RAPID COMMUNICATION

Factor V Cambridge: A New Mutation (Arg<sub>306</sub>→Thr) Associated With Resistance to Activated Protein C

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A new factor V mutation associated with resistance to activated protein C and thrombosis (factor V Cambridge, Arg<sub>306</sub>→Thr) was found in one patient from a carefully selected group of 17 patients with venous thrombosis and confirmed APC resistance in the absence of the common Gln<sup>506</sup> mutation. The Arg<sub>306</sub> mutation was also present in a first degree relative who also had APC resistance. Other potential causes of APC resistance, such as a mutation at the Arg<sub>679</sub> site and the factor V HR2 haplotype, were excluded. Subsequent screening of 585 patients with venous thromboembolism and 226 blood donors did not show any other individual with this mutation. Factor V Thr<sub>306</sub> is the first description of a mutation affecting the Arg<sub>306</sub> APC cleavage site and is the only mutation, other than factor V Leiden (Arg<sub>506</sub>→Gln), that has been found in association with APC resistance. This finding confirms the physiologic importance of the Arg<sub>306</sub> APC-cleavage site in the regulation of the prothrombinase complex. It also supports the concept that APC resistance and venous thrombosis can result from a variety of genetic mutations affecting critical sites in the factor V cofactor.

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MATERIALS AND METHODS

Patients. APC sensitivity ratios were measured on plasma samples from 602 patients consecutively investigated after a diagnosis of deep vein thrombosis or pulmonary embolus. The patients were not taking warfarin at the time of blood sampling. Deep vein thrombosis was diagnosed by ultrasound and venography and pulmonary embolus by ventilation-perfusion lung scanning. Four hundred twenty-four patients had a diagnosis of deep vein thrombosis only and 178 had symptomatic pulmonary embolism.

Standard APC sensitivity ratio. Samples were centrifuged twice at 2,500g for 10 minutes and aliquots of platelet-poor plasma frozen at −80°C until assay. The APC sensitivity ratios were determined after filtration of plasma through a 0.2-µm syringe filter (Gelman Sciences, Northampton, UK) with Coatest APC Resistance-C kit (Chromogenix, Molndal, Sweden). Plasma was incubated with an equal volume of activated partial thromboplastin time (APTT) reagent for 5 minutes. Clotting was initiated by the addition of CaCl<sub>2</sub>. Clotting times were expressed as a ratio of the clotting time in the absence of APC divided by the clotting time in the absence of APC. The plasma filtration method greatly reduces resistance to APC as a result of platelet phospholipid contamination and increases the specificity of the assay for true APC resistance from 32% to 98%. In healthy controls, APC ratios determined by this method are greater than 2.2.

Modified APC resistance assay. APC resistance in the presence of factor V-depleted plasma was assessed using the Coatest APC Resistance-C kit and factor V-depleted plasma (Chromogenix). Plasma was prediluted 1 in 5 with factor V-depleted plasma and APC sensitivity ratios were determined as in the standard assay. Modified APC sensitivity ratios were less than 2.0 in 40 patients with the Gln<sup>506</sup> mutation who were tested (range, 1.29 to 1.96) and greater than 2.2 in 40 unselected patients without the mutation (range, 2.23 to 4.64).

Extended APC resistance assay. An extended APC resistance assay was performed as initially described by Dahlback et al. Platelet-poor plasma was incubated with an equal volume of APTT reagent for 5 minutes. Clotting times were recorded after the addition of CaCl<sub>2</sub> supplemented with APC over a final concentration range of 0 to 100 nmol/L (Diagnostica Stago, Assières, France).

