Real-Time Quantitative Polymerase Chain Reaction for Apolipoprotein E genotyping

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Abstract

Background : Apolipoprotein E (ApoE) is an important substance responsible for the lipoprotein metabolism and the transportation of lipid and known to play an important role in the body's physiological and pathological processes. Recently, ApoE genotyping test has been applied to the field of diagnostic methods for predicting high risk of Alzheimer's disease, cardiovascular diseases, as well as new diseases such as immune regulation disorders. Therefore, a faster and more accurate ApoE genotyping test is required for the prevention of related disease and the general public health.

Methods : We evaluated 220 serum specimens using two kinds of assays: one-stop Real-Q ApoE genotyping (BioSewoom, Seoul, Korea) and Seeplex ApoE ACE genotyping (Seegene, Seoul, Korea). The results were finally confirmed using sequencing as the reference. Statistical analysis was performed for the concordance rates between each assay and sequencing and the sensitivity and specificity were calculated with their 95% confidence intervals.

Results : Sequencing results of 220 DNA samples showed 1 case of *E2/E2*, 139 of *E3/E3*, 9 of *E4/E4*, 17 of *E2/E3*, 4 of *E2/E4*, 50 of *E3/E4*. The concordance rate between One-step Real-Q ApoE genotyping and sequencing was 100% (220/220), and that between Seeplex ApoE ACE genotyping and sequencing was 99.5% (219/220). Both sensitivity and specificity of One-step Real-Q ApoE genotyping for every genotypes was 100%. The sensitivities and specificities of Seeplex ApoE ACE genotyping were almost 100%, with two exception: 99.3% sensitivity in *E3/E3* and 99.5% specificity in *E2/E2*.

Conclusions : The performances of One-step Real-Q ApoE genotyping and Seeplex ApoE ACE genotyping were comparable overall. However, the former showed better sensitivity and specificity comparable with those of sequencing. Hence, One-step ApoE genotyping could be a faster and more accurate option for ApoE genotyping. **(J Med Life Sci 2015;12(1):30-34)**

Key Words : One-step ApoE Genotyping, Sequencing, Concordance rate

Introduction

Apolipoprotein E (ApoE) is an important substance responsible for the lipoprotein metabolism and the transportation of lipid and known to play an important role in the body's physiological and pathological processes. ApoE is synthesized mainly in the liver, but is also found in other tissues such as the brain, kidney, and spleen. In peripheral tissues, it is mainly produced by liver and macrophages and mediates cholesterol metabolism and in the central nervous system, it is mainly produced by hepatic stellate cells and transports the cholesterol to the nerve cells via a member of the ApoE receptor of low density lipoprotein receptor gene family. The importance of ApoE was recognized for the first time in lipoprotein metabolism and cardiovascular disease. Recently, it has been studied for its role in various biological processes associated with Alzheimer's disease and immune regulation. ApoE gene is located in a cluster made apolipoprotein C1 and C2 on Chromosome 19, with four exons and three introns composed of a total of 3,597 base pairs. ApoE is activated by the X receptor and nuclear receptors to form heterodimers with peroxisome proliferator-activated receptor ã and retinoid X receptor[1]. ApoE gene has three type of single allele, E2, E3, E4 and six genotypes, *E2/E2*, *E2/E3*, *E2/E4*, *E3/E3*, *E3/E4*, *E4/E4*[2,3]. ApoE gene shows polymorphism and has three subtypes, Apo*E2* (cys 112, cys 158), Apo*E3* (cys112, arg158), and Apo*E4* (arg112, arg158)[4]. *E2* is found in approximately 7% of the population[5] and this type of apoprotein has a weak affinity for surface receptors compared to *E3* and *E4*, and associated with atherosclerosis. *E3* is found in about 79 % of the population and regarded as "neutral" ApoE genotype. *E4* is found in about 14% of the population and related with

