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RAPID AND DIRECT DETECTION OF APOLIPOPROTEIN E GENOTYPES USING WHOLE BLOOD FROM HUMANS

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Polymerase chain reaction (PCR) is a powerful molecular biological tool in the field of toxicity testing and diagnostics. The use of PCR for large-scale genetic testing requires an effective method of sample processing. Unfortunately, isolation of PCR-quality DNA is time-consuming. PCR performed directly on whole blood is preferred because of time efficiency, cost of the procedure, and possible automation for large-scale toxicity evaluation and diagnosis. The apolipoprotein E (APOE) gene contains two single-nucleotide polymorphisms (SNP) located at codons 112 and 158, producing three APOE protein isoforms known to be associated with the risks of developing cardiovascular disease and susceptibility to Alzheimer’s disease. In the present study, an attempt was made to use the AnyDirect solution for APOE genotyping by PCR using whole blood directly without DNA purification. Results for two PCR methods, (1) conventional PCR using purified DNA and conventional buffer and (2) direct PCR using whole blood and AnyDirect solution, were compared in four different PCR-based APOE genotyping methods including PCR restriction-fragment-length polymorphism (PCR-RFLP), allele-specific PCR, SNaPshot mini-sequencing, and multiplex tetra-primer amplification refractory mutation system (T-ARMS) PCR. There was complete concordance in the APOE genotypes between conventional PCR and direct PCR, in all four different PCR-based APOE genotyping methods. Data demonstrated that the four different PCR-based APOE genotyping methods are able to determine the APOE genotypes successfully using whole blood directly with the use of AnyDirect solution. The direct multiplex T-ARMS PCR using whole blood may be the most rapid, simple, and inexpensive method for detecting APOE genotypes among four different APOE genotyping methods.

Apolipoprotein E (APOE) is a 34-kD glycoprotein that plays a key role in lipid metabolism and transportation (Mahley, 1988; Choi et al., 2004). APOE is encoded by a 3.6-kb gene containing 4 exons located on the long arm of chromosome 19 (Das et al., 1985). The APOE gene is polymorphic, possessing three alleles, designated ε2, ε3, and ε4, that differ in the presence of either C or T nucleotides at codons 112 and 158 in the fourth exon of APOE. These genes code for three APOE protein isoforms: E2 (Cys112/Cys158), E3 (Cys112/Arg158), and E4 (Arg112/Arg158), which vary by a cysteine–arginine interchange at the 112 and 158 sites of the polypeptide chain (Utermann et al., 1980; Emi et al., 1988). This polymorphism leads to
the presence of six different genotypes in the human population: three homozygous (ε2/ε2, ε3/ε3, ε4/ε4) and three heterozygous ones (ε2/ε3, ε2/ε4, ε3/ε4).

The APOE isoforms are associated with several pathological processes, including high cholesterol levels and coronary disease at an early age (Menzel et al., 1983), type III hyperlipidemia (Breslow et al., 1982), and development of late-onset Alzheimer’s disease (Rose et al., 1994; Molloy et al., 2007). The identification of APOE isoforms is important, as they may be considered as biomarkers for coronary heart and Alzheimer’s diseases.

Several methods were developed to identify APOE isoforms. These methods can be divided into two types: (1) separation of the lipoprotein isoforms by isoelectric focusing either with subsequent immunoblotting using an anti-APOE antibody or by direct immunofixation (Cartier and Sassolas, 1992; Hackler et al., 1994); and (2) polymerase chain reaction (PCR) techniques that directly determine the genotypes. The methods for PCR-based APOE genotyping include PCR restriction-fragment-length polymorphism (PCR-RFLP) (Hixson & Vernier, 1990), allele-specific PCR (AS-PCR) (Donohoe et al., 1999; Lee & Park, 2001), single-stranded conformational polymorphism (SSCP) (Tsai et al., 1993), SNaPshot mini-sequencing (Ben Avi et al., 2004), real-time PCR (Papp et al., 2003; Park et al., 2007), mass spectrometry (Srivivasan et al., 1998), and multiplex tetra-primer amplification refractory mutation system (T-ARMS) PCR (Yang et al., 2007a).