Natural anticoagulants, lupus anticoagulant activity, and the FIII20210 mutation. Levels of natural anticoagulants and lupus anticoagulant activity were measured as previously described, with normal ranges as previously determined. Restriction enzyme analysis with HindIII after DNA amplification using a mutagenic primer was used to screen...
samples for the recently reported G to A transition at position 20210 in the 3'- untranslated region of the prothrombin gene.\textsuperscript{22,23}

**Plasma factor V coagulant activity.** Plasma factor V coagulant activity was measured in a one-stage APPT assay on an MDA180 coagulometer (Organon Teknika, Cambridge, UK) using factor V-deficient plasma and Platelitin LS phospholipid substitute (Organon Teknika). In healthy controls, the normal range was 70% to 170%.

**Factor VGIN\textsuperscript{24}**. Restriction enzyme analysis for detection of the FVGIN\textsuperscript{26} mutation (factor V Leiden) was performed as previously described.\textsuperscript{24} A 147-bp fragment encoding the APC cleavage site was amplified by polymerase chain reaction (PCR), and the DNA product was digested with Mnl I and analyzed by agarose gel electrophoresis.

**Factor V exon 7 amplification.** A 228-bp DNA fragment containing exon 7 of the factor V gene was amplified from genomic DNA by PCR. The amplification primers were synthesized corresponding to intronic sequences upstream (5'-TGTCCTTCTGCTCTAAC-3') and downstream (5'-TCTGAACTTCCACCCCA-3') of the exon 7 sequence. Each amplification reaction (50 µL) contained 0.5 µg of genomic DNA, 200 µmol/L of each deoxynucleotide triphosphate, 25 pmol of each amplification primer, and 1.25 U AmpliTaq in 10 mmol/L Tris-HCl, 50 mmol/L KCl, of each deoxynucleotide triphosphate, 50 pmol of each amplification reaction (50 µL) contained 0.5 µg genomic DNA. A total of 200 µmol/L and Platelin LS phospholipid substitute (Organon Teknika). In healthy (Organon Teknika, Cambridge, UK) using factor V-deficient plasma - untranslated region of the prothrombin gene.\textsuperscript{22,23}

**Factor V exon 13 amplification and DNA sequencing.** A 400-bp fragment of the 3' end of exon 13 encoding the Arg\textsuperscript{26} gene was amplified with an intronic upstream primer (5'-GCCAAGATGATTTTGTG-3') and a downstream exonic primer (5'-AGAAGAATTCAGTAGGCCCAT-3'). With the exception of an annealing temperature of 50°C, PCR conditions were the same as for exon 7 amplification. A nested sequencing primer, corresponding to the noncoding strand sequence 5'-TAGGGCAGTAAGTGAACCT-3', was used to sequence codons 631 through 727.

**Factor V HR1/HR2 haplotype analysis.** A 420-bp fragment was amplified using exon 13 exonic primers 5'-CAGACCTCAGCTCAT-CA-3' and 5'-CTGACTGATTTCTGCCA-3'. Each amplification reaction (50 µL) contained 0.5 µg genomic DNA. A total of 200 µmol/L of each deoxynucleotide triphosphate, 50 pmol of each amplification primer, and 1.25 U AmpliTaq in 10 mmol/L Tris-HCl, 50 mmol/L KCl, pH 8.3, and 1.5 mmol/L MgCl\textsubscript{2}. Amplification was performed with an initial denaturation step at 94°C for 10 minutes, followed by 35 cycles of 94°C for 20 seconds, 50°C for 20 seconds, and 74°C for 20 seconds followed by a final extension step at 74°C for 10 minutes. After amplification, 5 µL of the reaction mixture was analyzed by electrophoresis in a 1% agarose gel.

Restriction enzyme digestion of the PCR product was performed by overnight incubation at 37°C with 10 U of Rsa I (New England Biolabs [UK] Ltd). The digestion products were analyzed by electrophoresis in a 2% agarose gel. The HR1 haplotype (HIs\textsuperscript{299}) is represented by an undigested fragment of 420 bp. The HR2 haplotype (Arg\textsuperscript{299}) is represented by fragments of 121 and 299 bp representing the presence of an Rsa I site.