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atherosclerosis[6], Alzheimer's disease[7], cognitive impairment[8], reduced hippocampal volume[9], the rapid disease progression in multiple sclerosis[10] and ischemic brain there vascular disease[11]. As genotypes, *E2/E2* homozygote is observed in more than 90% of patients with type III hyperlipoproteinemia and *E4/E4* homozygote has been known as a major risk factor for coronary heart disease and Alzheimer's disease. Recently, ApoE genotyping test has been applied to the field of diagnostic methods for predicting high risk of Alzheimer's disease, cardiovascular diseases, as well as new diseases such as immune regulation disorders. Therefore, a faster and more accurate ApoE genotyping test is required for the prevention of related disease and the general public health. Many laboratories use a PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) method[12] and PCR-SSP (PCR-Sequencing Specific Primer) method[13]. The former is a time-consuming and may have incomplete action or the read error of the restriction enzymes electrophoresis, the latter, there is a limit to the number of samples that can be tested at a time. There are the exact method such as capillary electrophoresis method[14] and PCR-Sequencing Method[15], however, they have costly disadvantage. Therefore, we aim to apply the real-time quantitative polymerase chain reaction for ApoE genotyping, while relatively reducing the time and cost of obtaining accurate test results.

Material and Methods

Study population and Design

We evaluated 220 serum specimens and the biospecimen and data used in this study were provided by the Biobank of Jeju National University Hospital, a member of Korea Biobank Network, which is supported by the Ministry of Health and Welfare.All samples obtained were from Korean individuals and were collected together with written informed consent according to protocol approved by the Institutional Review Board of the Jeju National University Hospital. We performed three kinds of assays: Real-Q ApoE genotyping (BioSewoom, Seoul, Korea) and Seeplex ApoE ACE genotyping (Seegene, Seoul, Korea). The results were finally confirmed using Sequencing (BigDye® Terminator v1.1 Cycle Sequencing Kit, Applied Biosystems) as the reference.

Nucleic acid extraction and ApoE genotyping

Genomic DNA was extracted from EDTA-anticoagulated whole blood using Quick Gene (Kurabo, Co, Japan) according to the manufacturer' s instructions. For the multiplex PCR based on the DPO (Dual Primer Oligo) technology, the Seeplex ApoE ACE genotyping Assay (Seegene, Seoul, Korea), 17μL of master mix were remixed with 3μL of genomic DNA. After a pre-heating step at 94℃ for 15 minutes, 35 reaction cycles (denaturation at 94℃ for 30 seconds, annealing at 65℃ for 30 seconds, extension at 72℃ for 60 seconds), and post-step at 72℃ for 10 minutes using MyCycler Thermal Cycler System (Bio-Rad Laboratories, CA, USA).

For the one-stop Real-Q ApoE genotyping assay, we mixed 21μL of master mix (including 17.5μL of reaction mixture, 3μL of a primer mixture for codon 112 and 158, and 0.5μL of sterile water) with 4μL of genomic DNA. After pre-heating step at 95℃ for 10 minutes, 40 reaction cycles (denaturation at 95℃ for 20 seconds, annealing at 60℃ for 30 seconds, and extension at 72℃ for 30 seconds) which were performed on the 7500 Real-Time PCR System (Applied Biosystems, CA, USA) and the results were analysed by the standard curve method.

For sequencing analysis, an aliquot of 2μL of the amplicon product from above reaction was used with BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA). The amplified fragments were analyzed using an ABI PRISM® 377 Analyzer (Applied Biosystems) and Chromas v 2.33 software and analyzed using the Basic Local Alignment Search Tool (BLAST) from The National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST).

Statistical analysis

The concordance rates between each ApoE genotyping assay and sequencing were obtained. For each assay, sensitivity and specificity were calculated with their 95% confidence intervals (CI). The proportions between the two ApoE genotyping assays were compared using chi-squared test. Statistical analysis was performed using Analyse-it Software (Analyse-it Software, Ltd, Leeds, UK) and MedCalc Software (version 12.1.4, MedCalc Software, Mariakerke, Belgium). P values of equal to or less than 0.05 were considered statistically significant. Statistical analysis was performed for the concordance rates between each assay and sequencing and the sensitivity and specificity were calculated with their 95% confidence intervals.

