The A −844G Polymorphism in the PAI-1 Gene Is Associated With a Higher Risk of Venous Thrombosis in Factor V Leiden Carriers

P.E. Morange, M. Henry, D. Tregouët, B. Ganel, M.F. Aillaud, M.C. Alessi, I. Juhan-Vague

Abstract—Identification of combined genetic factors in factor V Leiden carriers is important for a more accurate risk assessment for venous thrombosis (VT). Among these individuals, we evaluated the role of polymorphisms of the plasminogen activator inhibitor-1 (PAI-1) gene in the thrombophilic phenotype. A total of 382 factor V Leiden carriers were included in the study. This population was divided into 3 groups. Group 1 (n = 168) included individuals with a personal history of VT; group 2 (n = 140) included individuals without personal VT but with a familial history of VT; and group 3 (n = 74) included individuals without VT and with a fortuitous discovery of the factor V Leiden mutation. We compared the genotype distribution of 2 polymorphisms, A −844G and −675 4G/5G, located in the promoter region of the PAI-1 gene among these 3 groups of individuals. The A −844G allele frequency differed significantly among the 3 groups (P = 0.048), the A allele being more frequent in patients who suffered from VT (61%) than in subjects without VT (52%, P = 0.015), whereas no difference was observed between the 2 groups of asymptomatic individuals. The prevalence of genotype AA carriers was higher in patients with VT (38%) than in asymptomatic individuals (21%, P = 0.015), leading to an odds ratio of 1.74 (95% confidence interval, 1.3 to 3.8). Carrying the AA genotype conferred a risk of deep VT of 2.08 (95% confidence interval, 1.28 to 3.40), whereas it did not seem to significantly influence the risk of pulmonary embolism. Concerning the −675 4G/5G polymorphism, no significant difference was observed among the 3 groups, the 4G allele frequency being 0.54 (in group 1), 0.49 (in group 2), and 0.45 (in group 3). These data suggest a role for the −A844G PAI-1 gene polymorphism in the thrombophilic phenotype of factor V Leiden carriers. (Arterioscler Thromb Vasc Biol. 2000;20:1387-1391.)

Key Words: PAI-1 ■ genetic polymorphisms ■ venous thrombosis ■ factor V Leiden

Factor V Leiden (FV Leiden), the cause of most cases of resistance to activated protein C, is a common mutation present in 4% to 6% of the general population. Heterozygosity for this mutation leads to a 3-fold increase in relative risk of venous thrombosis (VT; reviewed in Reference 1). However, there is a heterogeneity of thrombotic risk among carriers of this mutation, suggesting that there are additional factors, hereditary or acquired, contributing to the thrombotic phenotype. It has already been shown that in patients afflicted by heterozygous deficiencies of protein C, protein S, or antithrombin, VT is more common and occurs at a younger age when associated with FV Leiden.2–5

Among candidate genes that could contribute to the thrombotic phenotype in FV Leiden carriers, plasminogen activator inhibitor-1 (PAI-1) is of interest. Because of its key role in the inhibition of fibrinolysis, PAI-1 is probably involved in cardiovascular disease. In the promoter region of the PAI-1 gene, 2 polymorphisms, −675 4G/5G and A −844G, have been described.6–8 The first 1 affects the binding of nuclear proteins involved in the regulation of PAI-1 gene transcription,6,9 homozygosity for the 4G allele being associated with increased transcription of the PAI-1 gene. The substitution at position −844, included in a consensus sequence binding site Ets nuclear protein, is also potentially implicated in the regulation of the PAI-1 gene.7,8,10 Although several studies have suggested a contribution from the −675 4G/5G variant in myocardial infarction development,6,9,11 very few data are available on the relations between PAI-1 polymorphisms and the occurrence of VT. Except for 1 study,12 most have not found any relationship between the −675 4G/5G polymorphism and the development of VT in unselected patients.7,13,14 However, the −675 4G/4G genotype has been shown to be linked to an increased risk for VT in patients with protein S deficiency15 or in small series of FV Leiden carriers.16,17 Concerning the A −844G polymorphism, only 1 study was performed, without observing any relation between this polymorphism and the risk of VT in nonselected patients.7

The aim of the current study was to determine the role of the 2 polymorphisms, A −844G and −675 4G/5G, in the PAI-1 promoter in the thrombophilic phenotype observed in FV Leiden carriers.