PCR is a powerful and useful molecular biological tool employed in the field of toxicity testing and diagnostics (Medina et al., 2009; Cui et al., 2009; Xiao et al., 2009). Although PCR is technically simple, it is labor-intensive and time-consuming. The rate-limiting step is usually the extraction of DNA from whole blood samples. PCR performed directly on whole blood is a more rapid and simple approach; however, direct PCR amplification of DNA from whole blood is not reliable and is difficult due to the presence of numerous PCR inhibitors. Recently, Yang et al. (2007c) developed a novel PCR reaction buffer, termed AnyDirect solution. This solution prevented inhibition of PCR by potent PCR inhibitors and effectively enhanced PCR quality and quantity from whole blood without prior DNA isolation (Yang et al., 2007b, 2007c, 2007d).

The aim of the present study was to (1) use the AnyDirect solution for APOE genotyping by PCR using whole blood directly without DNA purification and (2) compare the results between two PCR methods of conventional PCR using purified DNA and conventional buffer versus direct PCR using whole blood and AnyDirect solution, using four different PCR-based APOE genotyping methods: PCR-RFLP, AS-PCR, SNaPshot mini-sequencing, and multiplex T-ARMS PCR.

MATERIAL AND METHODS

Human Whole Blood and Genomic DNA

This study was approved by the Institutional Review Board of Seoul National University Bundang Hospital. In total, 45 peripheral blood samples from aged subjects were obtained from Seoul National University Bundang Hospital (Table 1). The genotypes of the 45 samples were ε3/ε3 (n = 5), ε4/ε4 (n = 2), ε2/ε3 (n = 12), ε2/ε4 (n = 3), and ε3/ε4 (n = 23). Genomic DNA was extracted from 0.2 ml of each blood sample by QIAamp DNA Blood mini kit (Qiagen, Hilden, Germany).

APOE Genotyping by AS-PCR

APOE genotyping with AS-PCR was performed with specific Cys primers (Cys112 and Cys158), Arg primers (Arg112 and Arg158), and a common reverse primer AS-R (Table 2). Conventional PCR was performed with 20 ng of genomic DNA, 0.4 μM of Cys primers or Arg primers, 0.4 μM of AS-R primer, 200 μM of dNTP mixture, 1 M of betaine, 2 mM of MgCl₂, 2.5 U of Taq DNA polymerase (BioQuest, Seoul, Korea), and reaction buffer in a 25-μl reaction volume. The amplification was initiated with denaturation at 95°C for 2 min, amplification by 35 cycles of 95°C for 30 s,
62°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 7 min. When direct PCR was performed, 20 ng genomic DNA and reaction buffer was replaced with 1 µl whole blood and AnyDirect solution (BioQuest), and the amplification was initiated at 95°C for 10 min, followed by 50 cycles of 95°C for 30 s, 68°C for 30 s, and 72°C for 60 s, and then final extension at 72°C for 7 min. The resulting PCR products (20 µl) were treated with 5 U HhaI (NEB, Ipswich, MA), for 3 h. The restriction fragments were identified with 12.5% discontinuous polyacrylamide gel electrophoresis (Haas et al., 1994) and ethidium bromide staining (0.5 mg/L).

**APOE Genotyping by PCR-RFLP**

Conventional PCR was performed with 100 ng purified genomic DNA, 0.5 µM of apoEf1 and apoEr1 primers (Table 2), 200 µM of dNTP mixture, 1 M betaine, 2 mM MgCl2, 2.5 U Taq DNA polymerase (BioQuest), and reaction buffer in a 50-µl reaction volume. The amplification was initiated at 95°C for 2 min, followed by 45 cycles of 95°C for 30 s, 68°C for 30 s, and 72°C for 30 s, and then a final extension at 72°C for 7 min. When direct PCR was performed, 100 ng of genomic DNA and reaction buffer was replaced with 1 µl whole blood and AnyDirect solution (BioQuest), and the amplification was initiated at 95°C for 10 min, followed by 50 cycles of 95°C for 30 s, 68°C for 30 s, and 72°C for 60 s, and then final extension at 72°C for 7 min. The resulting PCR products (20 µl) were treated with 5 U HhaI (NEB, Ipswich, MA), for 3 h. The restriction fragments were identified with 12.5% discontinuous polyacrylamide gel electrophoresis (Haas et al., 1994) and ethidium bromide staining (0.5 mg/L).