**RESULTS**

**Determination of APC resistance and selection of patients.** Samples for sequencing of the factor V Arg\textsuperscript{26} APC-cleavage site were selected from 602 patients with venous thromboembolism. Four of these patients were homozygous for the Glm\textsuperscript{26} mutation and had APC sensitivity ratios between 1.2 and 1.9. A total of 112 patients were heterozygous with APC sensitivity ratios from 1.5 to 3.3 (median, 2.2). Four hundred eighty-six patients had a normal genotype and ratios of 1.3 to 6.0 (median, 3.4), including 22 who had sensitivity ratios of less than 2.2. These 22 patients were investigated further.

Three of the 22 patients had detectable lupus anticoagulant activity and free protein S levels were low in 2 others (9% and 25%; normal, >55%). The remaining 17 patients had normal levels of antithrombin, protein C, and free protein S: no detectable lupus anticoagulant activity; and the prothrombin gene 201G/A mutation was not present. Plasma from these patients was therefore subjected to the modified APC resistance assay after dilution in factor V-deficient plasma and exon 7 of the factor V gene sequenced to identify any mutations affecting the Arg\textsuperscript{26} APC-cleavage site.

Of the 17 selected patients, 6 had a modified APC sensitivity ratio of less than 2.0, despite the absence of the Glm\textsuperscript{26} mutation.

**DNA sequencing and restriction enzyme analysis.** One of the 17 selected patients was found to be heterozygous for a mutation (G to C) changing the codon for amino acid 306 from AGG (arginine) to ACG (threonine) (Fig 1).

The new G to C mutation at codon 306 removes a recognition site for the restriction enzyme BstNI. The 228-bp amplified fragment from a normal FV gene has a single cutting site, resulting in fragments of 162 and 66 bp after enzyme digestion. As expected, digestion of exon 7 DNA from the patient with the Thr\textsuperscript{306} mutation showed bands of 228, 162, and 66 bp arising from the products of the mutant and normal alleles (patient II.1 in Fig 2).

Restriction enzyme analysis using BstNI was performed on the remaining patient population. A normal digest pattern was detected in all 601 patients, including the 16 selected patients with APC resistance in the absence of the Glm\textsuperscript{26} mutation. In addition, BstNI digestion of DNA from 226 anonymous blood donors was normal.

**Family study.** The index patient (II.1, Fig 2) with the factor V Cambridge mutation (Arg\textsuperscript{306}—Thr) is a 49-year-old man who developed a spontaneous proximal deep vein thrombosis at the age of 47 years. He was treated with unfractionated heparin for 5 days and warfarin (target INR 2.5) for 6 months. He has now been off anticoagulant therapy without recurrence of venous thromboembolism for 2 years. His only living relatives are a
44-year-old sister and his mother and father, aged 73 and 74 years, respectively, none of whom have suffered a thrombotic event. Blood samples were obtained from the family members for DNA analysis and phenotypic study.

The patient’s mother was also found to have the Arg306 = Thr mutation (I.2, Fig 2). His father and sister did not have the mutation. The Arg679 site was sequenced in all four family members and was normal. Plasma factor V levels were normal in all four (94% to 134%) and all were homozygous for the HR1 haplotype.

The patient and his affected sister had APC resistance in both standard and modified APC resistance assays, whereas the unaffected father and sister were not APC resistant (Fig 2). The APC resistance phenotype of the patient and his affected mother was the same as that found in patients with the Gln506 mutation (Fig 3).

The patient’s mother has not been exposed to any high-risk situation for thrombosis other than two pregnancies.

**DISCUSSION**

We have described a new factor V mutant (factor V Cambridge, Arg306 = Thr) associated with APC resistance and thrombosis. It is, to date, the only mutation other than factor V Leiden associated with this phenomenon and the first description of a mutation affecting the Arg306 APC cleavage site. The degree of APC resistance associated with Thr306 is the same as that found in patients with the Gln506 mutation (Fig 3).

The patient’s mother has not been exposed to any high-risk situation for thrombosis other than two pregnancies.

