Efficient mispriming during apolipoprotein E genotyping

Iniciação errônea eficiente durante a genotipagem de apolipoproteína E

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abstract

The determination of ApoE genotypes is a common clinical laboratory procedure. The most frequently used method involves a PCR amplification of the target region followed by a restriction enzyme digestion of the resultant amplicon to obtain a genotype-specific restriction fragment pattern. We describe an unexpected and surprising difficulty that was encountered during our routine laboratory casework.

key words

Polymerase chain reaction
Apolipoprotein E
ApoE genotyping

Introduction

Apolipoprotein E (ApoE) is a polymorphic protein consisting of a single polypeptide chain, 299 amino acids long. The three major isoforms of the protein are ApoE2, ApoE3 and ApoE4. These differ from each other by cysteine-arginine substitutions at amino acid residues 112 and 158. These interchanges result from single-base changes of a thymine to a cytosine at the relevant position in the ApoE coding region. The biosynthesis of each protein isoform is under the control of three independent codominant alleles, ε2, ε3, and ε4, located at a single ApoE gene locus on chromosome 19q13. Depending on the inheritance of any two alleles, six common ApoE genotypes are possible. ApoE is an integral surface constituent of triglyceride-rich chyomicrons, chyomicron remnants and the lipoproteins VLDL and HDL, and acts in the receptor-mediated metabolism of these particles. The ApoE genotype alters risk predisposition to cardiovascular disease and to Alzheimer’s disease and may influence longevity and cognitive function.

Until now several different ApoE genotyping techniques have been described; these include allele-specific oligonucleotide probes, minisequencing, restriction isotyping using HhaI or AluI/HaeIII with gel electrophoresis and with capillary electrophoresis, the amplification refractory mutation system (ARMS), single-strand conformation polymorphism, oligonucleotide ligation assays, and heteroduplex analysis. More recent technologies have permitted genotyping by amplicon melting curve analysis using real-time thermocyclers, by electrochemical biosensors and by LNAs.

Central to most of these methods is an initial PCR amplification of the region housing the two polymorphic
sites in exon 4 of ApoE. We have been using the restriction isotyping procedure described by Hixson and Vernier(11) which is simple to carry out providing that complete restriction enzyme digestion of the amplicon is assured. We describe an unexpected difficulty that we observed when the amplicon could not be digested after the purchase of a new pair of primers to replace the nearly exhausted supply of the pair used routinely. The new primers had been desalted by reverse phase column chromatography and quality had been controlled by trityl analysis during synthesis. Upon receipt the primers were diluted in water, divided into aliquots and frozen.

Material and method

Anticoagulated (EDTA) blood samples were collected and 300μl were used to extract genomic DNA using Puregene® reagents (Gentra Systems) following the manufacturer’s protocol. The sequences of the oligonucleotide primers used to amplify this region are F4: 5’-ACAGAATTCCGCCCC GGCCTGGTACAC-3’ and F6: 5’-TAAGCTTGGCGCGCTG TCCAAGGA-3’ as described previously(11).

PCR amplification was performed using 360ng genomic DNA, 17pmol of each primer, 200nmol of dNTPs, 5% DMSO, 1.5U of Taq DNA polymerase (Biotechs – B & M Labs) together with the buffer supplied by the manufacturer in a final volume of 50μl. Amplification was carried out in a GeneAmp 9600 thermocycler (Applied Biosystems) using the following conditions: 5min of denaturation at 95°C, 34 cycles of denaturation at 94°C for 15s, annealing at 55°C for 28s and extension at 72°C for 45s. 30μl of PCR product were digested using 5U HhaI and the appropriate buffer (New England Biolabs) together with 10μg BSA in a total volume of 40μl for at least 5 hours at 37°C. An aliquot of 20μl of the restriction digest and 4μl of formamide loading buffer were electrophoresed in 12cm-long non-denaturing 10% polyacrylamide gels at 15V/cm during approximately 75min. The fragments were visualized in UV light following staining with 0.005% ethidium bromide for 15min. Sequencing of the PCR product was performed directly using 2.5μl of PCR product, 2.4pmol of F6 primer, 5μl of BetterBuffer® (Microzone), 1μl of ABI Prism® dye-terminator cycle-sequencing kit (Applied Biosystems), in a final volume of 15μl, using conditions as described by the buffer manufacturer. The sequencing products were electrophoresed and analyzed using a 377 DNA Sequencer (Applied Biosystems).

