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FACTOR V LEIDEN MUTATION INVESTIGATED BY AMPLIFICATION CREATED RESTRICTION ENZYME SITE (ACRES) IN PNH PATIENTS WITH AND WITHOUT THROMBOSIS

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ABSTRACT

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired chronic hemolytic anemia characterized by intravascular hemolysis, often associated with neutropenia and thrombocytopenia. Venous thrombosis, including the Budd-Chiari syndrome, is one of the major complications of PNH, but not all PNH patients develop thrombosis. The basis for the high risk of thrombosis in PNH is not known. Recent reports have shown that Factor V Leiden mutation is a common cause of increased tendency to develop thrombosis. Fifty-six PNH patients were tested for Factor V Leiden mutation using *Amplification Created Restriction Enzyme Site* methods. PNH patients do not show an increased frequency of Factor V Leiden mutations.

Keywords: paroxysmal nocturnal hemoglobinuria, activated protein C, factor V Leiden, point mutation, thrombosis

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hemolytic anemia characterized by intravascular hemolysis of red blood cells deficient in CD55 (*decay accelerating factor*), CD59 (*membrane inhibitor of reactive lysis*, MIRL) and other proteins bound to the membrane through a glycosyl phosphatidyl inositol (GPI).[1] Venous thrombosis, including the Budd-Chiari syndrome, is one of the major complications of PNH, but not all PNH patients develop thrombosis.[2] The basis for the high risk of thrombosis in PNH is not known, but the fact that platelets are abnormal may play a major role. Recent reports have shown that an inherited defect in anticoagulant response to activated protein C (APC-resistance) is a common cause of an increased tendency to develop thrombosis.[3] APC-resistance is mostly caused by a G1691A mutation (506 Arg→Gln) in coagulation Factor V [Factor V Leiden mutation].[3-5] We investigated the possibility that the coexistence of Factor V Leiden mutation in patients with PNH may increase their risk of thrombosis.

Patients and Methods

Patients

We tested 71 control subjects (non-PNH group) and 56 PNH patients (Budd-Chiari syndrome, 3 cases; deep vein thrombosis, 1 case; portal vein thrombosis, 1 case; thrombosis of IVC, 1 case; abdominal pain without proven thrombosis, 4 cases; no evidence of thrombosis, 20 cases; and no information on thrombosis, 26 cases).

Amplification created restriction enzyme site (ACRES) technique

Genomic DNA was extracted by standard methods.[6] For the amplification of genomic

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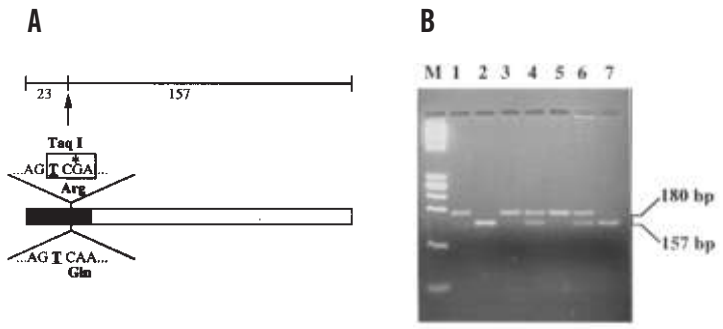

Figure 1. Factor V analysis by *amplification created restriction enzyme site* (ACRES).

A. Diagram of the amplified fragment consisting of a portion of exon 10 (hatched) and a portion of intron 10 (white). The *Taq I* restriction site (T↓CGA) is boxed. The asterisk indicates the position of the Factor V Leiden mutation (G1691A). The fragment amplified from the normal Factor V gene will be cut by *Taq I* into 2 fragments of 23 and 157 bp, respectively. The fragment amplified from Factor V gene with the Leiden mutation will not be cut.

B. Restriction enzyme analysis of amplified Factor V exon 10-intron 10 fragment on 3% agarose (NuSieve) gel electrophoresis. Lane 1, undigested PCR product; lanes 2 and 7, normal homozygote 506 Arg/Arg; lanes 3 and 5, mutant homozygote 506 Gln/Gln; lanes 4 and 6, heterozygote 506 Arg/Gln; lane M, size markers (ΦX174 *Hae* III digestion).

fragments containing the end of exon 10 and a portion of intron 10, we first used PR-990 nt and PR-6967 primers followed by *Mnl I* digestion as described.[3] Secondly, we designed a specific oligonucleotide in exon 10 of the Factor V gene that we called FVL (5'-GTAAGAGCAGATCCCTGGACAGTC); it contains a deliberate mismatch (G→T, underlined), such that a *Taq I* site will be created in the normal Factor V gene but not in the mutated gene during PCR amplification (Figure 1B). This is an adaptation of the so-called *amplification created restriction enzyme site* (ACRES) technique.[7] PCR was performed in a total volume of 50 µL containing 54 mM Tris-HCl, pH 8.8, 5.4 mM $MgCl_2$, 5.4 mM EDTA, 13.3 mM $(NH_4)_2SO_4$, 8 mM β-mercaptoethanol, 0.4 mg/mL BSA, 0.5 mM dNTP, 200 ng each of primers FVL and PR-6967, and 2 U of Taq polymerase. PCR conditions were as follows: first denaturation 94°C (5 min), 30 cycles of 94°C (45 sec), 60°C (45 sec), 72°C (1 sec) and final elongation at 72°C (7 min). The PCR product (15 µL) was digested with 0.4U *Taq I* (Biolabs) for 3 hours and then subjected to electrophoresis on 3% agarose gel (NuSieve).

Results

Digestion of the 180 bp amplified fragment with *Taq I* gives fragments of 157 and 23 bp in normal Factor V. The fragment amplified from Factor V gene with the Leiden mutation is not cut (Figure 1B). Using this ACRES method, we found the Factor V Leiden mutation in 2 out of the 71 control subjects (2.8%) and in 1 out of the 56 PNH patients (1.8%). This patient was one of the 4 who had abdominal pain without proven thrombosis.

Discussion

Before developing this metodology, we used a technique based on the fact that the Factor V Leiden mutation abolishes one of the *Mnl I* restriction sites within a PCR-amplified fragment.[3] However, we found that partial digestion often made interpretation of the results difficult. After this work had been carried out,[8] we learned that Rabès *et al.* (1995) independently developed the same method[9] for detecting the Factor V Leiden mutation. By creating an artificial *Taq I* restriction site, testing for the Factor V Leiden mutation becomes cheap, reliable and applicable to large population screening.[8-10]

We conclude that PNH patients with thrombotic complications do not have an increased frequency of the Factor V Leiden mutation and that the relationship between GPI-anchor deficiency[11] and susceptibility to venous thrombosis still remains to be established. From the point of view of patient management, we believe that if a patient with PNH shows APC-resistance there would be a strong case for long-term prophylactic anticoagulation, even before any thrombotic complications occur.

Note added in proof

The Factor V Leiden mutation was not found in 10 additional PNH patients (retinal vein

thrombosis, 1 case; no evidence of thrombosis, 4 cases; no information, 5 cases).

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