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Validation of the LightCycler-Apo E mutation detection kit using hybridization Probes for the genotyping of the apolipoprotein E point mutations (C3932T) and (C4070T)

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Introduction

Apolipoprotein E (Apo E) plays a major physiological role in the metabolism of chylomicrons and very-low-density lipoprotein remnants, which are rapidly removed from circulation by receptor-mediated endocytosis in the liver. The human Apo E gene is located on chromosome 19 and spans 3.7 kilobases, including four exons, yielding a molecule containing 299 amino acid residues (1). In humans, the structural gene for Apo E is polymorphic. It contains 3 alleles that encode for 6 different protein genotypes (Apo E 2/2, 2/3, 2/4, 3/3, 3/4, 4/4), of which Apo E 3/3 accounts for ~ 60% of the population's genotype in the United States (2). The E4 isoform (Arg_{112}/Arg_{158}) is often associated with increased concentrations of cholesterol and a higher risk of coronary disease (3). Furthermore, this polymorphism in the Apo E gene has been associated with an increased risk for Alzheimer's disease. Moreover, the age of onset to develop Alzheimer's disease is likely to be about 10-15 years earlier in carriers of the E4 allele than that in the general population (4). Traditionally, Apo E genotyping has been performed using conventional polymerase chain reaction (PCR) followed by enzymatic digestion of amplicons. This is a time consuming process, which sometimes yields unsatisfactory results. Another approach is a technique, which uses real-time rapid-cycle PCR and fluorescence resonance energy transfer (FRET) with the LightCycler (5). We have recently introduced real-time PCR in our laboratory. The aim of our study was to compare and evaluate the two different methods for Apo E genotyping by means of PCR.

Materials and Methods

Genomic DNA was isolated from 200 µl whole blood using *High Pure Template Kit* (Roche) according to the manufacturer's instructions. DNA isolates were stored at -20 °C until analysis.

Apo E genotyping using RFLP analysis was performed as described previously (6). Briefly, DNA amplification was performed on a *Perkin Elmer 2400* thermal cycler (Applied Biosystems) using the following primers: P3-apoE: 5'-CTCgCggATggCgCTgAgg and P5-apoE: 5'-CgggCACggCTgTCCAAgg.

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PCR (total volume 25 ml) cycling conditions were as follows; 5 at 95°C for DNA denaturation, 35 cycles (60 sec at 95°C (denaturation), 45 sec at 65°C (annealing), and 120 sec at 72°C (extension)), and finally 10 minutes at 72°C. Using the aforementioned primer combination, an amplicon size of 270 bp is expected (6). The amplicons (11 µl) were digested with 5 Units of Cfo1 (Roche) for 24 hours at 37°C in a total volume of 16 ml containing 1X SuRE/Cut buffer L (Roche). Fragments were separated on 4% agarose gel (Biozym) and visualized using ethidium bromide.

In the LightCycler –Apo E mutation detection kit (Roche Diagnostics), a 265 bp fragment of the Apo E gene was amplified which encompasses the codons 112 and 158 containing the polymorphic sites. The amplicon was detected by fluorescence using specific probes that hybridize during the annealing phase of the PCR cycles. One probe is labeled on the 5'-end with a LightCycler Red fluorophore and the other probe is labeled at the 3'-end with fluorescein. During the annealing phase, the probes hybridize to the amplified DNA fragment in a close head-to-tail arrangement. When the light from the LED excites fluorescein, it emits green fluorescent light, transferring the energy to the LightCycler Red, which then emits red fluorescent light. For genotyping, a melting curve was recorded by initially allowing maximum hybridization of the probes to the target DNA and then heating slowly to 80°C. The (decay in) fluorescence was monitored continuously to measure the dissociation of the detection probes. The corresponding melting peaks were calculated by plotting the negative derivative of the fluorescence with respect to the temperature ($-dF/dT$ vs. T).

Results

To perform RFLP, we amplified a region of 260 bp containing the codons 112 and 158 and this was digested with Cfo1, which recognizes the 5'GCG↓C3' sequence. By running known control DNA samples, genotyping is done by comparing the fragment sizes to those of unknown samples (results not shown). The average total analysis time was ~1.5 days. In our hands, gel electrophoresis of these relatively small fragments (< 91 bp) in a high (4%) percentage agarose gel sometimes resulted in faint bands and/or melting of the agarose gel, which made interpretation of the results difficult.

