Haematologica 1999; 84:204-207 original paper

Simultaneous detection of FV Q506 and prothrombin 20210 A variation by allele-specific PCR

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ABSTRACT

Background and Objective. **Factor V Leiden is the most important risk factor for hereditary thromboembolism, whereas the mutation in the 3'-untranslated region of the prothrombin gene seems to be only a mild risk factor for thrombotic events. On the other hand the factor V mutation (Arg 506) is frequently coinherited with the prothrombin 3'-untranslated region G20210A variant and there is increasing evidence that the co-segregated prothrombin variant is an additional risk factor for venous thromboembolism, contributing to thrombotic manifestations. A rapid, simple and cost-effective screening method is, therefore, required for the detection of both factor V Leiden and the prothrombin variant A20210G.**

Design and Methods. **Eighty-eight patients were enrolled in this study. Forty-four had a previously identified factor V and / or prothrombin mutation, the remaining 44 patients served as negative controls. A multiplex allele specific oligonucleotide PCR was established for the simultaneous detection of the two genetic risk factors for thrombophilia. To test the specificity of the simultaneous ASO PCR approach, the mutated and physiological factor V and prothrombin amplification products were sequenced.**

Results. **The factor V Leiden mutation and the prothrombin variant were correctly identified in all of 44 patients with known mutations. Furthermore the test was able to detect the mutated factor V and the II variant alone, as well as in the cosegregated pattern. Five patients with a homozygous pattern of factor V Leiden or prothrombin variant were also correctly identified. The sensitivity of the test is therefore 100 %. In none of the 44 control cases were false positive results seen.**

Interpretation and Conclusions. **The ASO PCR test is a rapid, simple and cost-effective screening test for thrombophilia.**

Key words: factor V Leiden, prothrombin 20210A

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In the past few years new risk factors for venous thrombosis have been discovered.[1] Besides acquired risk factors, such as hyperhomocysteinemia and high levels of factor VIII,[1,2,7] genetic abnormalities, such as factor V Leiden (FVQ506)[3,4] and the polymorphism in the prothrombin gene (20210 G to A),[5] are the most important causes of hereditary thrombosis. Among Caucasians the prevalence of factor V Leiden ranges from 3-7%,[6,8] whereas the prothrombin variant is present in approximately 2% of the population.[9]

As a functional test for thrombotic events APC resistance can be performed, but unfortunally this test cannot be carried out in patients receiving anticoagulant therapy and, furthermore, it does not discriminate between the FQ506 mutation and the recently discovered mutation at the 306 position.[10,11] No functional test is so far available for the prothrombin variant. Molecular biology techniques, such as RFLP analysis, the PCR approach, followed by restriction enzyme digestion, heteroduplex analysis or PCR based direct sequencing are time-consuming and labor-intensive. Therefore simple and rapid systems for the detection of factor V mutation and prothrombin variant by allele specific PCR amplification have been introduced.[12,13]

We present a new, simple, rapid and cost-effective approach for the simultaneous detection of factor V Leiden and the prothrombin variant 20210 A, using an allele specific PCR approach, without the need for restriction analysis.

Design and Methods

DNA samples and primers

DNA samples were obtained from a total of 88 patients. High molecular weight DNA was extracted from the white blood cell fraction, using DNAzol Reagent (Life Technologies, Grand Island, NY, USA).

For the detection of the factor V Leiden mutation the following three primers, described by Blasczyk *et al.*,[12] were used: sense 5' CTT TCA GGC AGG AAC AAC ACC 3', antisense 5' GGA CAA AAT ACC TGT ATT GCT C 3' and antisense primer for the amplification of the mutated gene 5' TGG ACA AAA TAC CTG TAT ACC TT 3'.

The following sense primer, described by Poort *et*

al.,[13] was used for the detection of the prothrombin variant: 5' TCC GCC TGA AGA AGT GGA TA. Additionally new primers were generated for the detection of the mutation: 5' CCA ATA AAA GTG ACT CTC AGC A 3,' and the physiological prothrombin gene, respectively: 5' CCA ATA AAA GTG ACT CTC AGC G 3', differing only in the last nucleotide, i.e. the 20210 G/A base variation.