**APOE Genotyping by SNaPshot Mini-Sequencing**

PCR reaction was performed with 20 ng genomic DNA or 0.5 µl whole blood, 0.3 µM of FO and RO primers (Table 2), 0.2 mM of dNTP mixture, 0.75 mM betaine, 1.5 mM MgCl2, 1.5 U HotStarTaq DNA polymerase (Qiagen), and reaction buffer (Qiagen) or AnyDirect solution in a total 25-µl reaction volume. The program used was an initial denaturation step of 95°C for 15 min followed by 40 cycles of 94°C for 30 s, 66°C for 30 s, and 72°C for 60 s. The final extension step was carried out for 7 min at 72°C. After PCR, the presence of a 514-bp product was confirmed on a 2% agarose gel. To avoid participation in the subsequent primer extension reaction, primers and unincorporated dNTP of the PCR reaction were removed before genotyping as...
follows. Five microliters of PCR product was incubated with 2 U ExoSAP-IT (USB, Cleveland, OH) for 45 min at 37°C, followed by 15 min at 80°C for enzyme inactivation. The primers SP-112 and SP-158, corresponding to the two single-nucleotide polymorphisms (SNP) at positions 112 and 158, which are used for the extension reaction, are outlined in Table 2. These primers were specifically designed with different lengths in order to introduce sufficient separation on capillary electrophoresis. Reactions were carried out in a final volume of 10 µl containing 1 µl purified PCR product, 5 µl SNaPshot multiplex mix (containing Taq polymerase and fluorescent labeled deoxy-NTPs) (Applied Biosystems, Foster City, CA), and 0.2 µM SP-112 and SP-158. The cycling program was 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 30 s. After cycling, the unincorporated fluorescent deoxy-NTP were removed by adding 1 U shrimp alkaline phosphatase (Fermentas, Hanover, MD) and incubating for 30 min at 37°C, followed by 15 min at 85°C for enzyme inactivation. One microliter of SNaPshot reaction with 9 µl formamide (Sigma, St. Louis, MO) was loaded on an ABI 3100 Genetic Analyzer (Applied Biosystems). Samples were run using POP 4 polymer (Applied Biosystems) and analyzed using Genescan (version 3.1) software.

### RESULTS

**APOE Genotyping by Multiplex T-ARMS PCR**

PCR reaction was performed with 20 ng human genomic DNA or 1 µl whole blood, all 6 primers (0.2 µM FO, 0.15 µM RO, 0.15 µM Fl-1, 0.5 µM RI-1, 0.15 µM Fl-2, and 0.4 µM RI-2) (Table 2), 0.75 M betaine, 2.0 mM MgCl2, 0.2 mM of dNTP mixture, 1 U HotStarTaq DNA polymerase (Qiagen), and reaction buffer (Qiagen) or AnyDirect solution in 20 µl reaction volume. The amplification was carried out with an enzyme activation step for 15 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 68°C, and 30 s at 72°C, and a final extension for 7 min at 72°C. The amplicons were separated by 2% agarose II (Amresco, Solon, OH) gel electrophoresis and stained with ethidium bromide (0.5 mg/L).

**APOE Genotyping by AS-PCR**

When APOE genotyping with AS-PCR was performed by conventional PCR using purified DNA and compared to direct PCR using whole blood, the results demonstrated no discrepancies between genotypes for the two PCR methods (Figure 1). The homozygote ε3/ε3 genotype, which contains Cys at codon 112

### TABLE 2. PCR Primers for APOE Genotyping

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys112</td>
<td>5'-GGCGAGATGGAGGACACGTC-3'</td>
</tr>
<tr>
<td>Cys158</td>
<td>5'-ATGGCATGCTCGACAGAAAT-3'</td>
</tr>
<tr>
<td>Arg112</td>
<td>5'-GGCGAGATGGAGGACACGTC-3'</td>
</tr>
<tr>
<td>Arg158</td>
<td>5'-ATGGCATGCTCGACAGAAAT-3'</td>
</tr>
<tr>
<td>AS-R</td>
<td>5'-GTTCAGTGATTGTCGCTGGGCA-3'</td>
</tr>
<tr>
<td>FO</td>
<td>5'-ACTCACCCCCCTGGCGCGAGGA-3'</td>
</tr>
<tr>
<td>RO</td>
<td>5'-CAGCGTATCTGTCCGCTTGTC-3'</td>
</tr>
<tr>
<td>Fl-1</td>
<td>5'-GGCCGGGACATGGAGGACACGTC-3'</td>
</tr>
<tr>
<td>Fl-2</td>
<td>5'-GGCGATGCCGATGCTCGACAGACGC-3'</td>
</tr>
<tr>
<td>RI-1</td>
<td>5'-CCCAGCTGATGCTGCACTGCA-3'</td>
</tr>
<tr>
<td>RI-2</td>
<td>5'-GCCACCATGCGTACCTGCACTGCA-3'</td>
</tr>
<tr>
<td>SP-112</td>
<td>5'-CCAAGGACCTGCAAGCGCGGCA-3'</td>
</tr>
<tr>
<td>SP-158</td>
<td>5'-GCCCGGCGCTGTCCGACAGAAAT-3'</td>
</tr>
</tbody>
</table>

