Polymorphisms in the Prothrombin Gene and their Association with Plasma Prothrombin Levels

H. Ceelie1, R. M. Bertina1, A. van Hylckama Vlieg1,2, F. R. Rosendaal1,2, H. L. Vos1

1Haemostasis and Thrombosis Research Centre, Dept. of Haematology, 2Dept. of Clinical Epidemiology, Leiden University Medical Centre, Leiden, The Netherlands

Key words
Prothrombin, G20210A, A19911G, venous thrombosis

Summary
To find genetic causes of high plasma prothrombin levels, an established prothrombotic risk factor, we searched for sequence variations in the prothrombin gene. We selected subjects with the 20210-GG genotype (since the 20210-A allele is already known to be associated with high levels) and elevated prothrombin levels (≥ 130 U/dl) from the Leiden Thrombophilia Study (LETS). No mutations were found in the 1 kb promoter region of the prothrombin gene in seven individuals with an isolated high prothrombin level. Comparison of the allelic frequencies of four different polymorphisms in the prothrombin gene in healthy volunteers and in the control subjects among the selected LETS individuals indicated a higher frequency of the 19911-G allele in the latter group (allele frequency 52 vs. 78%, respectively). Homozygous carriers of the 19911-G allele had 8 U/dl higher plasma prothrombin levels than 19911-AA carriers. This difference in prothrombin levels did not affect the thrombotic risk in 20210-GG carriers. In heterozygous 20210-A carriers the odds ratio increased from 1.6 (95% CI: 0.6-4.3) in subjects with 19911-A to 4.7 (1.6-14.0) in subjects with 19911-G on the other prothrombin allele.

Introduction
Prothrombin is a key component in the blood coagulation pathway. It is the precursor of the serine protease thrombin, which exhibits pro-coagulant, anticoagulant, as well as anti-fibrinolytic activities [for a review, see Mann (1)]. The prothrombin gene is located on chromosome 11 at position 11p11-q12 (2). It has a length of 21 kb and includes 14 exons and non-coding regulatory sequences (3).

Recently, a G to A transition at nucleotide position 20210 in the 3' untranslated region of the prothrombin gene was described, which is associated with a threefold-increased risk of deep vein thrombosis (DVT) (4). The importance of the 20210-A allele as a risk factor for venous thrombosis was confirmed in several independent studies (5-12). In these studies, the 20210-A allele was found in 4-8% of patients with previous venous thromboembolism (28%/10 than non-symptomatic, asymptomatic carriers of the 20210-A allele (4), indicating the presence of additional causes of high prothrombin levels, possibly genetic in origin. This idea is further supported by a study of Simioni et al. (19), in which it was shown that even among carriers of the 20210-G (wild-type) allele, many more patients with previous venous thromboembolism (28%) than non-symptomatic individuals (7%) had prothrombin antigen levels above the cut-off value of 115 U/l.

In the present study we looked for sequence variations in the prothrombin gene associated with elevated plasma prothrombin levels in subjects with the 20210-GG genotype. Whenever such an association was found, we also investigated the relation with thrombotic risk.

Subjects, Materials and Methods

Subjects
To estimate the frequency of prothrombin gene polymorphisms in the normal population, 64 healthy volunteers, not using oral contraceptives, were recruited from hospital personnel. Carriers of the 20210 A allele were excluded (n = 3). From one subject no plasma sample was available.

A second group of subjects came from a population-based case-control study, the Leiden Thrombophilia Study (LETS), which has been described in detail before (12). Briefly, consecutive patients with a first episode of DVT were selected from three anticoagulation clinics in the Netherlands. All patients were younger than 70 years of age and had not been diagnosed with cancer. The patients were asked to find their own healthy control subject; if they were unable to do so, partners of other patients served as controls. Controls were matched for age ± 5 years and sex with the cases. The study included 474 patients and 474 controls. From these 948 individuals, we selected all subjects with the 20210 GG genotype and prothrombin levels ≥130 U/dl (28 patients and 18 controls). Only 7 subjects (2 patients and 5 controls) had an isolated elevated prothrombin level, i.e. all, or all but one, of the other coagulation factors measured, i.e. FX, FVII, FIX, FX, FXI, FXIII, Protein S, Protein C, AT, and antithrombin were <1.50.

