Semi-Automated Detection of the Factor V Mutation by Allele Specific Amplification and Capillary Electrophoresis


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Summary

Recently a point mutation (G1691A) in the coagulation factor V gene was shown to cause resistance for cleavage by activated protein C. The mutation is associated with an increased thrombotic risk and thus the most common genetic cause of thrombophilia. Current techniques to investigate the single base pair mutation at the DNA level use an assay based upon the polymerase chain reaction followed by restriction enzyme digestion or Southern blotting and allele specific probing. The method we describe here consists of a single PCR in which two specially designed allele specific primers and two consensus primers were used in one reaction to distinguish between homozygous normal, heterozygous and homozygous mutant individuals. Amplification products were analysed using Capillary Electrophoresis and on line UV monitoring.

The Allele Specific Amplification Protocol and subsequent CE analysis (ASAP-CE) is a convenient, fast, automated and highly reproducible method that can be used in a routine laboratory setting.

Introduction

In the anticoagulant pathway the role of Activated Protein C (APC), a vitamin-K-dependent serine proteinase, is to selectively inactivate procoagulant factors Va and Vila (1). Using a new APC-resistance assay, Dahlbäck et al. (2) recently showed that an autosomal dominant trait is associated with venous thrombosis. Bertina et al. (3), Voorberg et al. (4) and Greengard et al. (5) showed that a guanine to adenine point mutation at nucleotide 1691 of the gene coding for coagulation factor V (G1691A) is strongly associated with venous thrombosis. The mutation leads to substitution of the arginine residue at position 506 of the protein by glutamine. The presence of arginine 506 is essential for the proteolytic inactivation of factor V by APC and the conversion to glutamine is therefore responsible for the resistance to APC cleavage. The factor V mutation is estimated to be present in 2-4% of the Dutch population (3) and in 7% of the Swedish population (6). It was shown to be associated with a three-fold increase in thrombotic risk (3, 7). About 40-50% of patients with familial thrombophilia show APC resistance, this makes factor V G1691A mutation the most common genetic cause of thrombophilia (3, 6).

APC resistance can be detected using an assay in which the clotting time of blood plasma is measured in the presence or absence of exogenous APC. When the ratio of the clotting times is below 0.84 × the normalised APC ratio, the patient is regarded as APC resistant. Because overlap in the APC ratios of patients and normal individuals can complicate diagnostics, an assay that allows direct detection of the factor V mutation can be of advantage. That way patients who are being treated with anticoagulants and for whom no proper APC ratio can be measured, can also be screened for the G1691A mutation.

Current methods to determine the G1691A mutation include DNA sequencing, polymerase chain reaction (PCR) and subsequent restriction digestion with Mnl I or Southern blotting of the PCR products and subsequent probing with allele specific oligonucleotides (3). For routine screening of patient samples a simple, fast and cost-effective method is important. The method we describe here is based upon a single PCR followed by direct analysis by Capillary Electrophoresis (CE) and on line UV detection. The semi-automated protocol is applicable for the molecular diagnosis of other diseases that are based on the presence of a point mutation.

Materials and Methods

DNA Isolation and Amplification

Genomic DNA was isolated from whole EDTA blood by salt extraction (7) and 1 μg was amplified by PCR in 50 μl containing 50 mM KCl, 20 mM Tris pH 8.4, 1.5 mM MgCl₂, 0.001% gelatine, 125 mM dNTPs, 15 pmol of each primer (see Table 1) and 2.5 U Taq polymerase (Gibco BRL, Gaithersburg, USA) and overlaid with 80 μl mineral oil.

PCR amplification was performed in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) starting with a denaturation step at 95°C for 5 min, followed by 35 or 40 cycles of 95°C for 30 s, 60 or 55°C for 30 s, 72°C for 1 min 30 s. The number of cycles and the annealing temperature depends on the primer set used: the first set shown in Table 1A uses 35 cycles and 60°C and the second set in Table 1B uses 40 cycles and 55°C. After the last cycle, the extension phase was prolonged to 10 min to allow full extension of all products. A sample of 15 μl was loaded on a 2% agarose gel stained with ethidium bromide, the remainder was used for analysis with capillary electrophoresis.

Capillary Electrophoresis

Instrumentation: An uncoated fused silica capillary, 200 mm effective length and 0.075 mm internal diameter (SGE, Ringwood, Vic, Australia) was mounted in a User Assembled Cartridge and placed in a Biofocus 3000 CE instrument (Bio-Rad, Hercules, USA).

CE analysis: Prior to all analyses the capillary was purged, using a pressure of 100 psi, with water (15 s), 1 N NaOH (10 s), 0.1 N HCl (10 s), 1×TBE (89 mM Tris-Borate, 0.2 mM EDTA, pH 8.3) (10 s), and then filled with Non Gel Sieving Buffer (kindly provided by Bio-Rad, Hercules, USA) for 90 s. This was followed by injection with 0.01 N acetic acid for 4 s for stacking purposes.
Samples were pressure injected (400 psi·s) without pretreatment and separated at reversed polarity (cathode at injection side) at 12.5 kV in NGSB as running buffer. The temperature of the cartridge and of the sample was 20°C and 15°C respectively. On line UV absorption at 260 nm was used for detection.

