CORRESPONDENCE

Rapid Simultaneous Screening of Factor V Leiden and G20210A Prothrombin Variant by Multiplex Polymerase Chain Reaction on Whole Blood

To the Editor:

The mutation in factor V (FV) G1691A, known as factor V Leiden,1 and the recently described genetic variation in the prothrombin (FII) gene G20210A2 are the two most prevalent known causes of inherited thrombophilia. Several polymerase chain reaction (PCR)-based methods have been described for the detection of each of them, separately. Although PCR is technically easy, it is rather expensive and time-consuming; the rate-limiting step is usually the DNA isolation. These arguments are important when considering the cost-benefit of largescale prophylactic testing in individuals at risk. PCR amplification of FV Leiden from whole blood has been reported to be feasible and reliable.3,4 In addition, a method for the combined detection of both abnormalities by using purified DNA has been recently published.5 An ideal method would be the combination of both advantages, ie, a multiplex PCR for the simultaneous detection of both genetic abnormalities using whole blood as DNA source.

We report here a very rapid, simple, and cost-saving method for the genotypic diagnosis of both risk factors. This is achieved by multiplex PCR amplification of the involved region of both genes using whole blood as DNA source, followed by combined restriction digestion and agarose gel electrophoresis.

First, we compared the feasibility of multiplex amplifications on purified DNA using conditions for FII (Poort et al2 and R.M. Bertina, personal communication, December 1996) and FV1 with minor modifications. Despite the different conditions, both gene products were amplified using either of these two methods. However, the FII program gave stronger amplifications (not shown). FII amplifications were performed using 67 mmol/L Tris-HCl, pH 8.8; 16.6 mmol/L (NH4)2SO4; 6.7 mmol/L MgCl2; 10 mmol/L 2-mercaptoethanol; 100 µg/mL bovine serum albumin; 10% dimethylsulphoxide; 1.5 mmol/L of each dNTP; 2.5 U Taq DNA polymerase (Amplitaq; Perkin Elmer Cetus, Norwalk, CT); 500 ng of each FII; and 400 ng of each FV primer1,2 in a total volume of 50 µL. The sequence of the reverse FV primer (5’-CTTGAAGGAAATGCCCCCATTA-3’) is different from that described.1 The FII program we use is as follows: After a denaturation step at 95°C for 4 minutes, thermal cycling was 1 minute of denaturing at 94°C, 1 minute of annealing at 53°C, and 2 minutes of extension at 67°C, with cycles repeated 32 times.

Subsequently, different amounts of whole blood (0.5, 1.0, 2.0, 5.0, and 10.0 µL) were assayed by adding ddH2O up to 20 µL before denaturation. The samples were denatured by heating the blood at 95°C for 5 minutes and then cooling at 30°C for 30 seconds, which was repeated three times. When using 1.0 or 2.0 µL of whole blood, both gene products were amplified using either the FII or the FV program. Again, the FII program resulted in stronger amplifications as compared with the FV program (Fig 1, lanes 1 and 2). Occasionally, low yields were obtained when using program FII. Therefore, we increased the number of cycles from 32 to 40. Because this resulted in consistent high yields (Fig 1, lane 3), we favor the use of 40 cycles for the multiplex amplification. Identical results were obtained when either fresh or frozen blood was used. When using half or one quarter of the amount of each of the primers, the yields were significantly lower (not shown).

For the genotype analysis, the 220-bp FV and 345-bp FII PCR products obtained by multiplex PCR were simultaneously digested by adding 6 U Mnl I and 10 U HindIII (New England Biolabs, Beverley, MA) to 28 µL of PCR product, incubated for 2 hours at 37°C, and subsequently separated on agarose gel. The wild-type FII fragment contains two Mnl I sites but no HindIII sites, resulting in fragments of 15, 58, and 272 bp, whereas the 20210A (mutated) product contains two Mnl I sites and one HindIII site, resulting in fragments of 15, 23, 58, and 249 bp after double digestion. The FV fragment contains no HindIII sites, resulting in Mnl I fragments of 37, 67, and 116 bp for the wild-type allele and 67 and 153 bp for the mutated allele. Consequently, when

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Fig 1. Multiplex detection of wild-type and mutated FII (G20210A) and FV (G1691A) using whole blood (2 µL) as the DNA source in PCR. Lanes 1 through 3, undigested multiplex PCR products using the described conditions for FII (lane 1 [32 cycles] and 3 [40 cycles]) and for FV (lane 2). Lanes 4 through 9, undigested and Mnl I and HindIII double-digested PCR product electrophoresis of a patient double heterozygote for FII and FV; undigested and digested factor FV fragment (lanes 4 and 5, respectively); undigested and digested FII fragment (lanes 6 and 7, respectively); undigested and digested FII and FV fragments obtained after multiplex PCR (lanes 8 and 9, respectively). Lanes 10 through 13, double-digested products obtained after multiplex PCR from a normal individual (lane 10), an FII and FV Leiden double heterozygote (lane 11), an FII heterozygote (lane 12), and an FV Leiden heterozygote (lane 13). Products were separated on a 2.5% agarose gel (per lane, 20 µL of the digestion mix is loaded). Marker is a 100-bp size marker (GIBCO BRL, Grand Island, NY). Relevant fragment sizes in basepairs are indicated.
both PCR products of a patient double heterozygote for FII and FV are digested, fragments of 272, 249, 153, and 116 bp are detected (Fig 1, lanes 9 and 11).

