.
9. Hoekma L, Castoldi E, Tans G, Girelli D, Gemmati D, Bernardi F, et al. Functional properties of factor V and factor Va encoded by the R2-gene. Thromb Haemost 2001;85:75-81.
10. Cargill M, Altshuler D, Ireland J, Sklar P, Ardlie K, Patil N, et al. Characterization of single-nucleotide polymorphisms in coding regions of human genes. Nat Genet 1999;22:231-8.
11. Margaglione M, Cappucci G, Colaizzo D, Giuliani N, Vecchione G, Grandone E, et al. The PAI-1 gene locus 4G/5G polymorphism is associated with a family history of coronary artery disease. Arterioscler Thromb Vasc Biol 1998;18:152-6.
12. Santacroce R, Bossone A, Brancaccio V, Fortina P, Margaglione M. In the presence of other inherited or acquired high-risk situations, the FV Cambridge mutation may be an additional thrombophilic risk factor, through its effect on APC sensitivity. Thromb Haemost 2000;83:963-4.
13. Koeleman BP, Reitsma PH, Allart CF, Bertina RM. Activated protein C resistance as an additional risk factor for thrombosis in protein C-deficient families. Blood 1994;84:1031-5.
14. Margaglione M, Brancaccio V, Giuliani N, D'Andrea G, Cappucci G, Iannaccone L, et al. Increased risk of venous thrombosis in carriers of the prothrombin A20210 gene variant. Ann Intern Med 1998;129:89-93.
15. 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 substitution in factor V. Br J Haematol 1997;99:257-61.
16. Norstrøm E, Thorelli E, Dahlbäck B. Functional characterization of recombinant FV Hong Kong and FV Cambridge. Blood 2002;100:524-30.
17. Yamazaki T, Nicolaes GAF, Sørensen KW, Dahlbäck B. Molecular basis of quantitative factor V deficiency associated with factor V R2 haplotype. Blood 2002;100:2515-21.

Pre-publication Report & Outcomes of Peer Review

Contributions

All authors (AB, FC, GD'A, VB, GC, LI, EG, and MM) gave substantial contributions to the conception and design of the study, analysis and interpretation of data, drafting and revising the article critically for important intellectual content, and gave the final approval of the present version of the manuscript. Responsibility for all Tables: MM.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Vicente Vicente, Deputy Editor. The final decision to accept this paper for publication was taken jointly by Professor Vicente and the Editors. Manuscript received October 8, 2002; accepted February 6, 2003.

In the following paragraphs, Prof. Vicente summarizes the peer-review process and its outcomes.

What is already known on this topic

Several FV genetic polymorphisms, other than FV Leiden, have been found to contribute to the APCresistance phenotype.

What this study adds

This paper suggests that the Asp79His polymorphism of the FV locus modulates FV plasma levels and also seems to affect APC sensitivity.