Reliable Genotyping of the G-20210-A Mutation of Coagulation Factor II (Prothrombin), Jan Danneberg, André P. Abbes, Ben J. M. Bruggeman, Henk Engel, Jan Gerrits, and Alexander Martens

Prothrombin (coagulation factor II) is the precursor of thrombin, which participates as a serine protease (factor II) in the coagulation cascade. Thrombin is essential in the processes of hemostasis and thrombosis [1–3]. The gene that codes for prothrombin is 21 kb in size and contains 14 exons [4]. The gene has been mapped on chromosome 11 at position 11p11-q12 [5].

Recently Poort et al. [6] reported a PCR-mediated site-directed mutagenesis method for the detection of the factor II G-20210-A mutation. This single-point mutation (G→A) at position 20210, the last nucleotide of the 3′-UT region [4, 6], has been shown to be associated with an increased risk of deep vein thrombosis. The prevalences for the heterozygous genotype 20210 AG found in the Leiden Thrombophilia Study were 2.3% in healthy controls and 6.2% in patients. A homozygous AA genotype was not found (expected prevalence 0.014%) [6]. The relative risk for thrombosis being associated with the heterozygote state AG was 2.8 [6].

The primers and enzyme were chosen in such a way that only the amplification product of the mutant allele was digested; the PCR product of the wild-type allele was not digested. The reverse primer PR 95–315 (5′-ATA gCA CTg gAg gCA TTg AA′ g C-3′) [6] contains a single-base mismatch G→A (asterisk), thereby creating a novel HindIII restriction site (A ↓ AgCTT) in the amplified product of the mutant allele. Because only the amplified product of the mutant allele is cut, no internal control for the digestion of HindIII is available in case of an amplified product of the wild-type allele. This could result in wrong conclusions in cases when digestion activity is decreased or absent, for example by influence of inhibitory factors in the reaction mix, and a false-negative result (wild-type) will be reported.

Our aim was to generate an internal positive HindIII digestion control. Because the reverse primer is the mutagenic primer, we designed a new forward primer upstream of the published primer PR 93–787 [6], so that a constitutive HindIII site was included in the amplified product.

The software program used was Primer-Designer© for Windows, version 2.0. A control site for HindIII was found at position 26400–4051. A new forward primer with best matching properties to PR 95–315 [6] was found at position 26302–3211. FAC2fw1: 5′-gCA CAg ACg gCT gTT CTC TT-3′. The location of PR 95–315 [6] was at position 26786–8071. The amplification product length before digestion was 506 bp. Digestion with HindIII generates in all cases a constant fragment of 100 bp; the remaining fragment was uncut in wild-type alleles (406 bp) and cut in two fragments (383 + 23 bp) in mutant-type alleles (Fig. 1A).

DNA isolation: Genomic DNA was isolated with the QIAamp® Blood Kit (Qiagen). A slight modification of the manufacturer’s procedure for whole blood was followed to eliminate the inhibitory effect of hemoglobin on Taq DNA polymerase. Briefly, whole blood was initially frozen to lyse red blood cells, and then 200 µL was centrifuged for 5 min at 400g. The supernatant lysate was removed by aspiration and the leukocyte pellet resuspended in 200 µL of PBS. The procedure was then continued according to the manufacturer’s instructions.

PCR conditions: PCR was carried out in a reaction volume of 50 µL, overlaid with 2 drops of mineral oil to prevent evaporation. The reaction mixture consisted of 100–250 ng of template DNA, 50 pmol of each primer FAC2fw1 and PR 95–315 (Pharmacia Biotech), PCR buffer (10 mmol/L Tris-HCl pH 8.3, 50 mmol/L KCl, 0.1 g/L gelatin, 1 g/L Triton X-100, 1.5 mmol/L MgCl2), 200 µmol of each dNTP (Pharmacia Biotech), and 1.5 U of Taq polymerase (SuperTaq HC; HT Biotechnology). This mixture was submitted to the following PCR temperature profile: initial denaturation at 95 °C for 2 min, 40 cycles with denaturation at 94 °C for 30 s, annealing at 55 °C for 15 s, extension at 72 °C for 1 min; final extension was at 72 °C for 5 min. The thermal cycler used was a Mastercycler 5330 (Eppendorf-Netheler-Hinz).

Each PCR run series included a wild-type and a heterozygous DNA control, and also a water control to exclude contamination.

Restriction enzyme digestion: Fifteen microliters of HindIII digest was digested with 0.5 µL of HindIII (10 U) in 1× NEBuffer-2 (New England Biolabs) in a final volume of 50 µL at 37 °C for 2 h.

Electrophoresis: Twenty-five microliters of the HindIII digest was electrophoresed in a 3% agarose gel (Agarose-NA; Pharmacia Biotech) that contained 0.5 mg/L ethidium bromide. Electrophoresis was performed for 3 h at 5V/cm in 0.5× TBE-buffer (1× TBE: 90 mmol/L Tris-borate, 2 mmol/L EDTA, pH 8.0). Control lanes consisted of 5 µL of uncut PCR product and a molecular mass marker (low ladder; BIOzym).

