

Activated Protein C Resistance: A Study among 60 Thromboembolic Patients in the Singapore Population

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Abstract

Resistance to activated protein C (APC-R) is the commonest inherited cause of thrombosis among Caucasians. Few studies have been carried out on its prevalence in Asians. We conducted a prospective study on 60 patients with thromboembolism to determine its prevalence in our local population. The Factor V Leiden (VaQ506) mutation associated with this condition was detected by amplification of the Factor V gene by polymerase chain reaction (PCR) and digestion of the fragment with Mnl I. Three patients were found to be heterozygous for this mutation. None of the 3 patients had other concomitant hypercoagulable states. In addition, we studied the prevalence of this condition in Malays which was found to be 0.5%. Our study suggests that the incidence of APC-R is much lower here compared to the West.

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Introduction

In 1993, Dahlback and colleagues described a new hereditary thrombophilia state-activated protein C resistance (APC-R).¹ Further studies showed this to be the commonest genetic cause of thrombophilia.² Similar to most inherited thrombotic disorders, it is transmitted in an autosomal dominant manner. Heterozygotes have a 5- to 10-fold increase in risk of thrombosis while for homozygotes the risk is 50- to 100-fold.³ However, due to incomplete penetrance of the genetic defect, patients with APC-R who develop thrombosis often have other concomitant genetic or exogenous factors such as protein C deficiency or oral contraceptive usage.

The underlying genetic defect causing APC-R was identified in 1994.^{2,4} More than 95% of cases are due to a point mutation in the Factor V gene (arginine 506 to glutamine) referred to as Factor V Leiden. Detection of this mutation can be carried out by various methods.⁵ Two of the methods that have been described include polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and allele-specific PCR. Both these methods were utilised in our study to determine the incidence of Factor V Leiden in 60 patients with thromboembolism.

Material and Methods

Subjects

Sixty patients referred to our department for management of thromboembolism between January 1995 to December 1996 were included in the study. Fifty-seven patients had either deep venous thrombosis alone (52 cases) or in combination with pulmonary embolism (5 cases). The remaining 3 patients had only pulmonary embolism with no evidence of concurrent deep venous thrombosis. The diagnosis of pulmonary embolism was based on the presence of definite mismatched defects on ventilation-perfusion lung scans while presence of deep vein thrombosis was confirmed by Duplex scan in all patients. All the patients were also evaluated for other concomitant hypercoagulable states-such as protein C and Sand anti-thrombin III deficiencies, anti-phospholipid syndrome, use of oral contraceptives, post surgery or trauma. Thirty-four patients had additional factors predisposing to a hypercoagulable state-19 were post surgical patients, 3 had anti-thrombin III deficiency, 3 protein C deficiency, 1 protein S deficiency, 4 underlying malignancy, 2 pelvic tumours and 2 anti-phospholipid syndrome.

The median age was 46.5 years (range 20 to 84 years).

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Of these, 26 were male patients. The racial profile was 41 Chinese, 12 Malays, 5 Indians and 2 Caucasians. Sixty normal controls were also studied initially-samples were obtained from healthy blood donors. The racial distribution was similar to patient cohort-40 Chinese, 12 Malays, 7 Indians and 1 Caucasian. A total of 200 Malay blood donors were also subsequently studied to determine the prevalence of APC-R in the Malay population. This figure was determined from statistical calculation based on sample size for population study.

PCR Assays

Genomic DNA was isolated from EDTA-blood by salting out method.⁶ For PCR-RFLP method, the FV Leiden mutation was detected by amplification of 287 bp fragment of exon 10/intron 10 of Factor V gene followed by digestion with the restriction enzyme *Mnl* I.⁷ The PCR conditions were 2 minutes of initial denaturation at 94°C followed by 35 cycles of denaturation at 94°C for 15 s, 15 s of annealing at 57°C and 30 s of extension at 72°C. The product was then subjected to *Mnl* I digest overnight at 37°C. The expected results as visualised on agarose gel are (Fig. 1a):

Normal person : 157, 93, 37 bp products

Heterozygous : 157, 130, 93, 37 bp products

Homozygous : 157, 130 bp products

The 37 bp band is difficult to visualise on agarose gels but a definite confirmation of the FV 506 mutation can be achieved without this information.