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We studied the genotype distribution of the polymorphisms A-844G and G-2221A of the Prothrombin gene in 382 individuals heterozygous for the FV Leiden mutation. Individuals were divided into 3 groups (Table 1). Groups 1 and 2 corresponded to all consecutive heterozygous FV Leiden carriers diagnosed through systematic screening for the FV Leiden mutation on the occasion of a health checkup examination at 3 different health centers (Marseille, Paris, and Lille). All of the study subjects were interviewed by a physician about their medical history, which emphasized manifestations of deep venous thrombosis (DVT), pulmonary embolism (PE), and superficial thrombophlebitis (STP). The date of occurrence and site of every thrombotic event were documented by venography, Doppler ultrasound, angiography, and/or ventilation/perfusion lung scan. Written, informed consent were obtained from each individual.

### DNA Analysis

Genomic DNA was prepared by standard salting-out techniques. Detection of FV Leiden (G→A) and genotypes of the PAI-1 G→A polymorphism was performed by using an allele-specific polymerase chain reaction. A polymerase chain reaction was performed for each allele determination according to the following described conditions. Amplification was carried out in 25 μL in a Perkin Elmer Thermocycler 9600 (Applied Biosystems). Each sample contained 62 ng of genomic DNA in 1× Taq polymerase buffer (2.5 mmol/L MgCl₂, 0.77 mmol/L dNTPs, 5 pmol of each primer (forward and reverse primers in each case plus the allele-specific primer that corresponded to the analyzed genotype), and 0.38 U Taq polymerase (Biotaq, Quantum Bioprobe). A first denaturation at 95°C for 2 minutes was followed by 40 cycles of the following conditions: 1 minute at annealing temperature (determined for each reaction); 72°C for 1 minute, 30 seconds (extension); 95°C for 45 seconds (denaturation); and then 72°C for 5 minutes. All primer sequences and annealing temperature are described in Table 2. Five microliters of each amplification sample was loaded onto a 2% agarose gel that was stained with ethidium bromide. The genotypes resulting from the G-to-A substitution at position -844 were assessed by XhoI endonuclease digestion as previously described.

### Statistical Methods

Because individuals within 1 family are not independent, conventional statistical procedures could not be used. Statistical analyses were carried out by using the estimating equations technique proposed by Liang and Zeger. We developed an application of this technique for studying associations between genetic markers and phenotypes by using family data. Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated by this technique. The linkage disequilibrium coefficient (D’) between the 2 PAI-1 polymorphisms was estimated by using log-linear model analysis. Statistical significance was taken at P<0.05.

### Results

Table 1 summarizes the main characteristics of the population. No significant difference in sex distribution was observed among the 3 groups (P=0.8). Whereas the mean age at which blood was drawn was significantly lower in patients from group 2 compared with those from group 1 (P<0.001).

### Methods

#### Patient Selection Criteria

We studied the genotype distribution of the polymorphisms A-844G and G-2221A of the Prothrombin gene in 382 individuals heterozygous for the FV Leiden mutation. Individuals were divided into 3 groups (Table 1). Groups 1 and 2 corresponded to all consecutive heterozygous FV Leiden carriers (n=308) from 220 unrelated families who were diagnosed in 1995 to 1998 at the Hematology Laboratory of Timone (Marseille, France) and who were without antithrombin, protein C, or protein S deficiency and lupus anticoagulant. Group 1 included patients with a personal history of VT. Group 2 included asymptomatic relatives of patients from group 1 or individuals with a familial but no personal history of VT. Group 3 consisted of 74 unrelated asymptomatic FV Leiden carriers diagnosed through systematic screening for the FV Leiden mutation at 3 different health centers (Marseille, Paris, and Lille).

All of the study subjects were interviewed by a physician about their medical history, which emphasized manifestations of deep venous thrombosis (DVT), pulmonary embolism (PE), and superficial thrombophlebitis (STP). The date of occurrence and site of every episode of VT and the presence of precipitating factors (such as surgery, trauma, prolonged immobilization, pregnancy or puerperium, and oral contraceptive intake) were collected. None of the patients had any evidence of neoplastic disease. The term DVT refers to deep leg VT as well as thrombosis at unusual locations such as the axillary, mesenteric, and cerebral veins. VT refers to DVT, PE, or STP. Venous thromboembolism (VTE) refers to DVT complicated or not by PE. The thrombotic events were documented by venography, Doppler ultrasound, angiography, and/or ventilation/perfusion lung scan.

