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Discrepancy in Genotyping of *Apolipoprotein E* between Allele-Specific PCR and Fluorescence Resonance Energy Transfer or Sequencing

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The human *apolipoprotein E* (*APOE*) gene contains several single-nucleotide polymorphisms (SNPs) that are distributed across the gene. The genotype of the *APOE* gene has important implications as a risk factor for various diseases. We observed 2 cases in which the results of allele-specific PCR (AS-PCR) of the *APOE* gene were not consistent with those of fluorescence resonance energy transfer (FRET) or sequencing analysis. In these cases, genotyping by AS-PCR showed that patients were ε2 homozygotes, while sequencing analysis and FRET showed that they were ε2/ε3 heterozygotes. Herein, we describe the causes of the errors in genotyping and describe the significance of these errors. *(Korean J Lab Med 2010;30:325-8)*

Key Words : *APOE, Allele-specific PCR, Fluorescence resonance energy transfer, Genotyping, Sequencing*

The human *apolipoprotein E (APOE)* gene contains several single-nucleotide polymorphisms (SNPs) that are distributed across the gene [1]. The 2 most common SNPs lead to changes in the coding sequence and yield 3 different polymorphic alleles which engender 6 different genotypes: ε2, ε3, and ε4, (Table 1). In addition, rare alleles such as ε1, ε5, ε7, etc., are also present. In contrast to the ε3 allele, the ε2 allele is associated with protection against late-onset Alzheimer's disease (AD) [2]. Most patients with type III hyperlipoproteinemia are also known to be homozygous for the ε2 allele [3]. However, the presence of the ε4 allele is a risk factor for AD and is associated with early onset of AD [2, 4-6]. Thus, the genotypes of the *APOE* gene have important implications as risk factors for AD, cardiovascular disease, etc.

Methods of *APOE* genotyping include sequencing analysis, allele-specific PCR (AS-PCR) [7, 8], rapid-cycle PCR, and fluorescence resonance energy transfer (FRET) which is performed using the LightCycler system [9, 10].

The FRET method appears to be rapid, simple, and accurate, thereby suggesting that it can be successfully used for diagnostic purposes. Multiplex AS-PCR analysis is also reliable, simple, and less time-consuming; however, it has a disadvantage-unexpected nucleotide polymorphisms or mutations located in the DNA template or adjacent to the 3′ end of the primer-binding site would hinder amplification, thereby leading to incorrect genotyping.

We report 2 recentlyobserved cases that showed discrepancies between the results obtained using AS-PCR and those obtained using FRET and sequencing analysis of the *APOE* gene.

APOE genotyping of a total of 673 subjects was performed from June to November 2009 (Fig. 1). Among these, 40 subjects (group 1) underwent genotyping with AS-PCR (Seeplex *ApoE* Genotyping Kit; Seegene, Seoul,

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Korea) and FRET (LightCycler *ApoE* Mutation Detection Kit; Roche Diagnostics, Mannheim, Germany). Further, 102 subjects (group 2) underwent genotyping with AS-PCR along with sequencing analysis of 4 exons of *APOE*. For the remaining 531 subjects (group 3), sequencing analysis was performed to confirm the presence of homozygous ε2 alleles, which was predicted by the results of previously performed AS-PCR.

One subject (case 1) from group 1 was shown to have the genotype ε2/ε2 and ε2/ε3 by AS-PCR and FRET, respectively. A sequencing analysis of this case showed that the subject had the genotype ε2/ε3 with 2 single-nucleotide G-to-A substitutions at the nucleotide positions 784 and 787, which both lead to the substitution of glutamic acid (GLU) to lysine (LYS) residue. In group 2, the results obtained using AS-PCR and sequencing analysis of 4 *APOE* exons were similar, and one of the ε3 homozygous alleles had the SNP. In group 3, 5 of the 531 cases were identified as ε2/ε2 by AS-PCR; among these, one subject (case 2) was identified as ε2/ε3 with SNP and 4 were identified as ε2/ε2. AS-PCR and sequencing analysis results of the two cases are shown in Fig. 2, 3, respectively.

The AS-PCR assay requires 3 sets of oligo primers, namely, wild-type, mutant, and common primers. The method involves 2 parallel experiments with the primer sets "wild+common" (WC experiment) and "mutant+common" (MC experiment). On the basis of the experimental results, 3 outcomes are possible: homozygous wild type, heterozygous, and homozygous variant type. The presence of a band in the WC experiment but not in the MC experiment indicates that the sample is homozygous

Table 1. Six different genotypes resulting from 3 polymorphic alleles (ε2, ε3, and ε4) of the *APOE* gene

Allele*	Nucleotide number	
	388†	526†
ε2/ε2	T/T	T/T
ε2/ε3	T/T	T/C
ε2/ε4	T/C	T/C
ε3/ε3	T/T	C/C
ε3/ε4	T/C	C/C
ε4/ε4	C/C	C/C

*ε2 (388T, 526T), ε3 (388T, 526C), ε4 (388C, 526C):†A of the ATG initiation codon was numbered+1 based on GenBank accession number NM_000041.

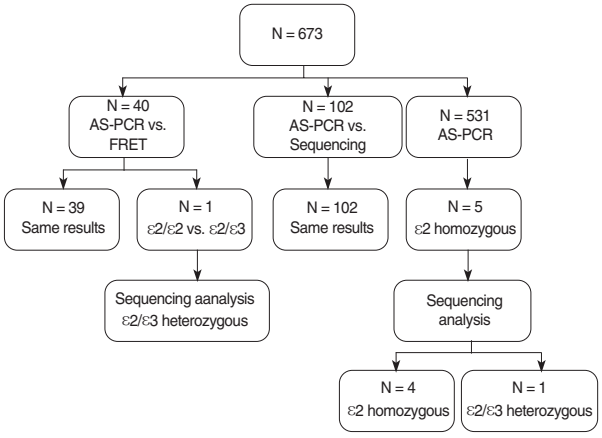

Fig. 1. A flowchart summarizing the analytic courses and results for the 673 cases.