As internal control of the multiplex PCR, two additional primers were included in each PCR mix, 5'-primer 5' GCC TTC CCA ACC ATT CCC TTA 3' and 3' primer 5' TCA CGG ATT TCT GTT GTG TTT C 3', amplifying a 429 bp fragment of the human growth hormone gene as described by Blasczyk *et al*.[12]

PCR conditions for simultaneous factor V and prothrombin variant ASO PCR

The PCR mixture in a final volume of 20 µL consisted of 100 ng genomic DNA, 60 mM Tris HCl (pH =8.8), 15 mM ammonium sulphate, 3.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 pmol of specific prothrombin primer, 3 pmol of the factor V Leiden primer and 1 pmol of each control primer. Taq polymerase was added at a concentration of 0.25 U. Amplification was carried out in the Hybaid Touch down PCR. After a first denaturation step at 94° for 2 min, 10 two temperature cycles, consisting of denaturation at 94°C for 20 sec and annealing/extension at 66°C for 40 sec were carried out, followed by 20 three temperature cycles with denaturation at 94°C for 20 sec, annealing at 62°C for 20 sec and extension at 72°C for 20 sec.

To confirm the results obtained by the multiplex PCR, all 88 samples were tested by a second method. For the Factor V Leiden mutation all samples were tested with the functional test (Biomerieux) as well as by the ASO PCR approach, described by Blasczyk *et al*.[12] The prothrombin variation was identified by a PCR and subsequent Hind III digestion as described by Poort *et al*.[5]

Sequence analysis

Sequence analysis was carried out using a direct sequence approach (Dye terminator ready reaction kit; Perkin Elmer) using an ABI373 automated sequencer (ABS). Briefly, for the prothrombin variant a 270 bp amplification product was generated, using the primer described by Poort *et al.*[13] Physiological and mutated factor V were directly sequenced, using the ASO PCR amplification products.

Results

Subjects

A total of 44 patients with a known factor V and/or factor II mutation were enrolled in this study. Twenty-four patients had a positive functional test for APC resistance (Biomerieux). The presence of the F VQ506 mutation was confirmed by ASO PCR for factor V Leiden, as described elsewhere.[12] Twelve patients with a heterozygous pattern for the prothrombin variant A20210G were identified according to the method described by Poort *et al.*[5] Using the same methods, 3 patients homozygous for factor V Leiden, 1 patient homozygous for the prothrombin variant and 4 patients with a combined factor V and prothrombin variant were identified.

Forty-four samples with negative results by APC resistance and for prothrombin variant served as negative controls.

Multiplex PCR

The primers used for the physiological FV and FV mutation generate a 233 bp and 234 DNA fragment respectively, whereas the primers used for physiological prothrombin gene and the prothrombin variant 20210 generate a 145 bp fragment. As a positive internal amplification control, a growth hormone gene was amplified, resulting in a 429 bp fragment (Figure 1 A and B).

The factor V Leiden and prothrombin variant were correctly identified in all 44 patients. The ASO PCR discriminated perfectly between the heterozygous and homozygous patterns of both factor V and/or prothrombin variant (Figure 1 lanes 1-4). The four patients with a known combination of factor V Leiden and prothrombin variant were also correctly identified (Figure 1 lane 5). No false negative results were seen in this group, indicating a sensitivity of 100%. In the control group consisting of 44 patients without mutation no false positive results were seen. To test the specificity of the combined ASO PCR

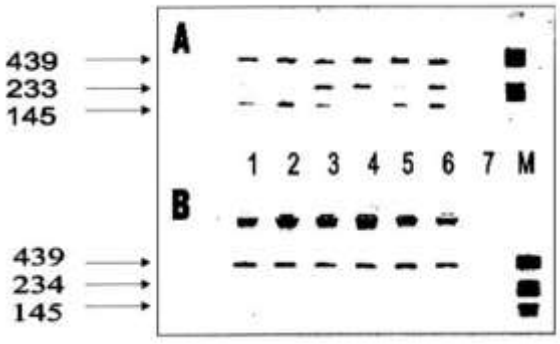

Figure 1. Simultaneous detection of factor V and prothrombin mutation FQ506 and G20210A by ASO PCR analysis. In A, the PCR products for the wild type factor V and the 3' untranslated region of the prothrombin variant are shown. In B, primers for mutated factor V and prothrombin have been used.
Lane 1: heterozygous for factor V; Lane 2: homozygous for factor V; Lane 3: heterozygous for prothrombin variant; Lane 4: homozygous for prothrombin variant; Lane 5: heterozygous for factor V and prothrombin variant.; Lane 6: patient without mutated Factor V and prothrombin; Lane 7: negative control; M Marker.

approach the mutated and physiological amplification products of 4 different patients were sequenced. The four sequence analysis confirmed the mutated or the wild type of the amplified fragment.