aThe boldface lowercase letters denote a deliberate mismatch, and the boldface uppercase letters denote allele-specific mismatch.
FIGURE 1. Electropherogram of AS-PCR using purified DNA and whole blood. AS-PCR was performed with Cys primers (lane A), containing Cys112 (588 bp) and Cys158 (451 bp) primers, or Arg primers (lane B), containing Arg112 and Arg158 primers. Every APOE genotype was amplified with allele specific primers, including \( \varepsilon_3/\varepsilon_3 \) (Cys112 and Arg158), \( \varepsilon_4/\varepsilon_4 \) (Arg112 and Arg158), \( \varepsilon_2/\varepsilon_3 \) (Cys112, Cys158, and Arg158), \( \varepsilon_2/\varepsilon_4 \) (Cys112, Cys158, Arg112, and Arg158), and \( \varepsilon_3/\varepsilon_4 \) (Cys112, Arg112, and Arg158). Lane M: 100-bp ladder.

and Arg at codon 158, produced a 588-bp product at Cys primers and a 451-bp product at Arg primers. The \( \varepsilon_4/\varepsilon_4 \) genotype, which possesses Arg at both codons 112 and 158, produced 588-bp and 451-bp products at Arg primers, but none at Cys primers. The heterozygote \( \varepsilon_2/\varepsilon_3 \) genotype, which contains Cys at codon 112 and both Cys and Arg at codon 158, generated 588-bp and 451-bp products at Cys primers and 451-bp product at Arg primers. The \( \varepsilon_3/\varepsilon_4 \) genotype, which contains Cys and Arg at codon 112 and Arg at codon 158, produced 588-bp product at Cys primers and 588-bp and 451-bp products at Arg primers. The \( \varepsilon_2/\varepsilon_4 \) genotype, which possesses both Cys and Arg at both codon 112 and 158, generated 588-bp and 451-bp amplicons at both Cys and Arg primers. AS-PCR of all 45 samples enabled distinction of APOE genotypes by both PCR methods. There was complete concordance in the genotypes between conventional PCR and direct PCR.

APOE Genotyping by PCR-RFLP

The first step in APOE genotyping with PCR-RFLP was amplification of APOE sequences containing amino acids 112 and 158 from genomic DNA. The purified genomic DNA or whole blood was first amplified by the consensus FO/RO primer pair (Table 2) and the 514-bp PCR products were subjected to SNaPshot mini-sequencing. Typical SNaPshot mini-sequencing profiles obtained by conventional PCR and direct PCR for the APOE genotyping on the three samples are shown in Figure 3. A single peak for each SNP marker was clearly evident. Data were easy to interpret, not reader biased, and there was complete concordance.

HhaI restriction endonuclease digestion. \( HhaI \) restriction endonuclease cleaved specific sites, resulting in a specific restriction pattern for each genotype (Figure 2). APOE genotypes were determined by the patterns of these fragments from electropherograms obtained from PCR-RFLP \( \varepsilon_3/\varepsilon_3 \) (91, 48, and 35 bp), \( \varepsilon_4/\varepsilon_4 \) (72, 48, and 35 bp), \( \varepsilon_2/\varepsilon_3 \) (91, 83, 48, and 35 bp), \( \varepsilon_2/\varepsilon_4 \) (91, 83, 72, 48, and 35 bp), and \( \varepsilon_3/\varepsilon_4 \) (91, 72, 48, and 35 bp). Figure 2 shows complete concordance in specific restriction pattern for each genotype between conventional PCR and direct PCR for five samples tested.

APOE Genotyping by SNaPshot Mini-Sequencing

The purified genomic DNA or whole blood was first amplified by the consensus FO/RO primer pair (Table 2) and the 514-bp PCR products were subjected to SNaPshot mini-sequencing. Typical SNaPshot mini-sequencing profiles obtained by conventional PCR and direct PCR for the APOE genotyping on the three samples are shown in Figure 3. A single peak for each SNP marker was clearly evident. Data were easy to interpret, not reader biased, and there was complete concordance.
FIGURE 3. APOE genotypes were analyzed by SNaPshot minisequencing using purified DNA and whole blood followed by capillary electrophoresis. The left and right peaks for each genotype represent single-nucleotide polymorphisms encoding for Cys/Arg at positions 112 and 158, respectively.

of genotypes for the two PCR methods (conventional PCR and direct PCR).

