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Apolipoprotein E Polymorphism in the Korean Population

Yong-Bin Eom[1], Yoon-Kyung Jo[2], Duk-Chul Lee[3] and Jee-Aee Im[4†]

[1]Department of Forensic Medicine, National Institute of Scientific Investigation, Seoul 158-707, Korea.
[2]Department of Clinical Laboratory Science, Dongnam Health Collge, Suwon 440-714, Korea.
[3]Departments of Family Medicine, College of Medicine, Yonsei University, Yongdong Severance Hospital, Seoul 135-720, Korea. [4]Department of Laboratory Medicine, MizMedi Hospital, Seoul 158-270, Korea

Apolipoprotein E (apoE) restriction isotyping used oligonucleotides to amplify apoE gene sequences containing amino acid positions 112 and 158. The amplification products were digested with *Hha*I and subjected to electrophoresis on 4% agarose gel. Each of the isoforms was distinguished by a unique combination of *Hha*I fragment sizes that enabled unambiguous typing of all homozygotic and heterozygotic combinations. *Hha*I cleaves at GCGC encoding 112arg (E4) and 158arg (E3, E4), but does not cut at GTGC encoding 112cys (E2, E3) and 158cys (E2). DNA was isolated from 72 study participants and apoE genotypes were determined utilizing the polymerase chain reaction and restriction isotyping. In the entire group of subjects, 38 (52.8%) had apo E4/4 or E3/4 (Group E4), 28 (38.9%) had the apo E3/3 genotype (Group E3) and 6 (8.3%) had apo E2/2 or E2/3 (Group E2). This genotypic information may help to identify individuals at increased risk for several diseases.

Key Words: Apolipoprotein E genotype, PCR amplification, Restriction isotyping

INTRODUCTION

The human apolipoprotein E (apoE) gene spans 3.7 kb including four exons (Das et al., 1985; Paik et al., 1989) and is located on chromosome 19 (Das et al., 1985) in a gene family that also contains the genes for apoC-I, C-I' (a pseudogene), and C-II (Myklebost et al., 1988).

ApoE plays a central role in cholesterol transport as reflected by the association of apoE with a variety of lipoprotein size classes, its synthesis in a variety of different tissues, and the ability of apoE to interact with two distinct hepatic receptors (LDL receptor and apoE receptor) (Mahley et al., 1988). ApoE is a constituent of lipoproteins with considerable variation due to cysteine-arginine exchanges. Genetic variation at the apoE locus in human populations is an important determinant of plasma lipid levels and relative risk of atherosclerosis (Davignon et al., 1988). Three common alleles of apoE encoding isoforms E2, E3, and E4 (Utermann et al., 1980; Zannis et al., 1981) and several rare variants have been identified (Mahley, 1988). E3 is the most common of these isoforms, and is distinguished by cysteine at position 112 (112cys) and arginine at position 158 (158-arg) in the receptor-binding region of apoE (Weisgraber et al., 1981). The E4 isoform (112arg and 158arg) is associated with increased levels of total cholesterol and betalipoprotein (Boerwinkle et al., 1989), and increased susceptibility to heart disease (Davignon et al., 1988; Utermann et al., 1984). The apoE4 polymorphism has been associated with dementia and hypercholesterolemia. Most patients with type III hyperlipidemia are homozygous for the E2 isoform (112 cys and 158 cys) that binds with reduced affinity to cellular receptors (Breslow et al., 1982; Weisgraber et al., 1982). In population studies, the E2 isoform is associated with decreased levels of cholesterol and betalipoprotein (Boerwinkle et al., 1989).

These reports of significant effects of apoE genotype on heart disease and known risk factors of heart disease have led to increased interest in rapid typing of apoE isoforms for population studies. Most methods for detection of allelic

*Received: November 21, 2005, 2005
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†Corresponding author: Jee-Aee Im, Department of Laboratory Medicine, MizMedi Hospital, 701-4 Naebalsan-dong, Gangseo-Gu, Seoul 158-270, Korea.
Tel: 82-2-2007-1359, Fax: 82-2-2007-1358
e-mail: jeaeim@hanmail.net

variation have relied on protein electrophoresis or nucleic acid hybridization with particular oligonucleotide or gene probes (Landegren et al., 1988). In this report, we describe restriction isotyping (restriction enzyme isoform genotyping) as a simpler and faster method for typing the common apoE isoforms, restriction isotyping uses polymerase chain reaction (PCR) amplification, but avoids the use of costly and time-consuming hybridization and sequencing techniques.

MATERIALS AND METHODS

1. Study population

Study subjects were recruited by advertisement from the health promotion center. All subjects signed an informed consent approved by the Ethical Committee of the hospital. 22 male and 50 female aged 47~72 years were included in this study. Exclusion criteria were high blood pressure (mean systolic blood pressure ≥150 mmHg or mean diastolic blood pressure ≥95 mmHg after two consecutive measurements), high fasting blood glucose (≥126 mg/dL), abnormal liver function test (AST>35 IU/L, or ALT>35 IU/L), and past medical history of illness (hypertension, diabetes mellitus, liver disease). Normal blood samples were hospital personnel who had not been on any medication including aspirin or nonsteroidal anti-inflammatory agents 21 days prior to venusection.