Recent studies have shown the existence of polymorphism at position 1691 leading to the possibility of a false positive result due to loss of *Mnl* I site arising from mutations other than the Factor V Leiden with the PCR-RFLP method.⁸ Hence, patients found to be abnormal by PCR-RFLP are also tested using allele-specific PCR.⁹ The primers used for allele-specific PCR are:

FV common primers (FVC) : ACC ATG ATC AGA
GCA GTT CA

FV for normal strand (FVN) : AAA AGT ACC TGT
ATT CCT C

FV for mutant strand (FVM) : AAA' AGT ACC TGT
ATT CCT T

Forward primer for beta : CGA TCT TCA ATA
globin (Lep2) TGC TTA CCA AG

Reverse primer for beta : CAT TCG TCT GTT
globin (Lep3) TCC CAT TCT A

An internal standard is necessary for allele specific PCR and we used exon 1 and 2 of beta-globin.

Internal control is added to each PCR reaction by amplifying part of beta exon using primer (Lep2 + Lep3) and is shown by the amplification of 915 bp PCR product. The cycling parameters are: initial denaturation at

94°C for 4 min followed by 30 cycles of 94°C, 58°C and 72°C for 20 s each, and final extension of 72°C for 10 min. The detection of a 212bp product using primer pair FVC + FVM indicates the presence of mutation while its absence indicates negative result. Detection of 212 bp using primer pair (FVC + FVN) indicates the presence of normal strand (Fig. 1b).

Results

Of the 60 patients with thromboembolism, 3 male patients were found to be heterozygous for Factor V Leiden mutation-2 were Indians and 1 Malay. The clinical profile of the three heterozygous patients is further detailed. Patient A is a 24-year-old Indian national who developed deep vein thrombosis of his right leg in October 1996 after a plane flight. He had no past history or family history of thromboses. There were no other risk factors for thrombosis. In spite of adequate anti-coagulation with warfarin, 5 months later he developed a second episode of venous thrombosis on his left leg. He was maintained on anticoagulation with warfarin and had no further episodes until last seen in December 1997. Patient B is also an Indian national working here. He presented in January 1995 at the age of 51 years with venous thrombosis of his left leg after surgery for fracture of the left neck of femur. There was no personal or family history of thrombosis or other hypercoagulable states. He was anti-coagulated for 6 months and discharged with advice to return should he develop symptoms. No family studies were performed for both these patients as their families were in India.

Patient C is the only Singapore citizen. He is a 29-year-old Malay who presented with idiopathic deep venous thrombosis in his right leg in September 1995. No other factors predisposing to thrombosis were found. He was anti-coagulated for 3 months and stopped after a repeat Duplex scan showed resolution of the thrombus. There have been no further episodes of thrombosis until September 1996 when he was last seen. Family studies confirmed that his mother was also heterozygous for APC-R though she was asymptomatic. His two siblings and father are normal.

Among the 60 normal blood donors initially tested, one Malay donor was found to be heterozygous for this mutation. The affected donor is a 30-year-old Singaporean with no personal or family history of thrombosis. As a result of these studies suggesting that APC-R might be more prevalent in Malays than expected, we went on to screen a total of 200 Malay blood donors. No other cases of APC-R were detected apart from that initial case. Hence, the prevalence of APC-R is estimated to be 0.5% in the local Malay population (confidence interval of 95%).

In all the 4 affected subjects, allele-specific PCR yielded

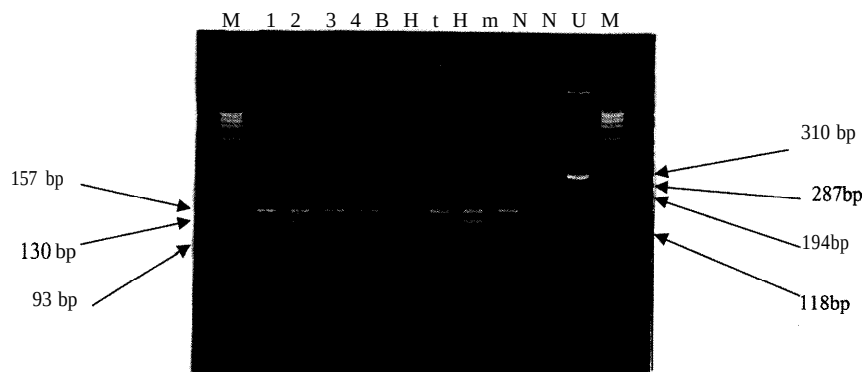


Fig. 1a. Detection of Factor V Leiden by PCR-RFLP. Lanes 1-4; 4 heterozygotes detected in the study; B: blank; Ht: heterozygous control; Hm: homozygous control; N: normal control; U: 287bp fragment of exon 10/intron 10 of Factor V gene; M: molecular marker (ϕ X174 DNA/HaeIII).

