Efficient mispriming during apolipoprotein E genotyping

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Iniciação errônea eficiente durante a genotipagem de apolipoproteína E

Gabriela Merheb de Azevedo Souza[1]; Nara Lúcia Romano[1]; Martin R. Whittle[2]

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

unitermos

Reação em cadeia de polimerase

Apolipoproteína E

Genotipagem de ApoE

resumo

A determinação do genótipo da apolipoproteína E (ApoE) é um procedimento clinicolaboratorial comum. O método mais freqüentemente utilizado envolve a amplificação por reação em cadeia de polimerase (PCR) de uma região alvo, seguida pela digestão com enzima de restrição do fragmento resultante, obtendo-se, assim, um modelo de fragmentos de restrição genótipo-específico. Nós descrevemos uma dificuldade inesperada que encontramos durante nossa rotina laboratorial.

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 chylomicrons, chylomicron 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[9] and to Alzheimer's disease[16], and may influence longevity[18] and cognitive function[18].

Until now several different ApoE genotyping techniques have been described; these include allele-specific oligonucleotide probes[20], minisequencing[19], restriction isotyping using HhaI or AflIII/HaeII with gel electrophoresis[11,13,22] and with capillary electrophoresis[5,8], the amplification refractory mutation system (ARMS)[21], single-strand conformation polymorphism[1], oligonucleotide ligation assays[4], and heteroduplex analysis[6]. More recent technologies have permitted genotyping by amplicon melting curve analysis using real-time thermocyclers[2,3], by electrochemical biosensors[15] and by LNAs[12].

Central to most of these methods is an initial PCR amplification of the region housing the two polymorphic

1. Bióloga da Genomic Engenharia Molecular Ltda.
2. Médico geneticista da Genomic Engenharia Molecular Ltda.

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′-ACAGAATTCGCCCC GGCCTGGTACAC-3′ and F6: 5′-TAAGCTTGGCACGGCTG 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 (Biotools – 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 10mg 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 nondenaturing 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 elctrophoresis. 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

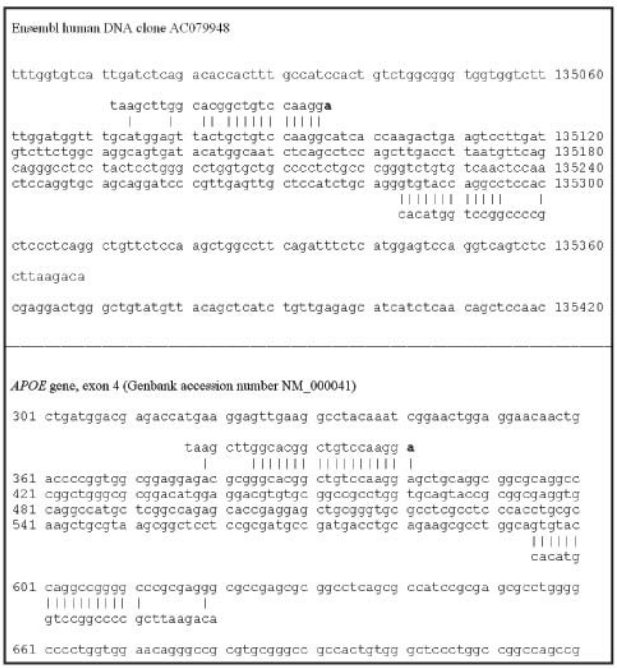

Figure – *The figure shows how primers F6 (superior) and F4 (inferior) hybridize to the keratin 18-like sequence in the upper panel and to the ApoE exon 4 sequence in the lower panel. The 3' terminal A nucleotide of primer F6 is shown in bold, and it is missing from this primer in the upper panel, permitting mispriming. The amplicon sizes are 238bp and 244bp in the upper and lower panels respectively*

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[13] and their sequences are: AEF: 5'-GAACAACTGACCCTGGTGGCGG-3' and AER: 5'-CGGTCCCCGGCCTGGTACACTG-3'.

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Mailing address

Martin R. Whittle
Genomic Engenharia Molecular Ltda.
Rua Itapeva 500/cj. 5AB
CEP 01332-903 – São Paulo-SP
Brasil
Tel.: (11) 288-1188
Fax: (11) 288-4962
e-mail: mwhittlt@genomic.com.br