Detection of apoB-100 R3500Q Mutation by Competitive Allele-Specific Polymerase Chain Reaction

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The apolipoprotein B-100 mutation R3500Q is one of the most common inherited defects causing abnormality of the lipid metabolism. We describe a one-step, single-tube PCR technique for detection of the mutation based on competition between allele-specific primers. Three oligonucleotides are used: two allele-specific primers differing in their 3' nucleotide (for the wild-type and the mutant allele) together with a common primer, resulting in simultaneous amplification of both alleles. This provided internal control of successful amplification and is expected to result in increased specificity. The allele-specific primers differ also in length, allowing us to distinguish both alleles by their size in a

single electrophoretic run. For optimization of the protocol, DNAs genotyped before by oligonucleotide ligation assay were used. The individual genotypes obtained by CAS-PCR coincided fully with the ones from a referent OLA test: seven heterozygous individuals were found, 4 of them among 150 unrelated hypercholesterolemic individuals studied and other three in the pedigrees of heterozygous carriers. On the overall 160 genotypes were determined, neither false-positive (0 out of 153 non-carriers) nor false-negative (0 out of 7 carriers) results were obtained. No homozygous mutant genotypes were identified in this sample. *J. Clin. Lab. Anal.* 15:256–259, 2001. ©2001 Wiley-Liss, Inc.

Key words: competitive allele-specific PCR; primer competition; apoB-100 R3500Q

The apolipoprotein B-100 (apoB-100) mutation Arg³⁵⁰⁰→Gln (R3500Q) causes reduced binding to the low-density lipoprotein (LDL) receptor and hypercholesterolemia (1). In most Caucasian populations studied so far, the underlying G→A substitution at nucleotide 10,708 in the apoB gene occurs with a frequency about 1:500 (2), and one in about 50 individuals with hypercholesterolemia is a carrier (3), thus, the mutation is one of the most common inherited defects causing abnormality of the lipid metabolism, and increased risk of atherosclerosis.

Methods based on the separation of the wild and mutant PCR amplicons, such as single-strand conformation analysis, heteroduplex analysis, denaturing gradient gel electrophoresis, are in use for apoB-100 R3500Q mutation screening (4–6). However, they all require additional confirmation steps. Methods, such as allele-specific oligonucleotide hybridization, oligonucleotide ligation assay (OLA), allele-specific PCR (AS-PCR), and mutagenic PCR with subsequent RFLP analysis detect the mutation unambiguously (7–10). Nevertheless, their further simplification offers potential for broader use by the routine clinical laboratories.

AS-PCR is suitable for routine biochemical diagnostic laboratory use, especially when a mutation is not amenable to restriction analysis unless mutagenic primers are used, because of its low costs per result and high laboratory throughput under minimal requirements for equipment and supply.

However, the inherent ability of the most commonly used *Taq* polymerases to elongate mismatched primers confers a risk for false positive results. The risk is highest when mutations G to A, respectively C to T antisense (the case with the apoB-100 R3500Q), are being discriminated, because the created mismatches are known to be among the most tolerated by the polymerase (11,12). On the other hand, it has been shown that competition between allele-specific primers can increase the specificity (13) and hopefully minimize the risk for false positives.

Here, we describe a one-step, single-tube PCR technique for detection of the apoB-100 R3500Q mutation based on competition between allele-specific primers. Three oligonucleotides are used: two allele-specific primers differing in their 3' nucleotide (for the wild-type and the mutant allele) together with a common primer, resulting in simultaneous amplification of both alleles, thereby providing internal control of successful amplification (at least one of the allelic prod-

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ucts should be visualized) and expected to have an increased specificity. The allele-specific primers differ also in length, allowing us to distinguish both alleles by their size in a single electrophoretic run.

MATERIALS AND METHODS

DNA samples from whole blood were extracted (14). To distinguish both alleles in a single electrophoretic run, competitive allele-specific primers with different length—5'ACTTACTTGAATTCCAAGAGCACACG3' (wild-type, #10,683-#10,708, 26 n) and 5'ACTTGAATTCCAAGAGCACACA3' (mutant, #10,687-#10,708, 22 n), together with a common primer (5'TATATGCGTTGGAGTGTGGCT3', #10,787-#10,807, 21 n)—were used in CAS-PCR, resulting in amplicons of size 125 and 121 bp (Fig. 1), respectively. The primers used in this study were synthesized by Microsynth, Switzerland.