Results and Discussion

In order to generate PCR fragments that were derived from the normal or mutated allele, we used consensus oligonucleotides (COs, primers 1 and 4 in Fig. 1A) as well as a set of specially designed allele-specific oligonucleotides (ASOs). The consensus oligonucleotides have a double function. Besides generating the allele specific products when used together with the allele-specific primers, the combination of both consensus primers generates a larger DNA fragment that serves as an internal control for quality and PCR efficacy of the patient sample. The allele-specific oligonucleotides have two important features. First, the sense or forward (F) primer (ASO-1F, primer 2 in Fig. 1A) has a guanine at the 3' end, whereas the antisense or reverse (R) primer (ASO-1R, primer 3 in Fig. 1A) has a thymine at the 3' end. This way, the primers were designed to be specific for the normal and mutant allele respectively. As shown in Fig. 1B, we expect the DNA from normal individuals to generate two products after PCR: a 202 bp internal control fragment and a 138 bp product specific for the normal allele. PCR on DNA from homozygous mutated individuals was expected to generate a mutant-specific 115 bp DNA fragment next to the internal control fragment. PCR applied to DNA derived from an individual that is heterozygous for the G1691A mutation was expected to result in the generation of all three fragments. As a second important feature, the allele-specific oligonucleotides have a stretch of mismatching bases at the 5' end (indicated in Table 1). This is necessary to prevent the generation of heteroduplex DNA molecules of the internal standard molecule as a result of priming of the upper strand of the mutant-specific PCR product (upper fragment in Fig. 1C) with the lower strand of the normal PCR product (lower fragment in Fig. 1C) and subsequent extension by polymerase. Heteroduplex molecules could negatively interfere with subsequent electrophoretic analysis.

We tested a first primer set consisting of ASO-1F, ASO-1R and CO-F, CO-R primers (Table 1A) in a PCR on DNA samples derived from a normal and heterozygous individual. The results after agarose gel electrophoresis are shown in Fig. 2A. The 115 bp mutant-specific PCR product is not visible when the four primers are used in equal concentrations (Fig. 2A lane 6 vs. lane 2). We therefore tested the influence of altering the ratio of primers CO-F and ASO-1R versus primers ASO-1F and CO-R (Fig. 2B). This way, the amplification of the normal DNA sample showed a decrease in the amount of 138 bp product at a ratio of 2:1 and 3:1 (Fig. 2B lane 3 and 4) while the amplification of the mutant DNA sample showed a 138 bp normal band at a ratio 1:1 and 2:1 (Fig. 2B lane 8 and 9). We concluded that this primer set gave rise to non specific amplification products which could lead to false test results.

We therefore changed the design of the mutant and normal oligonucleotide primers and included an extra mismatch at the 3' end of the allele-specific primers. The new set uses ASO-2F and ASO-2R primers which contain an extra mismatch directly adjacent to the allele-specific nucleotide at the 3' position (see Table 1B). To match the lower annealing temperatures of the newly developed allele specific primers, we also synthesised two new consensus primers (CO-2F and CO-2R) (Table 1B). The expected fragment lengths after DNA amplification with these primer sets is shown in Fig. 1B. The results of agarose gel electrophoresis of PCR products using the ASO and CO primers in one reaction is shown in Fig. 2C. Although the separation of the 137 bp fragment and 113 bp fragment is hardly visible after agarose gel electrophoresis, the new set seems to work in a specific manner. The identity of bands was confirmed by measuring their size after restriction digestion by Mnl I (data not shown). Because of the far better resolution, we subsequently analysed the products derived from controls and patient samples by capillary electrophoresis and on line UV detection.

Table 1A) Sequence and position of primers used. First set of primers used in the PCR

<table>
<thead>
<tr>
<th>primer</th>
<th>sequence</th>
<th>position nr</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASO-1F</td>
<td>5'-TTTACGAGATCCCTGGACAGGT-3'</td>
<td>1665-1691</td>
</tr>
<tr>
<td>ASO-1R</td>
<td>5'-AAATAGGACAATACTGTATTCCTT-3'</td>
<td>1691-1717</td>
</tr>
<tr>
<td>CO-F</td>
<td>5'-CATCTAGTGACCAGTACCATG-3'</td>
<td>1576-1601</td>
</tr>
<tr>
<td>CO-R</td>
<td>5'-TTTCTTGAAGGAAATGCCCCATT-3'</td>
<td>Intron 11</td>
</tr>
</tbody>
</table>