#### TABLE 1. Main Characteristics of the 382 Individuals Heterozygotes for FV Leiden

<table>
<thead>
<tr>
<th>Group</th>
<th>n=168</th>
<th>n=140</th>
<th>n=74</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, M/F</td>
<td>68/100</td>
<td>57/83</td>
<td>33/41</td>
</tr>
<tr>
<td>Age, y</td>
<td>Mean</td>
<td>47</td>
<td>35</td>
</tr>
<tr>
<td>Range</td>
<td>18–66</td>
<td>15–77</td>
<td>17–76</td>
</tr>
<tr>
<td>Age at first event, y</td>
<td>Mean</td>
<td>38</td>
<td>...</td>
</tr>
<tr>
<td>Range</td>
<td>9–76</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Type of VT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VTE</td>
<td>113</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>PE</td>
<td>42</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>STP</td>
<td>13</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Recurrence (&gt;1 VT)</td>
<td>78</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Prothrombin 20210 G→A, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>157 (94%)</td>
<td>132 (94%)</td>
<td>74 (100%)</td>
</tr>
<tr>
<td>AG</td>
<td>10 (6%)</td>
<td>8 (6%)</td>
<td>0</td>
</tr>
<tr>
<td>AA</td>
<td>1 (0.6%)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Group 1 included individuals with VT; group 2, individuals without VT but with a familial history of VT; and group 3, individuals without VT but with a fortuitous discovery of FV Leiden.

*Includes 9 individuals with thrombosis at an unusual location.

#### TABLE 2. Oligonucleotides and Annealing Temperatures Used for Detection of FV Leiden (G→A), Factor II (G20210A), and Genotypes of PAI-1 G→A Polymorphism

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Primer Sequences (5′–3′)</th>
<th>Primer Allele-Specific Sequences</th>
<th>Annealing Temperature, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>FV Leiden, G→A</td>
<td>F: GGGCTAATAGGACTACTCTTCAATC</td>
<td>G: GATCCCTGGACAGGGCA</td>
<td>63</td>
</tr>
<tr>
<td>G→1691A</td>
<td>R: TCTCTTGAGGAAATCCGTCAATT</td>
<td>A: GATCCCTGGACAGGGCA</td>
<td>63</td>
</tr>
<tr>
<td>Factor II</td>
<td>F: CTCTGACATATCTTCCTGCG</td>
<td>A: GAAAAGTGAACCTCAGCG</td>
<td>62</td>
</tr>
<tr>
<td>G+20210A</td>
<td>R: TCCAGTAGATTTACGGCTC</td>
<td>A: AAAAGTGACTCTCGACAG</td>
<td>62</td>
</tr>
<tr>
<td>PAI-1</td>
<td>F: TCAACGACAGCAGGTTGG</td>
<td>4G: GTCTGGACAGCGGGA</td>
<td>62</td>
</tr>
<tr>
<td>G-675 G/5G</td>
<td>R: TTTTCCCCAGGCGTCCCA</td>
<td>5G: GTCTGGACAGCGGGA</td>
<td>62</td>
</tr>
</tbody>
</table>

F indicates forward; R, reverse.
TABLE 3. Genotype and Allele Frequencies of PAI-1 Polymorphisms

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Individuals With VT (n=168)</th>
<th>Individuals Without VT but With a Familial History of VT (n=140)</th>
<th>Individuals Without VT (n=74)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>0.38</td>
<td>0.27</td>
<td>0.24</td>
</tr>
<tr>
<td>AG</td>
<td>0.45</td>
<td>0.50</td>
<td>0.53</td>
</tr>
<tr>
<td>GG</td>
<td>0.17</td>
<td>0.23</td>
<td>0.23</td>
</tr>
</tbody>
</table>

Frequency

| 4G/5G     | 0.54/0.46                   | 0.49/0.51                                                     | 0.45/0.55                     |

*P<0.05 for comparison among the 3 groups.