**APOE Genotyping by Multiplex T-ARMS PCR**

The multiplex T-ARMS PCR method for APOE genotyping was applied to 45 samples that were confirmed by AS-PCR and there was also complete concordance in the genotypes between conventional PCR and direct PCR (Figure 4). In multiplex T-ARMS PCR, two common outer primers (FO and RO) amplified the non-allele-specific common amplicon (514 bp), and each of two sets of allele-specific inner primers (FI-1 and RI-1 at codon 112, and FI-2 and RI-2 at codon 158) generated allele-specific amplicons (Figure 4). At codon 112, the ε2 and ε3 alleles, which contain TGC, produced an allele-specific amplicon with the FO and RI-1 primers (112 bp). However, the ε4 allele, which possesses CGC, was amplified by the FI-1 and RO primers to generate the ε4-specific amplicon (444 bp). At the other polymorphism site, codon 158, the ε2 allele, which contains TGC, formed an amplicon with the FO and RI-2 primers (253 bp), whereas the ε3 and ε4 alleles, which possess CGC, produced an amplicon with the FI-2 and RO primers (308 bp).

**DISCUSSION**

AS-PCR and PCR–RFLP are the most common and widely used methods in genotyping. Appropriately, these methods were used in this study as the primary basis of comparison for the other APOE genotyping methods. The amplification products using whole blood and AnyDirect solution were useful to perform AS-PCR, which yielded allele-specific amplicons of 588 and 451 bp at codons 112 and 158, respectively (Figure 1). Direct PCR with the use of AnyDirect solution also successfully amplified PCR products from whole blood, and the PCR products displayed specific restriction patterns following restriction endonuclease digestion at recognition sites in PCR-RFLP (Figure 2). Use of whole blood in PCR reactions resulted in a reddish or brownish color in the final PCR products; however, this did not interfere with detection of PCR products.
in restriction enzyme digestion and agarose gel electrophoresis.

The SNaPshot mini-sequencing approach consists of the single base extension of an unlabelled primer that anneals one base upstream to the relevant SNP with a fluorochrome-labeled dideoxynucleotide. The allele designation is then possible by separating extended products and detecting the fluorescence by capillary electrophoresis. The first step of SNaPshot mini-sequencing is amplification of a DNA region containing the SNP of interest. Presently, whole blood was successfully amplified by our direct PCR using AnyDirect solution, resulting in a 514-bp fragment containing both APOE SNP sites. A volume of 0.5 µl whole blood was sufficient to generate amplicons and yield sufficient detectable SNP extension products in the SNaPshot profile. As recommended in the SNaPshot manufacturer’s protocol, the straightforward and fast exonuclease I and shrimp alkaline phosphatase treatment were used as a PCR cleanup method. This enzymatic cleanup system also worked for our direct PCR method.

The SNaPshot mini-sequencing method is able to offer a reliable, sensitive, and convenient genotyping of a larger numbers of samples. However, the cost of SNaPshot analysis is high because it initially requires expensive equipment. Recently, a multiplex T-ARMS PCR method with six primers in a single reaction tube was reported for APOE genotyping (Yang et al., 2007a). The six primers used in this study were slightly modified from the previously reported primer sequences (Yang et al., 2007a). The modified multiplex T-ARMS PCR method using whole blood directly with the use of AnyDirect solution was able to determine the APOE genotypes successfully (Figure 4).

The direct multiplex T-ARMS PCR method does not require DNA purification, other enzymatic treatment steps, or expensive instrumentation, and can be carried out with one PCR step in a single reaction tube. APOE genotyping with the modified direct multiplex T-ARMS PCR can be achieved in approximately 2.5 h, including amplification of the APOE gene with multiplex T-ARMS PCR and identification of the genotypes with agarose gel. Therefore, the direct multiplex T-ARMS PCR using whole blood without DNA isolation with the use of AnyDirect solution may be the most rapid, simple, and inexpensive method for detecting APOE genotypes among the four different APOE genotyping methods of PCR-RFLP, ASPCR, SNaPshot mini-sequencing, and multiplex T-ARMS PCR.

REFERENCES

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