Results

Several PCR amplifications were performed using the previously established normal conditions and the expected 240-bp amplicon was generated, as visualised by agarose gel electrophoresis. However, upon digestion with HhaI, the expected restriction fragments were not produced. The activity of the restriction enzyme was shown to be normal. All the other components of both the PCR reaction, including the primer sequences and the restriction digest, were checked.

The identity of the amplicon was therefore sought by direct sequencing; the PCR product was purified and sequenced using the F6 primer. A BLAST search revealed that the PCR product comprised a segment of uncharacterized human genomic DNA, Ensembl clone AC079948, located on chromosome 12q14.3.

Discussion

This sequence has a very high similarity to the human keratin 18 gene (KRT18) situated on chromosome 12q13.13. To understand how this occurred, a careful scrutiny was made of the two primer sequences, upon which it was realized that the F6 primer was missing its last 3’ terminal base, an A nucleotide. We conclude, as shown in Figure, that this omission, together with other factors that are discussed below, was sufficient to permit incorrect priming.

A retrospective investigation showed that the oligonucleotide manufacturer had erroneously omitted the 3’ terminal nucleotide when setting up the synthesis procedure. A new correct primer supplied by the manufacturer allowed the ApoE genotyping to proceed as had been carried out prior to this event. We emphasize, however, that incorrect ApoE genotyping did occur because of the shortened F6 primer; it was not a degenerate primer.

The missing 3’ A terminal nucleotide of the F6 primer allowed the primer pair to anneal to the incorrect target and amplify a product of size nearly equal to that expected. The 3’ terminal nucleotide of primer F4 anneals correctly to the inappropriate target. Both primers had at least ten bases each at their 3’ ends hybridized to the complementary genomic DNA regions, sufficient to permit priming in the PCR reaction and thermocycling conditions employed. Other unknown factors may have also favored priming in this way.

In most cases correct annealing of the terminal 3’ base of a primer to the template sequence is essential to allow the DNA
polymerase to catalyze efficiently the extension reaction\(^{(14)}\).

Had the correct F6 primer been used, the 3’ A nucleotide would have been mispaired and have probably abrogated the amplification; however, DNA polymerases sometimes discriminate poorly certain primer-template mismatches, notably the C-A mismatch present in this case\(^{(7, 17)}\). The specificity of this primer pair is therefore compromised.

### Conclusions

The sequences of primers F4 and F6 were obtained from previous work\(^{(11)}\) and were adopted accordingly. However this primer pair had been specified previously in a different context\(^{(10)}\), such that their 5’ regions do not hybridize to the target region because the resultant amplicon was designed to be digested with two restriction enzymes and then subcloned. For these reasons this primer pair is not optimally suited for ApoE genotyping, even though subsequent workers have continued to use it in their modified assays. The primers P3 and P4 described by Kontula et al.\(^{(13)}\) are probably more appropriate for this purpose. Subsequently, we designed a new pair of primers to amplify reliably the region housing the two polymorphisms in exon 4 of the ApoE gene; these are very similar to primers P3 and P4\(^{(11)}\) and their sequences are: AEF: 5’-GAACAGCTACCCCTGTTGCGG-3’ and AER: 5’-GCCTCCCGCCCTGATACACTG-3’.

### References


13. KONTULA, K. et al. Apolipoprotein E polymorphism determined by restriction enzyme analysis of DNA amplified by polymerase chain reaction: convenient alternative to