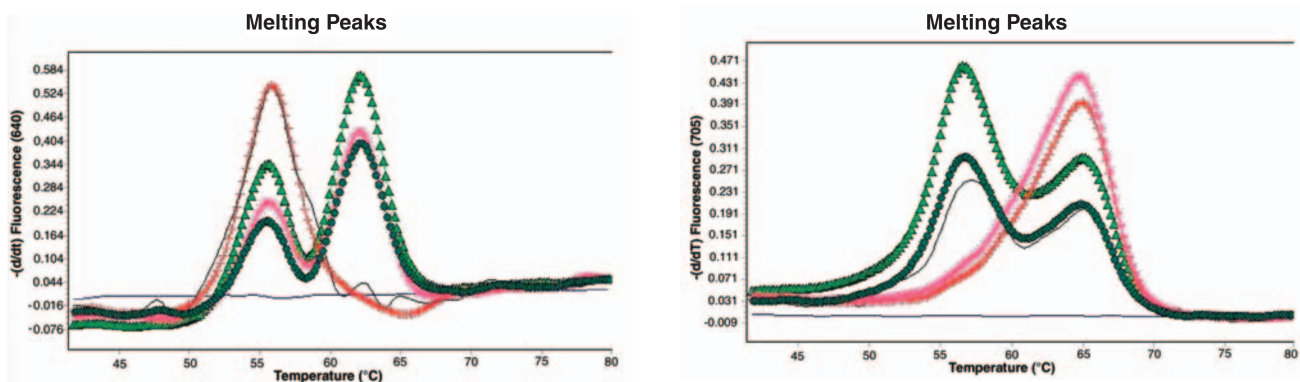

Figure 1. Apolipoprotein E genotyping using derivative melting curve plots for codon 112 (A) and codon 158 (B). Melting curve data for *A* and *B* were recorded at the end of the amplification cycles in channels 640 and 705, respectively. The temperature transition was programmed at 0.1 °C/s with continuous fluorescence acquisition for each sample from 42 °C to 80 °C. The melting curve analysis was performed by calculating a derivative melting curve plot of *–dF/dT* vs temperature. The melting curve plots are shown for the following genotypes: ε4/ε2 (dark green, -•-•-), ε4/ε3 (pink, -x-x-), ε3/ε3 (red, -+-+-), and xa sample with genotype ε3/ε2 (black, solid line). Melting analysis of a no-template control (horizontal line) was also performed.

The results of real-time PCR (followed by melting curve analysis) using the LightCycler kit were assessed by analyzing the melting peaks in two channels (LightCycler-red 640;channel 2; codon 112) and (LightCycler -red 705; channel 3; codon 158). By combining the results of the melting curve analysis in both channels, the allelic set-up can be determined. In all samples, unambiguous genotype of the individual codons was obtained since the difference in melting temperature (Δ between melting peaks) was on average 6.5 to 8.5°C (figure 1). The total analysis time (amplification + melting curve analysis) was approximately 45 minutes.

We genotyped 35 DNA samples and found the most common allele combinations (E4/E4, E4/E3, E4/E2, E3/E3, E3/E2) (table 1). Comparison between PCR-RFLP and the LightCycler Apo E genotyping method yielded complete concordant results.

Discussion and conclusion

The determination of Apo E genotype is of significant clinical value since certain polymorphisms, particularly the Apo E4 allele, have been associated with increased total serum cholesterol and greater risk for coronary heart disease. Furthermore, it constitutes a major risk factor for Alzheimer's disease.

Several methods have been described to determine the Apo E genotype/phenotype. These include isoelectric focusing (7), allele-specific oligonucleotide hybridization (8), restriction enzyme analysis (6), the amplification refractory mutation system (9), or single-strand conformation polymorphism (10). Such systems are time consuming and difficult to automate since they generally require enzyme digestion and electrophoresis procedures.

In the last decade, the introduction of real-time PCR has had a dramatic impact on qualitative and quantitative PCR applications. Recently, a LightCycler DNA assay for Apo E genotyping that uses rapid-cycle PCR and fluorescence resonance energy transfer (FRET) has become commercially available. We tested the Apo E polymorphism in 35 randomized samples with both RFLP and allelic discrimination on the LightCycler and found 100% concordance between the two methods. The use of the LightCycler was advantageous since it clearly reduced analysis time (45 minutes vs. 1.5 days for PCR-RFLP), and was also a safer method since it avoided the handling of acrylamide powders or ethidium bromide staining. Even though the reagent cost/test is slightly higher than conventional PCR, the reduced hands-on time ultimately results in a cheaper test.