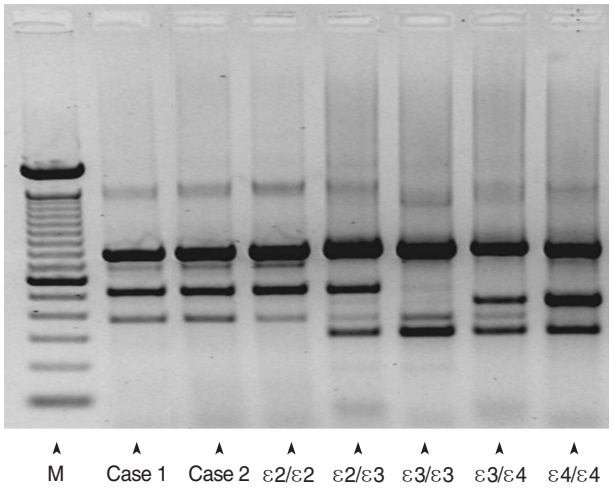

Fig. 2. Agarose gel electrophoresis of AS-PCR products. Case 1 and case 2 were ε2/ε2 homozygous.
Abbreviation: M, *APOE* marker.

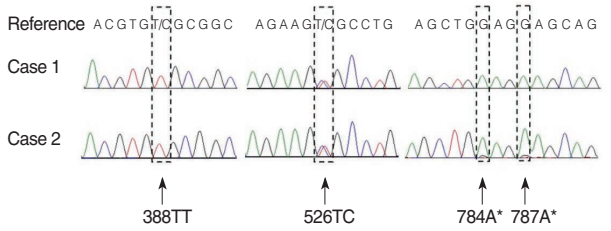

Fig. 3. Detection of mutations in the *APOE* gene by PCR-direct sequencing analyses. Case 1 and case 2 showed 388TT (left), 526TC (middle), and 2 point hemizygous mutations (784A* and 787A*) (right).
*Results of sequencing analysis of specific primers that target a 526C nucleotide are shown to the right in cases 1 and 2.

wild type. However, a band in the MC experiment but not in the WC implies that it is a homozygous variant type. Appearance of a band in both the WC and MC experiments indicates heterozygosity. However, if the nucleotides associated with the common primers show variation, no bands will be obtained in both the MC and WC experiments, thereby yielding inaccurate results.

Evaluation of the results from the AS-PCR and FRET analyses indicated a discrepancy in the results obtained using the 2 methods in 1 case from group 1 (AS-PCR, ε2 homozygous; FRET, ε2/ε3 heterozygous). Sequencing analysis for this case showed that the subject was ε2/ε3 heterozygous with SNP. We also performed AS-PCR and sequencing analysis on samples from the group 2 patients; however, no discrepancies in the genotyping of *APOE* were observed. We then performed sequencing analysis to confirm the ε2 homozygous results obtained by AS-PCR; and we identified an additional case of inaccurate genotyping. The SNP found in the ε3 allele yields ε7, a naturally occurring mutant of ε3 previously reported in humans [11]. The distance from position 526C of the ε3 allele to 784A of the ε7 allele was 258 bp. The size of the PCR product from 526C in AS-PCR is 311 bp. Thus, the common primer for 526C involves all or part of the 4 nucleotides between 784 and 787. Therefore, PCR analysis for the ε7 allele is expected to yield a band for 388T with no band for 526C. As a result of this phenomenon, ε3/ε7, ε4/ε7, and ε2/ε7 were interpreted as ε3/ε3, ε4/ε3, and ε2/ε2, respectively, in AS-PCR (Fig. 4).

The ε7 allele is associated with hyperlipidemia and atherosclerosis, and the *APOE* protein of this allele shows defects in its binding to the LDL receptor [12]. However, in a study conducted using the recombinant allele ε7 of *APOE*, Dong et al. [13] reported that the receptor-binding activity is not affected. Therefore, the association between ε7 and LDL receptor-binding activity is unclear. According to our data for group 2, in which sequencing of the entire *APOE* gene was performed, the relative frequency of the ε7 allele was 0.0049, and this was less than that of the ε2 allele (0.0833). Information about other rare alleles, including ε7, has been reported; however, this information is less important than that for the common alleles, such as ε2, ε3, and ε4.

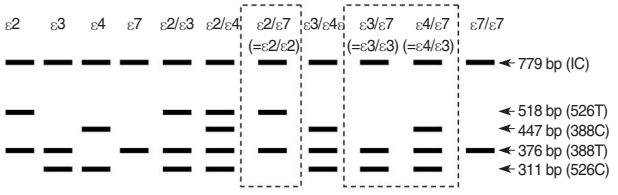

Fig. 4. PCR bands of each allele (ε2, ε3, ε4, and ε7) and their interpretations in AS-PCR. The ε7 alleles of genotypes ε3/ε7 and ε4/ε7 were interpreted as ε3, and the ε7 allele of genotype ε2/ε7 was interpreted as ε2 (dotted box).

In summary, sequencing analysis showed errors in genotyping when AS-PCR using the *ApoE* Genotyping Kit was performed. Moreover, we determined that the ε2 homozygous result requires further evaluation by sequencing analysis or other methods by using primers that can avoid the error caused by unexpected nucleotide polymorphisms or mutations located in the DNA template.

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