Patients: We included 200 patients referred to the Twenteberg Hospital with ultrasound-proven proximal deep vein thrombosis (median 44, range 16–83 years of age) in our study. Proximal vein thrombosis included thrombi involving the popliteal vein or above.

For all 200 patients tested for the mutation G-20210-A with the above-mentioned modified PCR method, the constitutive HindIII site was cut by HindIII, generating a 100-bp fragment (Fig. 1A and B) and proving a successful digestion. Eleven patients were found to be heterozygous and one patient was homozygous for the factor II mutation. In this way validating the constitutive restriction site is especially useful to confirm the absence of the factor II mutation.

1 EMBL data (ID: HSTHB, AC: M17262; M33691).
In general in an individual, testing for somatic mutations has to be performed only once. Therefore, reliable results are very important and can be obtained by using reliable controls. A constitutive restriction site is an appropriate control in most restriction digestion-based PCR techniques. When no usable control site for the specific restriction enzyme is available in the near region of the mutation, external digestion control could be performed by addition of 0.5 μg of uncut λ-DNA (Boehringer Mannheim) to the digestion reaction. The λ-DNA will be cut in the specific restriction fragments for the enzyme used (Fig. 1C). For example, primers (as described by Poort et al. [6]) were used that resulted in amplified product without a constitutive HindIII site. Digestion control in case of a wild-type was therefore not possible. The specific HindIII fragments of λ-DNA prove a successful digestion (λ-DNA: undigested = 48.5 kb, HindIII fragments of λ-DNA = 23.1 + 9.4 + 6.7 + 4.4 + 2.3 + 2.0 + 0.56 kb [7]). For adequate resolution of the factor II fragments (345 and 322 bp), electrophoresis was carried out in a 3% agarose gel. In this relatively high gel concentration, resolution of large DNA fragments will be poor (or nil) and only the smaller HindIII fragments of λ-DNA will be separated (the 0.56 kb band will not always be visible because of the low amount of nucleic acid). The 2.3-kb and 2.0-kb HindIII fragments of λ-DNA are clearly visible as two discrete bands in a 3% agarose gel, proving a successful digestion.

References

6. Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3'-untranslated region of the gene for coagulation factor II (prothrombin) point mutation G-20210A.

Primer PR 95-787 and PR 95-315 were previously published by Poort et al. [6]; primer Fac2fw1 is novel to this publication. (A) The HindIII/mutation site will be generated by the mutagenic primer PR 95–315 in case of a mutant-type allele; a wild-type allele will remain uncut at this site by HindIII. Only primer Fac2fw1 coamplifies the constitutive HindIII site, whereas PR 95–787 does not. This constitutive restriction site should be cut in all PCR products (wild-type as well as mutant-type); thus internal control for restriction digestion is obtained. (B) PCR was carried out with primers Fac2fw1 and PR 95–315. Even wild-type samples, where the HindIII/mutation site is not cut, will be digested at the constitutive HindIII site; the PCR product is reduced with the 1000 bp control fragment and a 406 bp wild-type band remains. Mutant alleles will also be cut at the HindIII/mutation site, which results in a 383 bp band (the remaining 23 bp band is not detectable on the gel). Lanes M, low ladder molecular mass marker; lane 1, undigested PCR product (506 bp); lane 2, wild-type pattern (406 + 100 bp); lane 3, heterozygous pattern (406 + 383 + 100 bp); lane 4, homozygous pattern (383 + 100 bp); lane 5, water blank. (C) PCR was carried out with primers PR 95–787 and PR 95–315. Primer PR 95–787 does not coamplify the constitutive HindIII site; external HindIII digestion control was performed by addition of 0.5 μg of λ-DNA to the digestion reaction. In each digested sample λ-DNA was cut into specific HindIII fragments. Wild-type samples further showed only the uncut PCR fragment of 345 bp; mutant alleles will also be cut at the HindIII/mutation site, which results in a 322 bp band (the remaining 23 bp band is not detectable on the gel). Lane M, low ladder molecular mass marker; lane 6, undigested PCR product; lanes 1–3 and 5, wild-type pattern; lane 4, heterozygous pattern.
Detection of CSF Leakage by Isoelectric Focusing on Polyacrylamide Gel, Direct Immunofixation of Transferrins, and Silver Staining, Freek W.C. Roelandse,* Nico van der Zwart, Jan H. Didden, Jenny van Loon, and John H.M. Souverijn (Dept. of Clin. Chem., Leiden Univ. Medical Centre, Albinusdreef 2, 2333 ZA Leiden, The Netherlands; *author for correspondence: fax + 31/71/5266753, e-mail roelandse@rulf2.medfac.leidenuniv.nl)