We analyzed the apoE genotype of 72 subjects who did not meet these exclusion criteria and divided them into three groups based on our results: apoE2 presenting group (E2 group), apoE2/2 and 2/3; wild group (E3 group), apoE3/3; apoE4 presenting group (E4 group), apoE4/3 and 4/4.

2. Anthropometric evaluation

Body weight was measured to the nearest 0.1 kg on an electronic scale. Subjects were weighed in light clothing and without shoes. Height was measured to the nearest 0.1 cm using a wall mounted stadiometer. Body mass index (BMI) was defined as weight/height^2 in kg/m^2.

3. Biochemical assays

Biochemical tests were estimated in blood venous samples withdrawn in the morning after overnight fasting. Serum levels of fasting glucose, total cholesterol, HDL-cholesterol, and triglycerides were assayed using an auto-analyzer ADVIA 1650 (Bayer, Tarrytown, NY, USA). LDL-cholesterol was calculated by Friedewald's formula (Friedewald et al., 1972). High-sensitivity C-reactive protein (hs-CRP) was measured by a latex-enhanced immuno-turbidimetric assay using an ADVIA 1650 Chemistry system (Bayer, Tarrytown, NY, USA), and the interassay and intra-assay reproducibilities were 2.70±1.13% and 2.55±1.0%, respectively.

4. Amplification of apoE gene from genomic DNA

Leukocyte DNA was extracted from 1 ml of whole blood with the QIAamp Tissue Kit 250 (Qiagen Inc., Valencia, CA, USA), according to the manufacture's instructions. Purified genomic DNA was diluted to a concentration of 10 ng/ul in TE buffer (10 mM Tris pH 8.0, 1 mM EDTA) and stored at -20℃. Oligonucleotide primer pairs were (5'-ACA GAA TTC GCC CCG GCC TGG TAC AC-3') and (5'-TAA GCT TGG CAC GGC TGT CCA AGG A-3'), described by Emi et al. (1988). On the basis of this sequence, a PCR product of 244 bp was expected. PCR reaction mixtures contained apoE specific primers at a concentration of 1 pmol/μl; dATP, dCTP, dGTP, and dTTP each at a concentration of 200 μM, 1.5 mM $MgCl_2$, 1.25 U of thermostable *Taq* polymerase (Takara, Japan), 1X PCR buffer (the buffer was supplied at 10X), 10% dimethyl sulfoxide and 2 μl (i.e., 20 ng) of template DNA. The final volume was filled to 20 μl with sterile distilled water. The amplification reaction was performed in a thermal cycler (GeneAmp® PCR System 2700, Perkin-Elmer Cetus, Boston, MA, USA). Cycling conditions started with a denaturation step at 95℃ for 5 min, which was followed by 30 subsequent cycles consisting of heat denaturation at 95℃ for 1 min, primer annealing at 60℃ for 1 min, and extension at 72℃ for 2 min. A final extension was performed at 72℃ for 7 min to complete the synthesis of all strands. The PCR products were visualized by ultraviolet illumination of ethidium bromide-stained 2% agarose gels (NuSieve GTG agarose; BMA, Rockland, ME, USA).

5. Restriction isotyping of amplified apoE sequences with *Hha*I and gel analysis

After PCR amplification, 5 units of *Hha*I (Takara shuzo, Kyoto, Japan) were added directly to each reaction mixture for digestion of apoE sequences (3 hr at 37℃). This process did not require purification of PCR products or addition of specific buffer components for *Hha*I digestion. Incomplete digestion was not a significant problem, most likely due to

the large amounts of *Hha*I enzyme relative to the small amount of amplified sequences in each reaction. Each reaction mixture was loaded onto an ethidium bromide-stained 4% agarose gel and electrophoresed for 1 hr under constant current. After electrophoresis, the DNA fragments were visualized by UV illumination.

6. Statistical analysis

Data are expressed as means ± SD. Clinical characteristics were compared between the apoE genotypes using ANOVA and t-test. Lipid levels were compared between the apoE genotypes using ANOVA. Significance was defined at the 0.05 level of confidence. All data were analyzed using the statistical program SAS 8.01 (SAS Institute, Cary, NC, USA).

RESULTS

1. Subjects characteristics

For the purpose of statistical analysis, subjects were categorized into three apoE genotype subgroups: (a) subjects carrying the ε2/ε2 or the ε2/ε3 genotypes (apoE2 group; 8.3%), (b) ε3/ε3 subjects (apoE3 group; 38.9%) and (c) subjects with genotypes ε3/ε4 or ε4/ε4 (apoE4 group; 52.8%). Due to their low frequency and the opposite effects on lipid levels of the ε2 and the ε4 alleles, subjects with the ε2/ε4 genotype were excluded from these groups and were not considered in the our analyses.

The clinical characteristics of male and female are shown in Table 1.