Since prothrombin levels increase the risk of thrombosis, analyses of the association between polymorphisms and plasma prothrombin levels were restricted to the control subjects of the LETS, excluding 20210-A carriers and
individuals using coumarins at the time of venepuncture. DNA was unavailable for two individuals, and 460 samples were studied.

The effect of polymorphisms in the prothrombin gene on the risk of thrombosis was studied by comparing the prevalence in patients and controls, in some of the analyses limited to those without the 20210-A allele.

Finally, we performed an analysis restricted to 20210-A carriers (29 patients, 11 controls) to assess the additional effect of the 19911-G allele on the risk of thrombosis and plasma prothrombin levels (excluding two patients using coumarins at the time of venepuncture for the latter analysis).

A third group of 22 homozygous carriers of the 20210-A allele was used to analyse the haplotype of the prothrombin 20210-A allele. Subjects came from Australia (n = 1), Austria (n = 2), France (n = 5), Germany (n = 3), Italy (n = 1), the Netherlands (n = 5), Spain (n = 4) and from the USA (n = 1).

Blood Collection and Laboratory Analysis

Blood was collected into tubes containing 106 mmol/l trisodium citrate. Plasma was prepared by centrifugation for 10 min at 2,000 g at room temperature and stored at -70°C. Genomic DNA was isolated from leukocytes using standard methods and stored at 4°C.

In the healthy volunteers, prothrombin activity was measured with a chromogenic method using S-2238 (Chromogenix, Milan, Italy) as a substrate and Ecarin (Sigma Chemical Co. St Louis, MO) as an activator on an ACL-200 following the instructions of the manufacturer. In the participants of the Leiden Thrombophilia Study coagulation factor levels had been measured as described previously (23-32). Prothrombin activity had been measured with a method comparable to that described above (4).

Sequencing of the Promoter Region of the Prothrombin Gene

To identify changes in the promoter region of the prothrombin gene in the seven subjects with isolated high prothrombin levels, a 947 bp fragment (nt -921 to 26 relative to the transcription start site, numbering according to Bancroft et al. [33]) was amplified by standard PCR (see below) using the following primers: 5'-CCG AAA GCT TGT TGT CCT CTT TGT CCC T-3' and 5'-ATG GAA GCT TGT CAG CTC CTG GGT CAC TGA G-3'. Fragments were sequenced using the same primers and an ABI PRISM BigDye Terminator Cycle Sequencing Kit (Perkin Elmer-Cetus). Two additional internal primers were used: 5'-CCA TGG ACA TTC CAT TCC TAA TCT CC-3' and 5'-CTG TGT GCC TCA GTT TCC TCA TC-3'.

Genotype Analysis

For the determination of genotypes of the prothrombin gene, four biallelic polymorphisms were used (Table 1). Three of them, the polymorphisms in intron 4 (T3728C), intron 5 (C4125G) and intron 13 (A19911G) have been previously described (34, 35). The polymorphism in intron 11 (T9832C) was identified in this study by direct sequencing of randomly selected healthy individuals. To study the haplotype of homozygous 20210-A carriers, an additional polymorphism in exon 10 (G8845A) was used (35). All polymorphisms were detected by PCR and allele-specific restriction analysis. PCR was performed according to standard procedures, using genomic DNA (0.1-1 μg), 200 mM of each dNTP (Pharmacia, Uppsala, Sweden), PCR buffer (67 mM Tris- HCl (pH 8.8), 6.7 mM MgCl2, 6.7 μM EDTA, 16.6 mM (NH4)2SO4 and 10 mM β-mercapto-ethanol), 100-200 ng of each primer, 0.1 mg/ml bovine serum albumin (Pharmacia), 10% dimethylsulfoxide and 1 U AmpliTaq DNA polymerase (Perkin Elmer-Cetus) in a final volume of 50 μl. For the detection of the polymorphism in intron 11 the PCR buffer did not contain bovine serum albumin and dimethylsulfoxide. Thermal cycling was carried out by incubation at 92°C for 2 min, followed by 33 cycles of denaturation at 92°C for 30 s, annealing for 30 s and extension at 72°C for 1 min and a final step of 4 min at 72°C. For restriction analysis 10 μl of product was digested with 2.5 to 5 U of enzyme (New England Biolabs, Beverly, MA) according to the manufacturer’s instructions.

