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SHORT COMMUNICATION

A Duplex PCR-RFLP Assay for Simultaneous Detection of FV Leiden and Prothrombin G20210A Mutations in Women with Recurrent Miscarriage

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Factor V Leiden (FV-Leiden) and prothrombin gene mutations (FII G20210A) are well-established independent risk factors for several pregnancy complications and may be associated with an increased risk of recurrent miscarriage. Molecular diagnosis testing for both mutations is widespread and need to be standardized. We have optimized a duplex polymerase chain reaction-restriction fragment length polymorphism assay (PCR-RFLP) for the simultaneous detection of both mutations in a single-tube single-enzyme digestion reaction for 35 women with recurrent miscarriage. This assay is a convenient method that should be applied in routine settings for detecting thrombophilia in women who are suffering from recurrent miscarriage.

1. Introduction

Factor V Leiden (FVL) and prothrombin gene mutation (FII G20210A) are well-established independent risk factors for thrombosis. In FVL (G1691A), arginine is substituted by glutamine at amino acid residue 506 in coagulation factor V.[1] Due to this substitution, factor Va becomes resistant to degradation by activated protein C, increasing the risk of venous thromboembolism, 3–5-fold in heterozygous individuals.[2] In FII G20210A, a G to A transition at position 20210 of the 3′ untranslated region of the factor II gene has been found to be associated with increased prothrombin levels and a 3-fold increased risk for venous thrombosis in heterozygosis individuals.[3]

In the present work, we described a novel duplex PCR–RFLP assay for the simultaneous detection of G1691A, G20210A, and the corresponding normal alleles. We determined that the method performed robustly and accurately.

2. Methods

Our study included 35 women with recurrent miscarriage; defined as at least two pregnancy losses at the first trimester. Genetic testing of thrombophilia was performed on blood samples of patients who consulted in our laboratory for genetic counselling. Written informed consent was obtained from all patients.

Genomic DNA was extracted from EDTA-anticoagulant blood samples according to a phenol-chloroform protocol. PCR amplification was performed using the forward and reverse primers for both regions of interest of factor V and prothrombin gene previously described.[4]

A 169 bp product from exon 10 of factor V (GenBank Accession Number L32764) and a 221 bp product from the 3′-untranslated region of the prothrombin gene (GenBank Accession Number M17262) were coamplified using PCR. Each 50 μL reaction contained approximately 60 ng genomic DNA, 20 pmoles/μL of each primer, 20 mM of dNTP and 2 U *Taq* DNA polymerase. The PCR program on the thermal cycler (MJ Mini Thermal Cycler, BioRad, USA) was as follows: a first denaturation step at 95°C for 8 minutes was followed by 35 cycles of 94°C for 1 minute, a touch down from 60°C to 54°C for 1 minute, and 72°C for 1 minutes, followed finally by 8 minutes at 72°C. A total of 10 μL from the PCR product was run on 2% agarose to check for any non specific bands.

An amount of the PCR product (10 μL) from each sample was digested with 1μL *MnlI* (fermentas). The mixture was incubated at 37°C for 4 hours and the digested products were electrophoresed on 2% standard agarose at 80 V for 30 minutes. The fragments were visualized by ethidium bromide under a UV transilluminator.

Simultaneously, every PCR product from each sample was sequenced, to compare the precision and accuracy among PCR–RFLP assay. After PCR, the reaction mixture was electrophoresed. The targeted PCR fragment was isolated and purified (Wizard SV Gel and

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PCR Clean-Up System, Promega, Madison, WI) to remove excess nucleotides and primers. After PCR product purification, sequencing reactions were performed with the DNA sequencing kit (Big Dye, Applied Biosystems, CA, USA). Products were analyzed on an ABI 9700 DNA Genetic Analyzer (Applied Biosystems).

3. Results

In the present study, we established a duplex PCR–RFLP assay to detect the Factor V Leiden and the prothrombin G20210A mutations. Several factors, such as primer concentration and PCR cycling condition, were optimized.

Simultaneous amplification of the Factor V exon 10 and the 3′-untranslated region of the prothrombin gene were performed. Amplicons resulted in 169 bp and 221 bp, respectively for the factor V and the prothrombin gene (Figure 1).

Simultaneous digestion of amplicons with MnlI, yielded to a complete digestion of both prothrombin and factor V wild-type and digested products resulted in 115 bp, 37 bp, 17 bp and 192 bp, 29 bp, respectively. Moreover, one restriction site disappeared in the presence of a mutation. In fact, the factor V Leiden homozygote gave fragments of 17 bp and 152 bp, and the prothrombin G20210A homozygote yielded fragments of 221 bp (Figure 1). Two women, among our series, were heterozygous for factor V Leiden and only one was heterozygous for the prothrombin G20210A mutation (Figures 2 and 3).

Using DNA sequencing for both prothrombin and factor V, among these 35 women, we confirmed the results obtained with duplex PCR-RFLP which validates our assay.

4. Discussion

Factor V Leiden and prothrombin G20210A are the most common thrombophilia. For diagnostic analysis of a large number of patients, the fast and economic assays are highly desirable. In fact, several methods for simultaneous detection of both mutations (FVL and FII G20210A) have already been described, using real-time PCR, Light Cycler or multiplex PCR ELISA.[5–7] As DNA sequencing, all of these techniques are fast and elegant, but they require expensive equipment. Because of their cost and complexity, they might be not used routinely in some medical environments.