Results

Sequencing results of 220 DNA samples showed 1 case of *E2/E2*, 139 of *E3/E3*, 9 of *E4/E4*, 17 of *E2/E3*, 4 of *E2/E4*, 50 of *E3/E4* (Table 1, 2). The concordance rate between One-step Real-Q Ap E genotyping and sequencing was 100% (220/220), and that between Seeplex ApoE ACE

genotyping and sequencing was 99.5% (219/220) (Table 3, 4). Both sensitivity and specificity of One-step Real-Q ApoE genotyping for every genotypes was 100%. The sensitivities and specificities of Seeplex ApoE ACE genotyping were almost 100%, with two exception: 99.3% sensitivity in *E3/E3* and 99.5% specificity in *E2/E2*.

Table 1. Results of two Apo E genotypings

Apo E geneotype	One-step Apo E	seeplex Apo E
	Cases	Cases
E2/E2	1	2
E3/E3	139	138
E4/E4	9	9
E2/E3	17	17
E2/E4	4	4
E3/E4	50	5
Total	220	220

Table 2. Results of Sequencing

Apo E geneotype	Count	112 TGC	112CGC	158TGC	158CGC	Sequencing
E2/E2	1	+	-	+	-	E2/E2
E3/E3	139	+	-	-	+	E3/E3
E4/E4	9	-	+	-	+	E4/E4
E2/E3	17	+	-	+	+	E2/E3
E2/E4	4	+	+	+	+	E2/E4
E3/E4	50	+	+	-	+	E3/E4

Table 3. Results of Apo E genotypes by two PCR assays and sequencing

	N (%) of results by each method		
	One-step Apo E	Seeplex Apo E	Sequencing
E2/E2	1 (0.5)	2 (0.9)	1 (0.5)
E3/E3	139 (63.2)	138 (62.7)	139 (63.2)
E4/E4	9 (4.1)	9 (4.1)	9 (4.1)
E2/E3	17 (7.7)	17 (7.7)	17 (7.7)
E2/E4	4 (1.8)	4 (1.8)	4 (1.8)
E3/E4	50 (22.7)	50 (22.7)	50 (22.7)
Total	220 (100)	220 (100)	220 (100)

Table 3. Results of Apo E genotypes by two PCR assays and sequencing

	N (%) of Concordence among the method		
	One-step Apo E vs Seeplex Apo E	One-step Apo E vs Sequencing	Seeplex Apo E vs Sequencing
E2/E2	1/2 (50.0)	1/1 (100)	1/2 (50.0)
E3/E3	138/139 (99.3)	138/138 (100)	138/139 (99.3)
E4/E4	9/9 (100)	9/9 (100)	9/9 (100)
E2/E3	17/17 (100)	17/17 (100)	17/17 (100)
E2/E4	4/4 (100)	4/4 (100)	4/4 (100)
E3/E4	50/50 (100)	50/50 (100)	50/50 (100)
Total	220/222 (99.1)	220/222 (99.1)	220/222 (99.1)

* Concordance rate(%) = (number of corresponding results by both mothods/number of corresponding results by either methods) x 100
Apo E, Apolipoprotein E, PCR, Polymerase Chain Reaction, *N*, Number

Discussion

Multiplex AS-PCR ApoE Genotyping assay is known to be also rapid, reliable, simple method, but, it has a disadvantage leading to incorrect genotyping due to the hinded amplification in the case of unexpected nucleotide polymorphisms or mutations located in the DNA template or adjacent to the 3′ end of the primer-binding site[17]. By Korean Institute of Genetic Testing Evaluation in 2013[18], 41 institutions are performing ApoE genotyping and Seeplex ApoE ACE Genotyping (Seegene, Seoul, Korea) was most commonly used (in 11 Korean institutions, 26.8%), followed by the Bio-Core ApoE genotyping PCR KIT (BioCore, Seoul, Korea), which was used by 7 institutions (17.1%). But, genotyping errors can still occur due to polymorphisms near codons 112 and 158 in the human ApoE gene, though ApoE genotyping may appear to be uncomplicated and a relatively easy test to perform[17].

The performances of One-step Real-Q ApoE genotyping and Seeplex ApoE ACE genotyping were comparable overall, However, the formershowed better sensitivity and specificity comparable with those of sequencing. The Real-Q ApoE genotyping kit yielded correct genotyping results for all ApoE genotypes and provided concordant results with the sequencing results. Hence, One-step Real-Q ApoE genotyping could be a faster and more accurate option for ApoE genotyping.

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