For optimization of the competitive allele-specific (CAS)-PCR protocol DNAs genotyped before by oligonucleotide ligation assay (OLA) (after reference 8) were used. Lacking homozygous mutant genomic DNA, we prepared mutagenized simulative test-templates (MSTTs). Two separate PCR reactions were run for 35 cycles (denaturation at 95°C, 30 sec, annealing at 54°C, 60 sec, elongation at 72°C, 20 sec) in PCR buffer (50 mmol/l Tris-HCl, pH 9.2, 0.01 mmol/l EDTA, 16 mmol/l (NH₄)₂SO₄, 1 g/l Tween 20), with 0.25 mmol/l each of dNTP, 2.0 mmol/l MgCl₂, and 20.0 g/l DMSO. Added to each reaction mixture (total volume 20 µl) were 1 U *Taq* polymerase, wild-type/common, and mutant/common primer sets (6.0 pmol each primer), respectively, and template genomic DNA (50 ng) from a heterozygous carrier genotyped beforehand by OLA. The PCR products were separated on 80 g/l polyacrylamide gel electrophoresis (acrylamide/bisacrylamide 19:1) in TBE (89

mM Tris-boric acid, 2 mM EDTA), using a vertical slab gel unit (80 × 70 × 0.75 mm, Mighty Small SE250, Hoeffer Scientific Instruments, USA), cooled by running tap water jacket. The electrophoresis was run under constant voltage and time (140 V, 60 min). The gel was silver stained (15) and dried with heating in a vacuum dryer (Hoeffer Scientific Instruments) for 40 min. Single PCR products, corresponding to the wild-type allele (125 bp) and to the mutant allele (121 bp), were obtained with each of the respective primer sets.

Under the conditions described above, PCR products were obtained and separated on polyacrylamide gels. The bands were visualized by ethidium bromide staining (0.5 µg/ml), and the amplicons with expected sizes were excised and eluted in PCR buffer. Three different MSTTs (homozygous wild-type, homozygous mutant, and heterozygous) were prepared and further used for optimization of the CAS-PCR conditions. For wild-type and mutant MSTTs, simulating the respective homozygous genotypes, 1.0 ng from the eluted PCR products was used for each PCR reaction. To simulate the heterozygous genotype a MSTT was prepared with equal quantities of both wild-type and mutant MSTTs and 1 ng of the mixture was used for each PCR reaction. The CAS-PCR mixtures (20 µl total volume, PCR buffer and common primer, 0.25 µmol/l) were used to analyze the following variations: *Taq* polymerase (STS, Bulgaria), 0.4–1.0 U; dNTPs, 0.25–0.15 mmol/l; dimethyl sulfoxide (DMSO), 20–100 g/l; MgCl₂, 1.0–2.0 mmol/l; and allelic primers between 0.1 and 0.4 µmol/l, with wild-type/mutant ratio from 1:1 to 1:3. The PCR was run for 35 cycles, with denaturation at 95°C (30 sec) and elongation at 72°C (20 sec), and the annealing was varied between 54 and 60°C (20–60 sec). The PCR products were separated on polyacrylamide gels, silver stained, and dried as described above.

The optimized conditions used for further analysis of ge-

Fig. 1. Genotyping of apoB-100 R3500Q mutation by CAS-PCR. (A) Principle of the technique. Allele-specific primers differing in their 3' nucleotide and in length are used simultaneously with a common primer. PCR

products are 125 and 121 bp, respectively. (B) Lane 1: molecular size markers (pBR/*Msp*I). Lanes 2, 4, and 5: heterozygous carriers. Lanes 3, 6, and 7: wild-type homozygous individuals.

nomeric DNA are those shown unambiguous patterns on the three simulative MSTTs. The established optimized CAS-PCR conditions for distinguishing the three MSTT genotypes (see Results) were tested on 160 human genomic DNA templates (50 ng/sample): 150 unrelated hypercholesterolemic individuals (total serum cholesterol >7.0 mmol/l) and 10 members of pedigrees of heterozygous carriers, all genotyped beforehand by OLA.