Our assay presents several main advantages. Firstly, the same restriction enzyme is used for the detection of both mutations, secondly, no special equipment is used, and thirdly the results are stable and reproducible in a single-tube reaction. This assay is convenient for detecting the most common genetic forms of inherited thrombophilia, factor V Leiden G1691A and prothrombin G20210A mutations. Every disorder causes several thrombotic events especially during pregnancy, since there are many pregnancy-associated changes in several coagulation factors that enhance the thrombogenic potential of these inherited disorders.

It has raised the implication of inherited thrombophilia in the pathogenesis of recurrent miscarriage, a devastating problem that affects 2%–5% of couples.[8] In fact, it has been postulated that maternal thrombophilia could be a risk factor for fetal loss because of the production of microthrombosis on placental bed vessels and placental infarctions, which produce a compromise in the feto-maternal circulatory system, that results in low placental perfusion and eventually in fetal loss.[9,10]

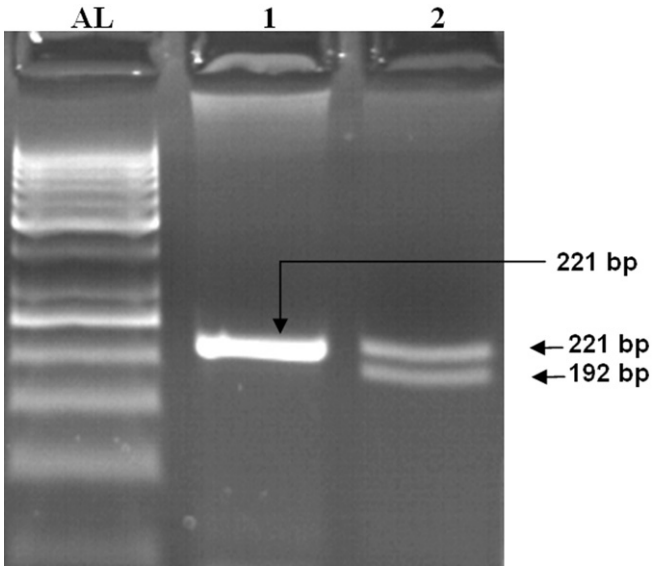

Figure 2 Simplex PCR RFLP profile of heterozygous FII G20210A mutation. AL: Allelic Leader (50bp); Lane 1: undigested wild-type FII (221bp); Lane 2: FII G20210A heterozygote (221 and 192bp).

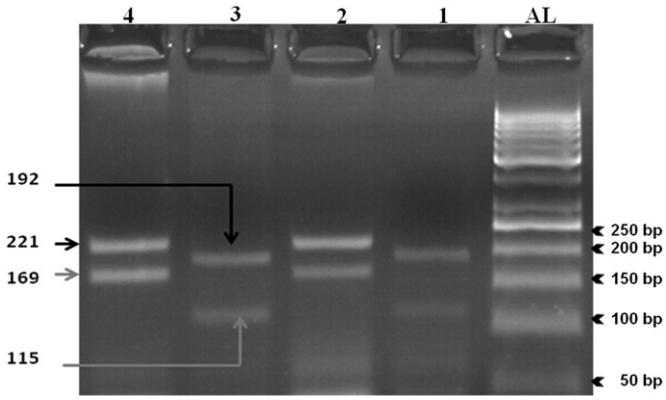

Figure 1 Duplex PCR and PCR RFLP profiles for FV and FII wild-types. AL: Allelic Leader (50bp); Lanes 1 and 3: digested wild-type FV(115bp) and FII (192bp); Lanes 2 and 4: undigested wild-type FV(169bp) and FII (221bp).

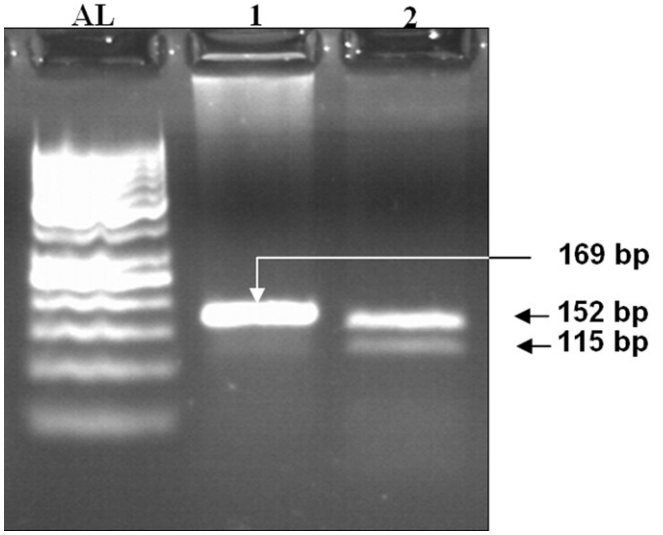

Figure 3 Simplex PCR RFLP profile of heterozygous FV Leiden mutation. AL: Allelic Leader (50bp); Lane 1: undigested wild-type FV (169bp); Lane 2: FVL GG1691A heterozygote (152 and 115bp).

5. Conclusion

In conclusion, duplex PCR–RFLP assay is an easy, accurate and reliable test that should be applicated in routine settings for the detection of thrombophilia in women who are suffering from recurrent miscarriage. For detected mutation, we could prescribe an antithrombotic therapy that may prevent fetal loss.

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