To determine haplotypes in individuals homozygous for both the 20210-A and 19911-G allele, allele specific PCRs for the 19911-A and 19911-G allele were performed (Table 1B). The two PCRs were done as mentioned above, with the exceptions that 7.5% DMSO and 100 ng of each primer were used and that thermal cycling was performed in 36 cycles with an extension time of 45 s. The 20210 genotype was subsequently detected by restriction analysis of the allele-specific PCR products with HinfIII, as described previously (4).

Statistical Analysis

Odds ratios were calculated as estimates of the relative risk of thromboas in the standard unmatched fashion. Ninety-five percent confidence intervals (95% CI) were constructed according to Woolf (36). Haplotype frequencies, used to calculate linkage disequilibrium coefficients, were estimated according to Ott (37). The standard disequilibrium coefficient (38) (Δ) and Lewontin’s D’ (39) were used as measures for linkage between nt 4125 and nt 19911.

Table 1: Location of single nucleotide polymorphisms in the prothrombin gene used in this study, primer properties, and restriction enzymes used for detection. A: Primers for the allele specific detection of the 19911 polymorphism

<table>
<thead>
<tr>
<th>Polymorphism (location)</th>
<th>primers (5'-3')</th>
<th>Nucleotide numbering</th>
<th>Tm (°C)</th>
<th>Product size (bp)</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>A) T3728C (intron 4)</td>
<td>TCCGACTGTTGCTGGATGGATCATG ACCCGAGCTGAGCAGCCATCACATTAC</td>
<td>nt 3474 - 3488</td>
<td>60</td>
<td>479</td>
<td>HpaII</td>
</tr>
<tr>
<td>C4125G (intron 5)</td>
<td>GAATGGGCGGTCGGCGCTTC CCGCGAGCTGAGCAGCCATCACATTAC</td>
<td>nt 4036 - 4056</td>
<td>62</td>
<td>206</td>
<td>Mnl</td>
</tr>
<tr>
<td>G8845A (exon 10)</td>
<td>CTAATGCTGAGTTGATTTAC GGACTTCTGCTGGCGCTTC</td>
<td>nt 8656 - 8675</td>
<td>53</td>
<td>360</td>
<td>Faul</td>
</tr>
<tr>
<td>T9832C (intron 11)</td>
<td>CTTGACGCTGACCACTTACCAGCTCTTGGGGCTTC</td>
<td>nt 9527 - 9550</td>
<td>60</td>
<td>621</td>
<td>Smal</td>
</tr>
<tr>
<td>A19911G (intron 13)</td>
<td>AGGATGACCTTCTTAAAGCCCGAGG CATTGGATACAGCGGTGGTAGTAAAGGGG</td>
<td>nt 19822 - 19845</td>
<td>62</td>
<td>178</td>
<td>Mnl</td>
</tr>
</tbody>
</table>

| B) 19911G                | GTATCCTGAAAAACAGGATCCTGAGGCGG | nt 26460 - 26485 | 68 | 348 | HindIII |
| 19911A                  | GTATCCTGAAAAACAGGATCCTGAGGCGG | nt 26460 - 26485 | 68 | 348 | HindIII |
| G20210A                 | ATAGGCGCTGAGGACATTTAGG | nt 26807 - 26786 | 26 | 178 | HindIII |

1 Nucleotide numbering is according to the original publication of Frezner Degen et al. (3)
Results

Sequence Variations in the Prothrombin Promoter Region

We screened seven individuals with an isolated high prothrombin level for mutations in the promoter region of the prothrombin gene by sequencing of almost 1 kb upstream of the transcription start site. This region contains all presently known transcription regulatory elements. Compared with the nucleotide sequence of Bancroft et al. (33), two variations, an insertion of an A at position -22 and an insertion of a G at position -646 were found in a homoyzygous state in all seven subjects and in control DNA, suggesting that they represent errors in the original sequence. The insertion at -646 has previously been described by Zivelin et al. (35). No other sequence variations in the promoter region were observed.