No significant difference was observed between the mean age at onset of the first thrombosis and the mean age of individuals from group 2 (P=0.7). The mean age at the time of blood drawing was similar in patients with and without VT and in those in whom a fortuitous discovery of the FV Leiden mutation was made (group 3).

The 20210 G→A mutation of the prothrombin gene was present in 11 (7%) patients from group 1, 8 (6%) from group 2, and none of the subjects from group 3. When patients with VT (group 1) were compared with subjects without VT (groups 2 and 3), the prevalence of the A allele carriers was not significantly higher in VT (7%) patients than in asymptomatic individuals (4%, P=0.21). There was a slight but nonsignificant difference in the mean age of the first VT episode between patients with the 20210 G→A mutation and those without (30 versus 37 years, respectively; P=0.2).

Distribution of A−844G and −675 4G/5G Genotypes in Patients With and Without VT

Results are presented in Table 3. The A−844G allele frequency significantly differed among the 3 groups (P=0.048), the A allele being more frequent in patients who suffered from VT (61%) than in subjects without VT (52%, P=0.015). No significant difference was observed in allele frequency between asymptomatic individuals with a familial history of VT and asymptomatic individuals in whom a fortuitous discovery of FV Leiden was made (A allele frequency of 0.52 and 0.51, respectively).

When we compared the −675 4G/5G allele frequency, a difference, though nonsignificant, was observed in allele frequency among the 3 groups, the 4G allele frequency being 0.54 in symptomatic and 0.45 in asymptomatic individuals with a fortuitous discovery of FV Leiden. The 4G allele frequency in asymptomatic individuals with a familial history of VT was intermediate (0.49).

After excluding subjects with the 20210A allele of the prothrombin gene, the distribution of A−844G and −675 4G/5G genotypes was not at all modified in symptomatic and asymptomatic individuals (data not shown). The 2 polymorphisms of the PAI-1 gene were found to be in nearly complete linkage disequilibrium (D*′=0.99, P<0.001), the G allele being almost always associated with the 5G allele.

Relative Risk for VT in −844 AA Carriers

The prevalence of the −844 AA genotype was significantly higher in symptomatic than in asymptomatic individuals (38% versus 26%, P=0.015), leading to an OR of 1.74 (95% CI, 1.11 to 2.71; Table 4). This prevalence was even higher in patients with DVT but without a history of PE (42%, P=0.003, compared with asymptomatic individuals), leading to an OR of 2.08 (95% CI, 1.28 to 3.40). When we reexamined the data for the group of patients with at least 1 objectively verified episode of PE, the prevalence of the AA genotype was not statistically different from that observed in asymptomatic individuals (33% versus 21%). Once again, exclusion of individuals with the 20210 G→A mutation did not modify the results, the prevalence of the −844 AA genotype being 39% in patients with VT and 27% in individuals without VT (P=0.015, OR=1.8). There was no difference in the mean age of the first episode of VT between patients with the −844 AA genotype and those with the AG or GG genotype (37 versus 38 years, respectively; P=0.6).

Discussion

It is now accepted that thrombophilia is a multigenic disorder. Indeed, VT episodes rarely occur in individuals with a single genetic defect but frequently in individuals with 2 or more genetic defects. The aim of this study was to evaluate the role of genetic polymorphisms located in the PAI-1 gene in the risk of VT in heterozygous FV Leiden carriers.

Because the asymptomatic subjects referred to our thrombophilic center belonged mostly to families with a history of VT, we decided to also select a group of individuals (group 3) in whom the FV Leiden mutation was discovered through systematic screening for the FV Leiden mutation on the
occasion of a health checkup examination at 3 different health centers. This procedure avoids selection bias due to the inclusion of asymptomatic individuals from thrombophilic families.

The 20210 A allele of the prothrombin gene was observed in 7% of patients with VT compared with 4% of asymptomatic individuals, this difference being not significant. This finding differs from previous studies that showed a higher risk of VT in individuals with combined defects compared with individuals carrying only FV Leiden.

However, this difference did not reach the level of significance. It is important to note that no individual from group 3 carried the A allele compared with 6% of individuals with a personal or familial history of VT.