RESULTS

We optimized the CAS-PCR conditions for detection of apoB-100 R3500Q mutation on MSTTs. The temperature, buffer components, polymerase, and dNTP concentration were optimized, and the best results were obtained under the following conditions: *Taq* polymerase, 0.45 U; dNTPs, 0.15 mmol/l; DMSO, 70 g/l; MgCl₂, 1.4 mmol/l; wild-type primer, 0.1 μmol/l; mutant primer, 0.275 μmol/l; common primer, 0.25 μmol/l; 35 cycles of denaturation at 95°C (30 sec) and elongation at 72°C (20 sec); annealing was optimal at 57°C (30 sec).

Human genomic DNA was successfully genotyped under the conditions established for MSTTs (Fig. 1B). The individual genotypes obtained by CAS-PCR coincided fully with the ones from the referent OLA test: seven heterozygous individuals were found, four of them among the 150 unrelated hypercholesterolemic individuals studied and the other three in the pedigrees of heterozygous carriers. On the overall 160 genotypes were determined, neither false-positive (0 out of 153 non-carriers) nor false-negative (0 out of 7 carriers) results were obtained. No homozygous mutant genotypes were identified in this sample.

DISCUSSION

Assays for detection of the apoB-100 R3500Q mutation based on allele-specific PCR have been developed in various formats: (i) two separate reactions with wild-type and mutant primers, respectively (9); (ii) one-tube amplification of fragments bearing both wild-type and mutant sequence, and simultaneous asymmetric PCR using a third internal mutant primer, thus providing control of successful amplification (16); (iii) one-tube amplification refractory system combined with competitive oligonucleotide priming, in which the amplification of the normal allele is prevented by a 3' dideoxynucleotide-terminated primer blocker, resulting in increased specificity from the competition between the allele-specific primers and thus avoiding false-positive results (17).

In the developed CAS-PCR protocol we aimed to combine the individual advantages of the above techniques: one-tube reaction (16,17), internal control of successful amplification (16), and increased specificity by creating competition between the allele-specific primers (17). Next, our intention was to add to the utilities of the above one-tube techniques (16,17)

the ability for discrimination between homo- and heterozygous carriers of the mutation.

Simplification of the electrophoretic detection can be achieved by different length of the allele-specific primers. In our protocol, one of the primers (wild type) was designed to be 4 bases longer than its competitor (mutant), and the common primer was positioned to keep the amplification products short enough (121 and 125 bases, respectively). The allele-specific primers used had a T_m of 74 and 62°C (mutant and wild type, respectively), whereas the common primer was designed to have a T_m in the range of the mutant one (62°C). These respective melting temperatures were supposed to restrict nonspecific polymerization, to provide easier temperature optimization (a small difference between T_m of allelic primers), and to avoid preferential amplification of any allele (T_m of the common primer closer to the T_m of the less stable, shorter primer). Primer concentration and ratio are important for obtaining the trade-off between specificity and efficiency. Different concentrations and ratios of allelic primers were tested, and optimal results were obtained using a 2.75-fold higher concentration of the shorter (mutant) allelic primer.

The annealing temperature was varied between 54 and 60°C, and an optimal range of 56–58°C was established; 57°C was the temperature with the best trade-off (clear detection signal and approximately equal amplification of both alleles for the heterozygous templates), with annealing times of 20 and 30 sec for extension.

Allele specificity and yield can be also affected by buffer components and especially by the magnesium ions and DMSO. It is well known that higher MgCl₂ concentrations enhance *Taq* polymerase activity at the expense of reduced specificity of the reaction, whereas addition of co-solvents, such as DMSO, can improve the allele selectivity. In our experiments, analyzing their combined effect in the range of 1.0–2.0 mM (MgCl₂) and 2–10% (DMSO), the effect of magnesium and DMSO concentration was found to be mutually dependent, and the most distinct detection was observed at 1.4 mM MgCl₂ and 7% DMSO.

Polymerase and dNTP concentration resulted in higher specificity upon lowering their concentrations, probably due to the competition conditions set. Decreasing the polymerase amount from 1.0 to 0.4 U/reaction and the dNTPs from 0.25 to 0.15 mM, we observed a slightly elevated specificity without significant change of the level of amplification.

The goal of this work was to develop a protocol to be used by routine clinical laboratory. The developed CAS-PCR protocol for detection of apoB-100 R3500Q mutation combines reliability (internal control with visualizing at least one of the allelic products), high specificity (due to competition between the allele-specific primers), simplicity (one-step, one-tube, single electrophoretic run without any labeling), and low cost per result. All these are valuable for routine diagnosis in risk groups and population screening.

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