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Simultaneous Genotyping for Factor V Leiden and Prothrombin G20210A Variant by a Multiplex PCR-SSCP Assay on Whole Blood

Dear Sir,

The understanding of the genetic basis for hereditary thrombophilia has been substantially expanded by the detection of common mutations in the genes for factor V (FV Leiden – G1691A) and prothrombin (G20210A mutation) associated with a remarkable increase in risk to develop thrombosis (1, 2). Therefore, genotyping for both factors has been included into routine diagnostic procedures. A variety of methods based on polymerase chain reaction (PCR) amplification of the corresponding gene fragments have been developed to detect either of these mutations separately. However, performing two different tests for large numbers of samples is time-consuming and cost-benefit calculations are critical, especially when considering prophylactic testing of individuals at risk. Thus, combining both mutations in a single genotyping procedure is of great practical impact. Two such approaches based on multiplex PCR and restriction analysis have been reported (3, 4). One of these procedures was further simplified by applying whole blood directly to the PCR reaction (4). Recently, two further assay procedures based on multiplex PCR and heteroduplex analysis (5) or on allele-specific multiplex PCR amplification (6) using isolated DNA have been described. Both test procedures allow the concomitant detection of a third possible risk factor for venous thrombosis, the methylenetetrahydrofolate reductase (MTHFR) C677T mutation.

We have developed an alternative assay for the simultaneous detection of factor V (FV) Leiden and prothrombin (PT) 20210 G/A dimorphism which can be also performed on whole blood. First, both gene fragments are amplified by multiplex PCR followed by testing for the type of alleles present by single-strand conformation polymorphism (SSCP). Primers used for the FV gene were essentially as described by Bertina et al. (1) with a slight modification in the reverse primer (forward: 5'-GATGCCCAGTGCTTAACAAGACCA-3', reverse: 5'-TGTTATC-ACACTGGTGCTAA-3'). The primers for the PT gene fragment were: 5'-GGATGGGAAATATGGCTTCTA-3' (forward – nt20032-20052) and 5'-GAATAGCA-CTGGAGCATTGA-5' (reverse – nt20235-20215). Sizes of the amplification products are 268 bp for the FV gene fragment and 204 bp for the PT fragment. For multiplex PCR, 20 pmol of each of the four primers were included into the 50 µl reaction mixture containing 10 mmol/l Tris (pH 8.7), 40 mmol/l KCl, 3.5 mmol/l $MgCl_2$, 17 mmol/l $(NH_4)_2SO_4$, 0.2 mmol/l of each deoxynucleotide triphosphate (dNTP) and 1.25 units of Taq polymerase. After denaturing at 96° C for 10 min, 35 cycles containing the following steps were performed: 1 min at 55° C, 3 min at 72° C, 45 sec at 97° C. After final extension at 72° C for 10 min samples were cooled to 4° C. For genotype analysis by SSCP an aliquot of the PCR sample was mixed with an equal amount of formamide, heated to 95° C for

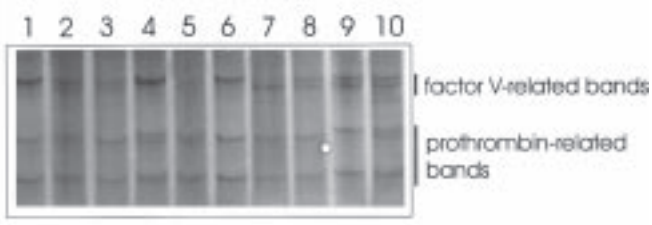

Fig. 1 Simultaneous detection of factor V Leiden mutation (G1691A) and the prothrombin G20210A variant by multiplex PCR and single-strand conformation polymorphism (SSCP) in blood samples. SSCP gel demonstrating band patterns for different genotypes. Lanes 1 and 6 – homozygotes for the two normal alleles (1691G and 20210G); lanes 2, 5 and 10 – samples heterozygous for both factor V Leiden and prothrombin 20210A variant; lanes 3 and 8 – samples heterozygous for factor V Leiden and homozygous for the normal prothrombin allele; lane 4 – homozygous for the normal 1691G allele and heterozygous for the 20210A allele; lane 7 – sample homozygous for factor V Leiden and the normal 20210G allele; lane 9 – sample from a patient heterozygous for factor V Leiden and homozygous for the prothrombin 20210A variant

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5 min and then placed immediately on ice. A 15 µl-aliquot of this sample was subjected to electrophoresis at constant voltage (120 V) for 18 hours at room temperature in a 10% (w/v) polyacrylamide gel, sized 190 × 245 × 0.6 mm and containing 5% (v/v) glycerol. Gels were stained with silver using a commercial kit (PIERCE).

When establishing the assay conditions, FV and PT gene fragments were amplified separately to locate the characteristic positions of single strand bands. DNA samples genotyped previously for FV Leiden by restriction with *Mnl*I (1) were used for this analysis. Samples homozygous for normal FV or FV Leiden, respectively, produced single bands with slightly different electrophoretic mobility, the mutated single strands migrating faster. Heterozygous samples gave the expected double band pattern. Occasionally, two additional faint bands moving slightly faster were seen, which did not interfere with the correct FV genotyping (see Fig. 1, lane 10).

The SSCP pattern for the PT gene fragment showed two clearly spaced bands for most samples; a few samples produced two closely spaced bands in the position of the slowly migrating single strand, whereas the fast migrating strands appeared somewhat diffuse. Direct sequencing on an Automated ABI 310 Genetic Analyzer revealed that the two-band pattern represents samples homozygous for the 20210G allele, samples with the three band pattern are heterozygous G/A. Then, genotyping for both risk factors after multiplex PCR was performed. SSCP patterns demonstrated, that the FV and the PT-related bands are clearly separated and can be evaluated easily (Fig. 1). A total of 380 samples mainly from patients with venous thrombosis previously tested for FV Leiden by restriction analysis (1) was genotyped by the multiplex PCR-SSCP assay (Fig. 1). There were eight samples typed heterozygous for FV Leiden by restriction analysis but normal by the SSCP assay. Repeated testing by restriction analysis and/or direct sequencing confirmed the SSCP results. Obviously, these samples had been previously misclassified because of incomplete digestion by *Mnl*I. In all other cases there was complete agreement between the results of restriction enzyme- and SSCP-based assays. Genotyping for PT 20210 G/A dimorphism revealed 15 heterozygous samples and one suspected homozygote for the mutated A allele (Fig. 1, lane 9). All the suspected heterozygotes, the homozygous sample and ten normal samples were subjected to DNA sequencing. The results of the PCR-SSCP assay were confirmed in all cases.

Finally, multiplex PCR was performed using 1-4 µl of whole blood as DNA source. As suggested by Gómez et al. (4), blood samples diluted to 20 µl with ddH2O were subjected to three temperature cycles consisting of two steps – 95° C for 5 min and 30° C for 30 sec – prior to addition of PCR reagents (primers, buffer, dNTPs, ddH2O and Taq polymerase) to a final volume of 50 µl and thermal cycling as described above. Best results were obtained with 2 µl of blood, when PCR produced FV- and PT-related bands of almost equal intensity.

In conclusion, the combination of multiplex PCR using blood samples and SSCP screening provides a very fast, simple, reliable and low-cost alternative for the simultaneous detection of the two most common genetic risk factors for venous thrombosis. Ambiguity of test results due to incomplete digestion by restriction enzyme is excluded. In a large series of samples there was no indication of false positive results due to the potential of the SSCP method to detect other sequence variations in the vicinity of the tested mutation sites.

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