In conclusion, the presented multiplex amplification on 2 µL of whole blood followed by a combined restriction digest of the obtained PCR products offers a very rapid, feasible, and cost-saving method for large-scale FII and FV genotype analysis.

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To the Editor:

Since the discovery of the factor (F) V Arg 506 to Gln mutation (FV:R506Q) as the most common inherited disorder associated to venous thrombophilia,1-6 and its apparent cosegregation with other well-established inherited prothrombotic risk factors,7-12 evidence is accumulating that the association of double or multiple hematostatic defects greatly increase the penetrance of thrombotic disease. This finding raises the question whether the novel sequence variation in the prothrombin gene (20210 G to A variant),13 which has been identified as a common but probably mild risk factor for venous thromboembolism (VTE),13-16 may also cosegregate with the FV:R506Q mutation and contribute to the thrombotic tendency in subjects being affected by activated protein C (APC)-resistance.

Therefore, we read with interest the recent report by Alhenc-Gelas et al17 about the rare association between the prothrombin 20210 A allele and FV:R506Q in thrombophilic families. These investigators looked for an association of the two risk alleles in 288 subjects belonging to 26 families; 151 carried the FV:R506Q mutation and 66 had had thromboses. However, no probands or family members had the 20210 A allele. Thus they concluded that the prothrombin variant does not frequently contribute to thrombosis in individuals with the FV mutation. The question is this: Are the findings reported by Alhenc-Gelas et al17 affected by the high percentage of asymptomatic subjects studied or by the selection of patients, respectively? Furthermore, because no separated and detailed data about age or clinical settings were given for the FV:Q506 carriers, their results are difficult to assess.

We report here different and intriguing data showing a highly prevalent coinheritance of the prothrombin variant 20210 A allele as an additional prothrombotic risk allele among young symptomatic FV:Q506 carriers.

After obtaining informed consent, FIIGenotyping was performed in 200 apparently healthy controls and unpreferentially in 200 carriers of FV:Q506, including 150 unrelated patients who had had an objectively confirmed VTE before 45 years of age. The FV genotype at nucleotide 1691 was determined by polymerase chain reaction (PCR) and Mnl I restriction analysis of PCR-amplified genomic FV DNA fragments.2-3 Screening of the prothrombin variant due to a G to A transition at nucleotide 20210 of the FIIGene was performed by HindIII cleavage of a 345-bp fragment amplified by PCR using a mutagenic primer as described previously.13 The 20210 A allele was found in 4 of 200 healthy subjects with a normal FV genotype (100 men and 100 women; age range, 18 to 47 years; median age, 26 years), corresponding to a prevalence of 2%, whereas among 50 asymptomatic heterozygous FV:Q506 carriers (22 men and 28 women; median age, 31 years; range, 24 to 64 years), the prothrombin variant was detected in 2 subjects (4%). Among 115 symptomatic subjects affected by the heterozygous FV: R506Q mutation (69 women and 46 men; median age at onset of VTE, 28 years; range, 18 to 45 years), 14 (12.2%) also had the FII 20210 A allele. In the presence of the 20210 A allele, the relative risk of juvenile VTE was additionally threefold increased in patients carrying the FV:R506Q mutation in a heterozygous form (95% confidence interval, 0.8 to 11.7), which itself was found to increase the risk of VTE approximately fourfold.18 Patients affected by double heterozygous defects presented with thrombosis at a slightly younger age (median age at onset of VTE, 27 years) as compared with patients suffering from either FII 20210 A (33 years) or FV:Q506 in a heterozygous (29 years) form. In the group of 35 symptomatic patients affected by homozygous FV:R506Q mutation (21 women and 14 men; median age at onset of VTE, 27 years; range, 18 to 33 years), a coexistence of the prothrombin 20210 AG genotype was detected in 5 subjects, corresponding to a prevalence of 14%.

Persons homozygous for the 20210 A allele were not found.

With respect to the coexistence of the prothrombin variant 20210 GA in carriers of the FV:R506Q mutation, the rate observed in the presented study of relatively young thrombophilic patients was clearly higher compared with the rare association published for other populations.14-17,19 However, assuming the theory that a high proportion of combined inherited hemostatic abnormalities predispose for thrombophilia already at a young age, the significance of the uncommon coinheritance of both FV:Q506 and prothrombin variant observed in previous studies is difficult to assess; either the age was not mentioned at all17 or the majority of patients investigated were over the age of 60 years,15 much older than our patient population.14,15 By contrast, Poort et al13 reported that the prothrombin variant was identified in 18% of selected patients, segregated in 40% with the FV:R506Q mutation.13 Furthermore, the 20210 A allele possibly has a similar distinctive racial and/or geographical distribution, as has been described for the FV mutant.20 These

REFERENCES

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