Table 1B) Second set of primers

<table>
<thead>
<tr>
<th>primer</th>
<th>sequence</th>
<th>position nr</th>
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<tr>
<td>ASO-2F</td>
<td>5'-CATGCGAGATCCCTGGACAGGT-3'</td>
<td>1669-1691</td>
</tr>
<tr>
<td>ASO-2R</td>
<td>5'-AAATAGGACAATACTGTATTCCTT-3'</td>
<td>1691-1717</td>
</tr>
<tr>
<td>CO-2F</td>
<td>5'-TACTACAGTGACGTGGACATCA-3'</td>
<td>1578-1599</td>
</tr>
<tr>
<td>CO-2R</td>
<td>5'-TCTTCTGAGGAAATTGCCCCAT-3'</td>
<td>Intron 11</td>
</tr>
</tbody>
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Allele-specific nucleotides are underlined, mismatches are depicted in bold, the position of the oligonucleotide primers is relative to the cDNA of factor V gene (8), the position and sequence of CO-R and CO-2R is derived from Zeller et al. (9).
A) Analysis of PCR products on ethidiumbromide stained 2% agarose gel; M: 100 bp molecular weight marker, lane 1 - 4 wild type DNA. Lane 1: Set of all four primers. Lane 2: primers CO-F and ASO-1R for amplification of mutant fragment. Lane 3: primers CO-R and ASO-1F for amplification of normal fragment. Lane 4: primers CO-F and CO-R for amplification of the internal standard fragment. Lanes 5 to 8: the same for heterozygous DNA. Lane 9: negative control.

B) The effect of different ratios of primer set CO-F and ASO-1R versus ASO-1F and CO-R. M: molecular weight marker. Lanes 1-3 for wild type DNA: ratios 1:1, 2:1 and 3:1 respectively. Lanes 4-6 for heterozygous DNA and lanes 7 to 9 for homozygous mutant DNA. Lane 10 negative control.

C) M: molecular weight marker. Lanes 1 to 3: Results of amplification with the new primer set (CO-2F, ASO-2R and ASO-2F and CO-2R). Lane 1: DNA derived from normal person. Lane 2: DNA derived from heterozygous person. Lane 3: DNA derived from homozygous mutant individual. Lane 4: negative control.

We then performed a double-blind screening of DNA derived from 20 patients and healthy control persons using the ASO-2F and 2R and CO-2F and 2R primer sets in one reaction. All resulting products were analysed by capillary electrophoresis. The results were 100% concordant with the results found with the Mnl I digestion protocol as described before (3).

The analysis by capillary electrophoresis showed good reproducibility although we observed a slight shift in migration times of peaks on the electropherogram when a large series of analyses were performed (n = 28, sd = 6.9%). This is caused by evaporation of Non Gel Sieving Buffer. When we determined relative migration times using the ratio of the migration times of the large fragment and the beginning of the PCR background material on the electropherogram (indicated in Fig. 3 as $t_0$), the standard deviation in the migration times was minimal (n = 28; sd = 1.1%). In our current protocol, one CE analysis including purge and refill of the capillary takes about 12 min. A large series of samples (up to 25 samples) is analysed over night.

These data show that ASAP can be used to screen for factor V point mutation (G1691A). A similar approach using an extra mismatch at base four from the 3' end was described by Norby et al. (11) to screen a mitochondrial DNA mutation causing Leber's hereditary optic neuropathy. If the sequence surrounding the site of the mutation allows the design of allele-specific primers without internal loops and without a strong homology to other DNA sequences, ASAP can be used to screen point mutations in other genes. ASAP is a simple and fast procedure and when used together with analysis by capillary electrophoresis it is highly suitable for routine analysis of point mutations. The use of capillary electrophoresis has additional advantages, i.e. it has excellent resolution and is highly reproducible, the performance is fully automated and the detection is non-radioactive. As part of good laboratory practice (GLP), the raw data derived from the assay can be stored, subsequent data acquisition and analysis is easy.
Acknowledgments

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References

6. Dahlbäck B, Hildebrand B. Inherited resistance to activated protein C is corrected by anticoagulant cofactor activity found to be a property of factor V. Proc Natl Acad Sci USA 1994; 91: 1396-1400.
9. cDNA sequence of factor V gene HSFAV, acc nr M 16967 (Genbank).

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A manual for every physician who is faced with skin lesions in daily practice. The malignant melanoma is increasing in incidence worldwide like no other malignant tumor and continues to entail high mortality. Therefore, early recognition is of literally vital importance. The authors have compiled a color atlas encompassing the clinical symptoms, histopathology and differential diagnoses of all the special forms of malignant melanoma, benign nevi and other pigmented skin lesions. At the head of every chapter a diagram is provided which represents an idealized cross-section through the skin showing the clinical and histopathological appearance of the individual skin lesions. A special chapter is dedicated to dermatoscopy. Didactically clear photographs of clinical findings and of histopathological specimens complete the visual material.

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