Allele Frequencies of Prothrombin Gene Polymorphisms in Selected Individuals and Healthy Controls

All control subjects from the LETS study with plasma prothrombin levels ≥ 130 U/dl and with the 20210-GG genotype (n = 18) were genotyped for four polymorphisms in the prothrombin gene (T3728C, C4125G and T9832C and A19911G) as were 61 healthy volunteers with the 20210-GG genotype. The allele frequencies are summarised in Table 2. For the polymorphisms at nt 3728, 4125 and 9832 no major differences were found. However, as to the A19911G polymorphism, we found that the 19911-G allele was more frequent among the LETS controls with plasma prothrombin levels ≥ 130 U/dl (allele frequency 78%) than in the group of healthy volunteers (allele frequency 52%). Moreover, it was striking that 19911-AA carriers were absent from the first group. These results suggested an association between the nucleotide at 19911 and plasma prothrombin levels. The 19911 genotypes of the seven subjects with isolated elevated plasma prothrombin levels (see previous paragraph) were: 19911-AG (n = 3; all controls) and 19911-GG (n = 4; 2 patients and 2 controls).

The A19911G Polymorphism and Prothrombin Level

Further support for the supposition that the 19911 polymorphism is associated with plasma prothrombin levels came from the observation that in the healthy volunteers mean values for plasma prothrombin levels for 19911-AA (n = 13), AG (n = 33) and GG (n = 14) carriers were 94.4 U/dl (95% CI, 86.9-101.8), 101.8 U/dl (97.0-105.6) and 108.6 U/dl (102.2-113.8), respectively. Despite the relatively small number of subjects this indicated an allele dosage effect on prothrombin levels. For instance, 19911-GG carriers all had similar prothrombin levels, independent of their genotype at nt 4125. The apparent association between the polymorphic site at nt 4125 and prothrombin levels is therefore the result of the existing linkage disequilibrium between this site and the polymorphism at nt 19911. (Δ = -0.66 and D’ = -0.98).

The A19911G Gene Polymorphism and the Risk of Deep Vein Thrombosis

Table 5 illustrates that the presence of the 19911-G allele in 20210-GG carriers was not associated with an increased risk of deep vein thrombosis, despite its association with higher prothrombin levels. Neither heterozygous nor homozygous carriers of the allele showed an increased risk of venous thrombosis. Apparently, the moderate increase in prothrombin levels does not influence the thrombotic risk.

We investigated whether the 19911-G allele may cause an additional risk of DVT in 20210-A carriers. It has previously been shown that 20210-A allele is in strong linkage disequilibrium with 19911-A (35).
We could confirm this observation in 22 homozygous carriers of the 20210-A allele, that were genotyped for five prothrombin gene polymorphisms. All subjects were homozygous for a prothrombin allele with the haplotype: 3728T-4125C-8845G-9832T-19911A-20210A (44 alleles).

Eighteen out of forty 20210-AG carriers from the LETS were homozygous for the 19911-A allele, so these individuals carried one 19911-A/20210-A allele and one 19911-A/20210-G allele. The other twenty-two 20210-AG carriers had the 19911-AG genotype. In these individuals haplotypes were determined by using a PCR specific for the 19911-A or -G allele, followed by restriction enzyme analysis to identify the nucleotide at position 20210. It appeared that all 22 individuals carried one 19911-A/20210-A and one 19911-G/20210-G allele.

