An assessment of the comparative utility of functional and molecular level analyses in the investigation of patients with thrombophilia

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Abstract

Aim—To determine the relation of the low anticoagulant response phenotype with the Factor V Q506 (Leiden) mutation in a cohort of patients with thrombophilia.

Methods—Fifty four patients with either a personal or family history of deep vein thrombosis were investigated both for their anticoagulant response by the activated protein C resistance test (APCR) and their genetic status in respect of the Leiden mutation by means of a PCR-RFLP method.

Results—Low APCR ratios do not necessarily predict possession of the Leiden mutation. Conversely, normal ratios do not exclude the mutation. Of 14 individuals with low APCR ratios, the Leiden mutation was absent in five. Of the remainder, three were heterozygous and six homozygous. Of nine heterozygote individuals, only three had low APCR ratios. All patients homozygous for the defect had low APCR ratios.

Conclusions—These results lend further weight to the hypothesis that the APC resistant phenotype results from more than one genetic defect and indicate the value of combined functional and molecular investigations in all patients with thrombophilia.

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Keywords: thrombophilia, activated protein C resistance, phenotype, genotype.

Activated protein C (APC) resistance is an inherited defect in anticoagulant response characterised by increased risk for thromboembolism. The main cause of the defect has been shown to be a point mutation in the Factor V gene (G to A), known as the Leiden mutation, resulting in an amino acid change from Arg to Gln.¹ This substitution destroys the APC cleavage site on Factor Va, removing an important control on thrombin formation. The prevalence of this mutation in the normal population has been shown to be between 2 and 4%.^{2,3}

The APC resistance test measures the prolongation of the clotting time in response to the addition of APC to patient plasma. A diagnosis of APC resistance is made when a significant reduction in clotting time is observed compared with healthy controls. The test provides a relatively simple and reliable method of diagnosis, but is of limited use in

patients on oral anticoagulant treatment. Modification of the test involving predilution of the patient sample has been reported to overcome this difficulty but has not been explored by this laboratory as yet. Patients can also be investigated at the DNA level for the presence of the Factor V (Leiden) mutation using a PCR-RFLP technique¹ and this test has the additional advantage of determining whether the individual is homozygous or heterozygous for the defect.

Methods

Blood samples for analysis were collected from 54 patients (22 men, 32 women; age range 18–68 years) with either a personal (n = 42) or an immediate family (n = 12) history of deep vein thrombosis. Plasma was prepared by centrifugation of the blood at 2000 × g for 20 minutes and stored at –70°C pending analysis. DNA was extracted by the method of Miller *et al.*⁴

APCR TEST

Full lupus anticoagulant screening was performed on all samples to exclude interference of the lupus inhibitor in the APCR test.

All analyses were performed using the Coatest APC resistance C kit. Briefly, one volume of plasma was mixed with APTT reagent and incubated at 37°C for five minutes. One volume of calcium chloride was added and clot formation timed. A second analysis was performed, replacing the calcium chloride with APC/calcium chloride (Human activated Protein C co-lyophilised with calcium chloride) and clot formation timed again. Clot formation was determined using an ACL 3000 instrument with time for detection extended to 150 seconds in accordance with the manufacturer's instruction.

The APC ratio, defined as clot time with APC plus calcium chloride/clot time with calcium chloride, was calculated for samples and controls. An APC sensitivity ratio ≤ 2.2 was taken to indicate a defect in the anticoagulant response to APC, being the assay cut off value defined by screening 63 healthy volunteers (27 men, 36 women; age range 18–52 years). In the case of ratios below the established cut off point, results were confirmed by repeat analyses on fresh samples.

DNA ANALYSIS

DNA analysis was carried out as described previously.¹ Briefly, a 267 base pair (bp) PCR product containing the mutation site was generated. The 267 bp fragment was then digested

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Table 1 Results of APCR and Leiden mutation analyses in patients with either a personal or family history of thromboembolism

Genetic status	APCR Ratio		Not tested*	Total
	≤ 2.2	> 2.2		
Normal	5	18	3	26
V Q506	3	6	6	15
V Q506	6	0	3	9
Not determined†	0	4	0	4
Total	14	28	12	54

*Patients on oral anticoagulant therapy.

†DNA extraction/PCR failure.

for 18 hours with Mnl I restriction endonuclease (Immunogen International). The PCR digestion product was electrophoresed in a 3% agarose gel containing 0.5 µg/ml ethidium bromide and visualised using ultraviolet illumination. Presence of the Leiden mutation was revealed by the loss of a restriction site resulting in fragments of 67, 37 and 163 bp with the normal Factor V allele and fragments of 67 and 200 bp with the Leiden allele.

Results

At the time of writing, we have performed 54 individual laboratory investigations on patients with either a personal or family history of thromboembolic disease (table 1). Of this group, APCR results were obtained for 42, the remainder being excluded from analysis on the basis of their anticoagulant treatment. Fourteen patients had an APCR result below the cut off point of 2.2, giving a 33.4% positive rate. Genetic analysis of 50 subjects from the same cohort revealed that 24/50 patients possessed the Leiden mutation, with nine of 50 being homozygous for the defect and 15/50 heterozygous. All homozygous samples had APCR values below the assay cut off point, but only three (33.4%) of nine heterozygotes had low ratios. Five patients defined as APC resistant by the APCR test lacked the Leiden mutation. Homozygous subjects had a mean (SD) APC ratio of 1.7 (0.3), heterozygous subjects of 2.4 (0.6) and normal subjects of 2.7 (0.5). Notably, three individuals identified as heterozygote on genetic analysis had APCR values below those obtained for some homozygotes.

Discussion

The lack of concordance between APCR results and genetic status is at odds with previous reports, which have shown good correla-

tion between the two,^{1 5 6} but is in accord with the view that the APC phenotype may result from more than one genetic defect.^{7 8} From the results obtained, the sensitivity of the APCR test for the Leiden mutation is 60% with a specificity of 78.3% and efficiency of 71.1%. Test sensitivity is increased to 100% in those homozygous for the defect, but reduced to 34% in heterozygotes. These findings indicate that sole reliance on APC resistance testing in the investigation of patients with thrombophilia is likely to result in failure to identify a high proportion of those in whom the Factor V Q506 mutation is present, but that direct gene analysis for the mutation is inappropriate as a first line test. Taken together, the results of our study would indicate the value of combined functional and molecular level testing in all patients with thrombophilia. With regard to the latter we find the PCR-RFLP approach used in the current study to be a method well suited for use in the routine diagnostic laboratory, being both relatively simple to perform and easy to interpret. Alternative approaches to analysis requiring probe hybridisation or direct DNA sequencing are limited in their application because of their technical complexity and requirement for more specialist facilities but could be used as adjuncts to PCR-RFLP or as stand-alone techniques if desired. We are currently involved in work to introduce sequence based analysis to our laboratory.

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