In unselected patients with DVT, no association between the 4G/5G polymorphism and the development of VTE was observed in several different studies, in contrast to the findings of Sartori et al, who reported a significantly lower 5G/5G prevalence in DVT patients compared with healthy individuals, without, however, any statistically significant difference in allele frequencies between the 2 groups. On the other hand, in selected populations with an established genetic risk factor for VT, such as protein S deficiency or FV Leiden, concurrence of homozygosity for the 4G allele led to an increased risk for VT. Indeed, in patients with protein S deficiency, Zöller et al reported that homozygosity for the 4G allele was a significant risk factor for PE but not for DVT. Concerning FV Leiden carriers, the association between the 4G/5G polymorphism and VT has been suggested only in relatively small studies. Junker et al found that of patients with cerebral sinus thrombosis and FV Leiden, 7 (87.5%) were homozygous for the 4G allele compared with 23 of the 97 (23.7%) controls without the FV Leiden mutation. Moreover, in a recent study conducted in children, patients with VT carrying FV Leiden more often showed the 4G/4G genotype compared with the controls, these results being reported as preliminary with respect to the small number of subjects investigated. These 2 latter studies led us to evaluate the role of this polymorphism in a larger population of FV Leiden carriers. In the study herein, the frequency of the 4G allele was higher in FV Leiden patients with a history of VT compared with asymptomatic individuals, particularly in individuals with a fortuitous discovery of FV Leiden. However, this difference did not reach the level of significance.

Concerning the A −844G polymorphism, the A allele frequency was significantly higher in patients with a personal history of VT than in asymptomatic individuals. This difference was not observed in the study of Gubric et al. The fact that we have studied the influence of this polymorphism in a population of thrombophilic patients could have magnified its importance. These data are in favor of a mild role played by the A −844G polymorphism that may be difficult to identify, because it individually conveys so little risk that clinical manifestations occur most of the time when it is combined with other genetic risk factors such as FV Leiden. Interestingly, we observed that the −875 AA genotype led to a 2-fold increased risk of DVT in FV Leiden carriers, whereas it did not seem to influence the risk of PE. This finding supports the hypothesis that phenotypic manifestations of VT depend on the thrombophilic trait carried.

A puzzling question concerns the role played by PAI-1 polymorphisms in VT. Despite the fact that the 2 polymorphisms studied might be involved in the promoter activity of the PAI-1 gene, we have previously shown that genetic polymorphisms explain only a small part of PAI-1 plasma level variations in healthy individuals compared with the effect of environmental factors. However, PAI-1 plasma levels might not reflect the expression of PAI-1 in the local environment within the thrombus. This latter hypothesis is supported by the fact that PAI-1 mRNA expression is increased in the endothelial cells located in the vicinity of pulmonary arterial thrombi, this overexpression not being correlated with plasma PAI-1 antigen levels. In the study herein, the 2 polymorphisms are in strong linkage disequilibrium, as was previously shown in other studies. The fact that the A −844G polymorphism was more closely associated with the risk of VT than was the 4G/5G polymorphism in the present study might be due to random fluctuations. It is not possible from these results to confirm a functional role for any of the 2 polymorphisms. They might also be markers of an as-yet-unidentified functional mutation.

In conclusion, the −844 AA genotype of the PAI-1 gene is a risk factor for VT in FV Leiden carriers. This finding could be important to assess more accurately the risk of VT and to better manage prophylactic and diagnostic measures, especially in the case of triggering conditions in FV Leiden carriers.

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7. Gubric N, Stegnar M, Peternel P, Kaider A, Binder BR. A novel G/A and C/G polymorphisms in VT. Despite the fact that the 2 polymorphisms studied might be involved in the promoter activity of the PAI-1 gene, we have previously shown that genetic polymorphisms explain only a small part of PAI-1 plasma level variations in healthy individuals compared with the effect of environmental factors. However, PAI-1 plasma levels might not reflect the expression of PAI-1 in the local environment within the thrombus. This latter hypothesis is supported by the fact that PAI-1 mRNA expression is increased in the endothelial cells located in the vicinity of pulmonary arterial thrombi, this overexpression not being correlated with plasma PAI-1 antigen levels. In the study herein, the 2 polymorphisms are in strong linkage disequilibrium, as was previously shown in other studies. The fact that the A −844G polymorphism was more closely associated with the risk of VT than was the 4G/5G polymorphism in the present study might be due to random fluctuations. It is not possible from these results to confirm a functional role for any of the 2 polymorphisms. They might also be markers of an as-yet-unidentified functional mutation.

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