Inactivation of factor Va occurs via sequential cleavage by APC at Arg306, followed by Arg679 with loss of cofactor activity. Cleavage at Arg306 is responsible for the loss of approximately 70% of this activity, whereas cleavage at Arg679 is responsible for the loss of approximately 30%.9,10,12,26 In vitro, cleavage at Arg306 has no direct effect on cofactor activity, but cleavage at this site is necessary for exposure of the inactivating cleavage sites at Arg506 and Arg679. The rate of inactivation of factor VaGln506 (factor V Leiden) is slower than that of normal factor VaArg506. On incubation with APC, factor VaArg506 is completely inactivated after 5 minutes, whereas factor VaGln506 retains approximately 50% of its initial activity.11 At low concentrations, the difference in APC inactivation of the mutant and wild-type proteins is even more pronounced.27 The mutant protein is associated with an increased thrombin potential in vitro,13 an eightfold increased risk of deep vein thrombosis in heterozygotes, and a 50- to 100-fold increased risk in homozygotes.14

Egan et al28 have recently studied factor V inactivation using site-directed mutagenesis to produce mutant recombinant factor V proteins. They have confirmed the importance of the Arg306 site by demonstrating that a recombinant factor V molecule with a mutated 306 site (replacement of arginine by alanine or glutamine) is inactivated at a slower rate than plasma or recombinant wild-type factor V and at a similar rate to recombinant FVaGln506.28 However, APC cleavage patterns of factor V with a threonine substitution at the 306 site have not yet been reported. Despite the presumed physiologic importance of cleavage at Arg306, no mutation affecting this site has been identified until now. We used a rigorous screening strategy to identify patients with venous thromboembolism who showed APC resistance in the absence of the factor V Leiden mutation. In a large cohort of patients, we used an optimized standard
APC sensitivity assay and a ratio of less than 2.2 as a high predictive value for the Gln 506 mutation. Patients with this degree of APC resistance in the absence of the Leiden mutation were therefore identified as candidates for other mutations. Because APC resistance has been demonstrated in the presence of lupus anticoagulant activity and thrombotic events may have been due to other forms of thrombophilia, only patients with isolated APC resistance were investigated further. Sequencing exon 7 of the factor V gene showed a normal sequence in all but one of the selected patients. This patient was found to have a G to C mutation altering the amino acid codon 306 from AGG (arginine) to ACG (threonine). This mutation would remove the primary APC cleavage-inactivation site from the mutant protein. Other potential causes of APC resistance, such as a mutation at the Arg679 site and the factor V HR2 haplotype, were excluded. APC resistance was also present in the modified APC resistance assay after dilution in factor V-deficient plasma, confirming that the phenotype is due to a defect in factor V and not in another protein such as factor VIII. Conclusive evidence of the relationship between the Thr306 mutation and APC resistance will require purification of the mutant protein and analysis of the APC cleavage pattern. Nevertheless, the presence of this mutation in a family with APC resistance is further evidence of the physiologic importance of the Arg306 cleavage site. It seems likely in the absence of any other form of thrombophilia or predisposing high-risk situation that the mutation and the associated APC resistance was the cause of deep vein thrombosis in the index patient.

In selecting patients, the APC sensitivity ratio of 2.2 was chosen for its high specificity for the Gln506 mutation, but the sensitivity associated with this cut-off is only 50%. It is possible that some patients with a ratio greater than 2.2, who were not selected by our screening strategy, could have the Thr306 mutation and it is possible that patients with the Gln506 mutation could also have the Thr306 mutation, particularly those with very low APC sensitivity ratios. However, we screened the whole patient cohort and did not find any other affected patient. Similarly, the mutation was absent in 226 blood donors. Therefore, the Thr306 mutation is not a common polymorphism and it is a rare cause of APC resistance. However, the finding confirms the physiologic importance of the Arg306 APC-cleavage site in the regulation of the prothrombinase complex. It also supports the concept that APC resistance and venous
thrombosis can result from a variety of genetic mutations affecting critical sites in the factor V cofactor.

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