In conclusion, the LightCycler Apo E kit seems to be a rapid, simple and accurate method for the determination of Apo E genotyping.

Table 1. Number of cases and frequency of all genotypes found in 35 subjects

Genotype	Number of cases	Frequency (%)
e3/e4	16	45.7
e3/e3	10	28.6
e2/e3	6	17.1
e4/e2	2	5.7
e4/e4	1	2.9
e2/e2	0	0

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Activatie van stolling tijdens hemodialyse is afhankelijk van de wijze van antistolling

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Inleiding

Tijdens hemodialyse (HD) is er een intensief contact tussen het bloed van de patiënt en de membraan van de kunstnier. Het contact kan resulteren in activatie van trombocyten en het genereren van trombine (1). Ten gevolge van obstructies in het dialysesysteem en veranderingen in de wand van het kunstniermembraan wordt de efficiëntie van HD beperkt. De mate van activatie van het stollingssysteem is afhankelijk van het type en de permeabiliteit van het kunstniermembraan. In vergelijking met polysulfon(PS)-'high-flux'- en cellulosetriacetaat(CT-)membranen induceren cuprofaan(CU)- en polyacrylnitril(AN69)-kunstniermembranen aanzienlijk meer activatie (2-4). Om activatie van stolling in het extracorporele circuit te voorkomen wordt heparine of 'low-molecular-weight heparin' (LMWH) toegediend. Applicatie van heparine en LMWH beperkt trombocytenaggregatie. De werking van antitrombine III wordt gepotentieerd en reeds gevormd trombine wordt geneutraliseerd, waardoor het ontstaan van een fibrinestolsel wordt tegengegaan. Als alternatief voor blootstelling aan heparine of LMWH is HD m.b.v. citraat een elegante wijze van antistolling. Lokale toepassing van antistolling m.b.v. citraat is voor wat betreft filtratie van elektrolyten en schadelijke stoffen even effectief gebleken als gebruik van heparine of LMWH (5). De complexe procedure voor toediening resulteert echter snel in verstoring van het elektrolyten- en zuurbase-evenwicht.

In dit onderzoek wordt bepaald of de wijze van antistolling invloed heeft op de activatie van trombocyten en stollingsfactoren. CD62p en plaatjesfactor 4 (PF4) komen na stimulatie vrij uit α-granulae van trombocyten. CD62p komt als gevolg van activatie tot expressie op het membraan van de trombocyt. PF4 komt in dergelijke omstandigheden in toenemende mate voor in plasma. Daarnaast wordt activatie van stolling aan de hand van vorming van trombine-antitrombine-III-complexen aangetoond. Glycoproteïne V is een subunit van het trombocyten-GP1b-V-IX-complex. Door binding aan von-Willebrand-factor fungeert het GP-1b-V-IX-complex als mediator bij adhesie van trombocyten aan het subendotheel van de vaatwand. Na activatie van trombine wordt 'soluble' glycoproteïne V (sGPV) afgesplitst van glycoproteïne V (6). Protrombinefragment 1 en 2 (F1+2) wordt afgesplitst van protrombine in geval van verhoogde FXa-activiteit.

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Patiënten en methoden

Acht patiënten (20-80 jaar) zijn gedurende 3 weken gedialyseerd op een 'high flux' polysulfon-F-60-membraan. Achtereenvolgens wordt heparine (2000-3500 IU bolusinjectie + continu 1000 IU/uur), LMWH (Fragmin®, 2000-5000 U bolusinjectie) en trinatriumcitraat (continue applicatie) als antistollingsmiddel gebruikt. Exclusiecriteria voor patiënten aan de studie betreffen het gebruik van salicylaten, warfarine, dipyrinamol of andere preparaten die de trombocytenfunctie beïnvloeden (Persantin®). Bij elke derde sessie zijn tijdens de HD-behandeling bloedmonsters afgenomen uit de arterielijn op t = 0 en vervolgens uit de efferente lijn op t = 5, 30, 60 en 150 minuten. Bloedmonsters zijn afgenomen in Vacutainer-buizen met K_2EDTA, 0,105 M natriumcitraat en CTAD (Becton Dickinson, Plymouth, UK).

Expressie van CD62p is bepaald m.b.v. een flowcytometer (Beckman Coulter, Mijdrecht, Nederland). Met behulp van ELISA methoden zijn zowel TAT en F1+2 (Enzygnost®, Dade Behring, Marburg, Duitsland) als PF4 en sGPV (Asserachrom®, Diagnostica Stago, Asnières, Frankrijk) bepaald.