Screening strategy

The multiplex PCR was carried out, using only the primers which recognize the mutated allele of both factor V Leiden and/or the prothrombin variation. Only patients with a positive factor V Leiden and/or prothrombin variant were tested further with the primers which recognize the physiological factor V and the 3'untranslated region of the prothrombin gene in order to investigate the heterozygous and homozygous patterns (Figure 2).

Discussion

Factor V Leiden mutation is the most important risk factor for venous thromboembolism,[1, 14] whereas the mutation in the 3' untranslated region of the prothrombin gene seems to be only a mild risk factor for thrombotic events.[5,9] It has been clearly shown that factor V mutation Arg 506 is frequently coinherited with the prothrombin variant.[15,16] The association of the two prothrombotic alleles indicate that the prothrombin variant is an additional risk factor for venous thromboembolism and might contribute to thrombotic manifestations.[16]

There is a high prevalence of the two described risk factors for venous thrombosis in the population. Therefore a rapid, simple and cheap test for the simultaneous detection of the point mutations for factor V Leiden and the prothrombin variation is required. Various different methods for the simultaneous detection are described in literature, among which a very elegant, but laboratory-intensive heteroduplex technique[17] and two simple and fast PCR approaches.[18,19] All these methods require further analysis of the amplification products, e.g. enzyme digestion. We describe here a fast and simple test for simultaneous detection of factor V Leiden and the prothrombin variant, using allele specific primers which specifically recognize the mutated genes. Time-consuming restriction analysis is not necessary. Using the allele specific PCR approach, pure DNA and standardized PCR conditions are required. Furthermore the temperature and/or primer concentration are crucial points for the specificity of the ASO PCR approach, as pointed out also by Poort *et al*.[13] Impure DNA or a minimal change of primer concentration can lead to unspecific amplification products, especially for the mutated factor II gene.

These technical problems can be easily overcome by using mastermixes to which only DNA and Taq polymerase must be added. Furthermore, we obtained an enormous reduction in costs using our strategy. The first step of the simultaneous factor V and prothrombin variation screening reduces the costs by more than 50%, because patients with a mutated allele for factor V and prothrombin variant are identified in a single step. The second step, the determination of normal factor V and prothrombin is carried out only in the patients with a mutated allele of factor V and/or factor II. We therefore achieve a nearly 90% reduction of costs compared to those of the single determination procedure, without any loss of sensitivity or specificity.

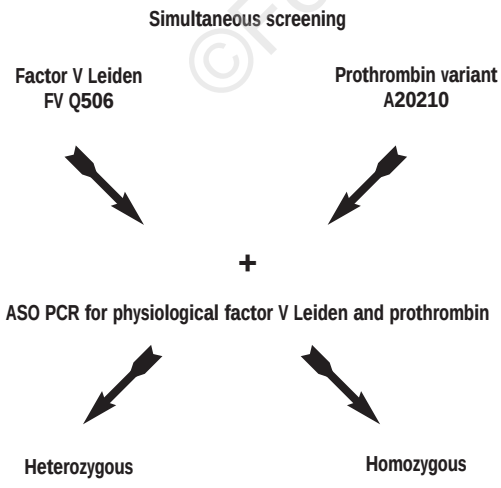

Figure 2. Screening strategy.

Contributions and Acknowledgments

MM was responsible for the conception of the study and for interpretation of the results; he also wrote the paper. AJL established the simultaneous ASO PCR approach, WM and KG carried out sample preparation, ASO PCR, functional test for the factor V Leiden and sequence analysis. PC was the senior author and responsible for the final version of the paper.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received September 7, 1998; accepted January 4, 1999.

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