Interestingly, among 19911-A/20210-A carriers 62% of the patients but only 36% of the controls carried the 19911-G/20210-G allele as the second allele (Table 6). Further analysis showed that, when using 20210-AG-carriers as the reference group, 20210-AG subjects with the 19911-G allele as the second allele, compared to those with 19911-A/20210-A and one 19911-G/20210-G allele to the thrombotic risk of 19911-A/20210-A carriers. However, it should be noted that the confidence intervals of the estimates of risk of DVT showed a large overlap. Within the group of 20210-AG carriers mean values of plasma prothrombin levels for 19911-AA (n = 17) and 19911-AG (n = 21) carriers did not differ: 131.0 U/dl (95% CI 121.1-140.8) and 132.7 U/dl (125.3-139.3), respectively.

Discussion

In this study, we have searched for additional genetic causes of high plasma prothrombin levels, an established risk factor for venous thrombosis. Our investigations have been focussed on the prothrombin gene itself. The approach was first to look in control subjects from the Leiden Thrombophilia Study (LETS) with the 20210-GG genotype and with an isolated elevated plasma prothrombin level (≥130 U/dl) for sequence variations in 1 kb of the promoter region of the prothrombin gene. We did not observe any relevant variation. We conclude that the high plasma levels of prothrombin in these subjects are not the result of genetic differences in the promoter region of the prothrombin gene.

Subsequently, we determined the allele frequencies of four prothrombin gene polymorphisms in the 20210-GG LETS controls with plasma prothrombin levels ≥130 U/dl and compared the observed allele frequencies with those observed in a group of healthy volunteers. By this approach we found an association between the 19911-G allele and prothrombin levels. The effect of this allele is small when compared to that of the 20210-A allele. This might explain why the 19911-G allele is not a risk factor for venous thrombosis in 20210-GG carriers. Interestingly, it was a risk factor when present together with the 20210-A allele. The risk associated with the 20210-A allele was the highest when the other prothrombin allele carried a 19911-G. Using a combination of allele-specific oligonucleotides and restriction analysis, it was confirmed that the 19911-G was indeed on the 20210-G allele and not on the 20210-A allele, so recombination resulting in the combination 19911-G/20210-A could be excluded. The confidence intervals of the estimates of risk of DVT show a large overlap and we can not exclude the possibility that the differences in risk between carriers and non-carriers of the 19911-G allele within this group are a result of chance. One would expect to find slightly higher plasma prothrombin levels in 20210-AG carriers with the 19911-G allele as the second allele, compared to those with 19911-A allele as the second allele; this is presently not the case, but to demonstrate a difference of 4 U/dl many more individuals will be needed.

There is no obvious explanation for the association of the 19911-G allele with increased plasma prothrombin levels. The 19911 polymorphism is located approximately in the middle of intron 13, the last and relatively small intron of the prothrombin gene. It has been shown that sequences in the last intron of a gene can influence 3'-end formation (40). However, with regard to the region of the 19911 polymorphism, no regulatory sequences have been identified so far. It is therefore by no means certain that this polymorphism itself influences prothrombin expression. Possibly, it is a marker for another variation in (part of) these prothrombin alleles. Further detailed haplotype analysis of the 19911-G alleles can help with the identification of the sequence variation responsible for the elevated plasma prothrombin. So far, we identified four different haplotypes of the prothrombin gene with a G at position 19911 in 20210-A carriers (data not shown). However, we were not able to detect significant differences in expression level between these alleles. In conclusion, our research has shown that there are at least two DNA variations in the prothrombin gene that are associated with increased prothrombin levels. Coexistence of the two changes (G20210A and A19911G) might increase the chance of thrombosis.

Acknowledgements

This study was supported by grant no. 97 001 from the Trombosestichting Nederland. The LETS study was originally supported by a grant from the Netherlands Heart Foundation (89-063).

We like to thank J. Thom, P.A. Kyrie, I. Jahan-Vague, K.H. Reuner, A. Siegmann, F. Bernardi, J. Danneberg, J. van der Meer, J.M. Soria and R. Wang who kindly provided DNA samples from homozygous 20210-A carriers and we like to thank H. de Ronde and C. Spaargaren for their technical assistance.

References


1069