Leakage of cerebrospinal fluid (CSF) from the subarachnoid space into the nasal or aural cavity creates a pathway for life-threatening central nervous system infection. Detection of the leakage can be cumbersome [1]. In former days the identification and localization of CSF leakage was almost entirely dependent upon the clinical history and methods involving intrathecal injection of radioactive material or a dye. Furthermore, various chemical tests, such as determination of glucose, protein, and electrolytes, performed on the secreted material have been advocated for the differentiation between CSF leakage and other secretions [2]. The injection methods are risk-bearing for the patient and the above methods yield a considerable chance of false-positive or false-negative results [3]. Nowadays O-sialotransferrin, also named β2-transferrin, asialotransferrin, or the τ fraction, is an accepted marker protein for the detection of CSF in excretions from the nose or the ear, or from head or neck wounds. O-Sialotransferrin is not strictly unique to CSF. The protein can also be demonstrated in human aqueous humor [4] and in perilymph fluid [5–7]. It is also found in the blood of patients with chronic liver diseases [8, 9], with an inborn error of glycoprotein metabolism [10], and with a genetic variant of transferrin [8, 11, 12]. The protein can be detected by direct immunofixation of transferrins after agarose electrophoresis (high-resolution electrophoresis, HRE) [3, 13] or after blotting [1, 14, 15]. The method we used up to now was the agarose/immunofixation method according to Zaret et al. [3]. We used the Paragon High Resolution Electrophoresis kit (HRE kit, Beckman Instruments) and an anti-transferrin reagent (Beckman Array® System, Beckman Instruments). One disadvantage of this fast method is the high protein concentration that is necessary for use: The secretion extract must be concentrated to a protein amount of 10 g/L. Therefore small samples cannot be analyzed. Furthermore, the location of the O-sialotransferrin band in secretions can be displaced, compared with the location of the O-sialotransferrin band in a control CSF. This phenomenon hampers detection. An admixture of large quantities of blood (hemoglobins) disturbs the pattern because of β2-transferrin [3].

Our currently described method improves the detection of O-sialotransferrin in the case of minor CSF leakages and even in small blood-containing samples. The described method is not meant to be a stat method. O-Sialotransferrin (pI 5.9) and other transferrin isoforms, such as monosialo- (pI 5.8), disialo- (pI 5.7), and trisialotransferrin (pI 5.6), are easily recognized. The method is suited for small samples, requires less protein, is sensitive, and gives more information compared with the agarose electrophoresis methods. Because in rare cases O-sialotransferrin can occur in blood, the necessity of involving a serum sample from the same patient for comparison is obvious.

In detail, the procedure is as follows: Nose and ear secretions from patients suspected of having a CSF leakage are absorbed with Merocel® tampons, cotton swabs, or gauze. Next the material is extracted with a small amount of distilled water. After centrifugation of the extract at 1900g for 10 min at room temperature, the total protein concentration of the supernatant is measured (Hitachi 911 analyser, Boehringer Mannheim). Blood samples to prepare serum are obtained through venipuncture. After centrifugation at 1900g for 10 min at room temperature, the total protein concentration of the serum is measured (Hitachi 747 analyzer, Boehringer Mannheim). Control CSF consists of a CSF sample that shows a clear fraction of the O-sialotransferrin by isoelectric focusing (IEF). Extract supernatant, serum, and control CSF are divided up and stored at –20°C before analysis. If required, extract supernatant and control CSF are concentrated in Fugisep® centrifuge concentrators (type PES 10, Intersep Filtration Systems) to a total protein amount of 250 mg/L. Serum and extract supernatant with a total protein amount of >250 mg/L are diluted to 250 mg/L with a 1:2 mixture of saline and distilled water. pH focusing experiments are performed on a Multiphor® II electrophoresis kit with a 2103 power supply (Pharmacia-LKB) equipped with a Kryostat WK5 cooling unit (Colora) adjusted to 8°C. Polyacrylamide gels are ready-to-use Ampholine® PAG plates for IEF, pH range 3.5–9.5 (Pharmacia Biotech). Sample volume is 20 μL. Samples of patients’ sera are placed adjacent to the prepared extract. Control CSF is incorporated in each assay. The power supply is limited to a maximum of 1500 V and 50 mA. Total focusing time is 90 min. After pH focusing the PAG plate is (gel-side down) placed on top of a 1-mm layer of a 50-fold dilution in distilled water of transferrin antibody (A0061, Dako Corp.) in a plexiglass tray. Incubation is for 60 min at 37°C. After the incubation several washings with distilled water are applied (overnight). Silver staining is performed at room temperature with a 1 g/L silver nitrate solution as coloring reagent for 30 min, a developer reagent containing 30 g of sodium carbonate and 500 μL of formalin per liter for 4–10 min (dependent on the visual band intensity/background ratio), a 480 g/L citric acid solution as stopping reagent for 10 min, and a 50 g/L glycerol solution as preserving reagent for 30 min (all chemicals for the silver staining are purchased from Merck). Finally the PAG plate is covered with a cellophane preserving sheet (Pharmacia Biotech), which is...