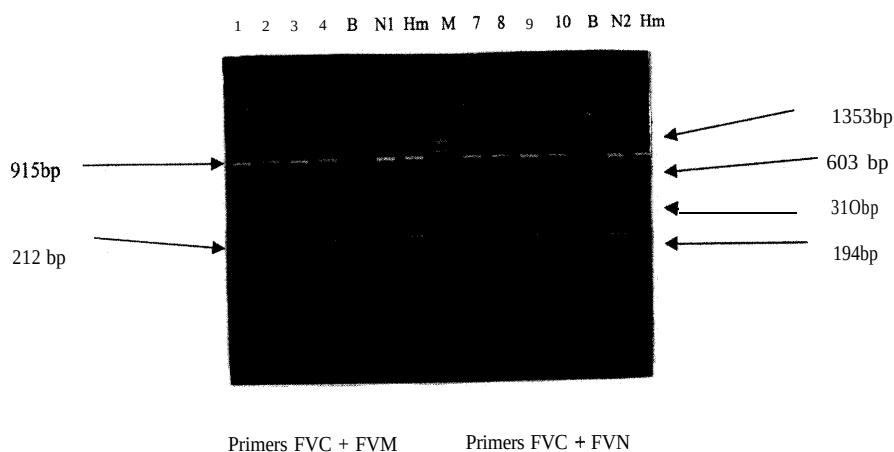


Fig. 1b. Detection of Factor V Leiden by allele-specific PCR. Lanes 1 to 4 and 7 to 10 are the 4 heterozygotes using primers FVC + FVM and FVC + FVN, respectively. Detection of 212 bp using primer pair (FVC + FVM) indicates the presence of mutation while its absence indicates negative result N1. Detection of 212 bp using primer pair (FVC + FVN) indicates the presence of normal strand (N2). Internal control is added to each PCR reaction by amplifying part of beta globin exon and is shown by the amplification of 915 bp PCR product. Lane B: blank; Hm: homozygous control.

the same results as the initial assay i.e. no false positives were found. No homozygous carriers of this mutation were detected in our study.

Discussion

Since the discovery of activated protein C resistance in 1993, it has been recognised to be the commonest genetic defect associated with hypercoagulability. It was found to account for 21% of deep vein thromboses in the under-70s¹ and for up to 50% of familial venous thromboses.¹⁰ The underlying defect in more than 95% of cases is a point mutation in the Factor V gene referred to as Factor V Leiden.⁴ The frequency of the Factor V Leiden allele in Europe is high at 4.4% with carrier rates of 15% among Greeks and 8.8% in UK whites.¹¹ In contrast, this disease has not been extensively studied in Asia. Studies available^{11,12} suggest it is rare in Asians especially in Chinese population. The prevalence of this mutation in India (Punjab) has been reported to be 1.3%¹³ while there are no studies reported in Malay population. From our

study, the incidence of APC-R is low in our local population. The estimated prevalence of 0.5% for this condition in the Malays is interesting. We postulate that this may have arisen as a result of intermarriage. Indeed, of the 2 Malays found to be heterozygous for APC-R, patient C's mother had a history of Indian ancestry.

How should we manage these patients? As the asymptomatic heterozygous individuals do not usually develop thrombosis except in risk situations, anti-coagulation should only be instituted in 2 settings.¹⁴ Firstly, prophylactic therapy with heparin should be given in risk situations and secondly, in the event of development of a thromboembolic episode. There is no definite data on the optimal duration of anti-coagulation in patients with APC-R and thromboembolism. However in general, chronic anti-coagulation should be considered in those who have recurrent or life-threatening episodes or patients with additional genetic defects (such as protein C, protein S or antithrombin III deficiency).

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