Table 1. Clinical characteristics of the subjects

Characteristics	Male (N=22)	Female (N=50)	*P*-value
Age (years)	57.2±6.4	55.6±5.7	0.309
BMI[a] (Kg/m^2)	25.0±2.9	24.0±3.0	0.260
Systolic BP[b] (mmHg)	128.5±12.2	128.98±18.5	0.897
Diastolic BP[c] (mmHg)	80.2±7.5	76.4±11.4	0.161
Fasting glucose (mg/dl)	94.8±11.3	89.6±9.5	0.047
Cholesterol (mg/dl)	184.8±30.9	207.8±40	0.019
Triglycerides (mg/dl)	136.7±75	120.6±70	0.385
HDL-cholesterol[d] (mg/dl)	47.5±12.4	55.1±13.4	0.026
LDL-cholesterol[e] (mg/dl)	110.0±32.4	130.1±38.2	0.034
HS-CRP[f] (mg/dL)	0.65±1.7	0.34±1.1	0.348

Data are shown as means ± the standard deviation.
P-values are calculated by t-test.
[a] Body mass index.
[b] Systolic blood pressure.
[c] Diastolic blood pressure.
[d] High density of lipoprotein cholesterol.
[e] Low density of lipoprotein cholesterol.
[f] High-sensitivity C-reactive protein

2. ApoE restriction isotyping by PCR amplification and cleavage with *Hha*I

ApoE restriction isotyping relies on cleavage at polymorphic *Hha*I sites to distinguish E2, E3, and E4 sequences. There were six *Hha*I cleavage sites (GCGC) in the amplified E4 sequence (Paik et al., 1989), including *Hha*I sites at codons for arginine residues (GCGC) at positions 112 and 158. The E3 sequence encodes a cysteine residue at position 112 (GTGC) which abolishes the *Hha*I cleavage site in the E4 sequence, resulting in a total of five *Hha*I cleavage sites. The E2 sequence encodes cysteines at positions 112 (GTGC) and 158 (GTGC) that abolish two cleavage sites relative to the E4 sequence, resulting in a total of four *Hha*I cleavage sites. Fig. 1 shows gel-separated products of apoE amplification and *Hha*I digestion using genomic DNA from human subjects representing each homozygotic and heterozygotic combination of common apoE alleles. With the exception of a shared 38 bp fragment (common *Hha*I site at position 38, the other shared fragments were not detected

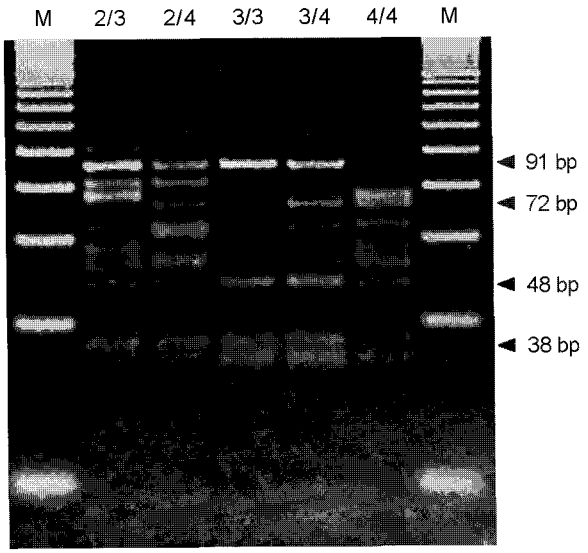

Fig. 1. Electrophoretic separation of *Hha*I fragments after gene amplification of DNA from subjects with known apoE isoforms. 4% agarose gel is shown after electrophoresis of *Hha*I fragments from an E2E3 heterozygote (lane markerd 2/3), E2E4 heterozygote (2/4), E3/E3 homozygote (3/3), E3/E4 heterozygote (3/4), E4E4 homozygote (4/4). The fragment sizes (in bp) of a DNA standard (lane marked M) are shown to the both side of the gel.

due to their smaller sizes), each genotype possessed unique combinations of *Hha*I fragment sizes. The E2/E2 sample contained 91 and 83 bp *Hha*I fragments reflecting the absence of sites at 112 cys and 158 cys. The E3/E3 sample also contained the 91 bp fragment (112 cys), as well as 48 and 35 bp fragments from cleavage at the *Hha*I site at 158 arg. The E4/E4 sample also contained these 48 and 35 bp fragments (158 arg), as well as a unique 72 bp fragment from cleavage at 112 arg (the 19 bp fragment was too small for detection). Each of the samples from heterozygotic combinations contained both sets of fragments from each apoE allele.

For the entire population the percentage of subjects with the various apoE genotypes were: E3/E4, 47.2%; E3/E3, 38.9%; E4/E4, 5.6%; E2/E3, 5.6% and E2/E2, 2.8% (Table 2).

Table 2. Frequency of apoE genotype among study subjects

ApoE genotypes[a]	Number of subjects (%)
E2 group	6 (8.3)
E 2/2	2 (2.8)
E 2/3	4 (5.6)
E3 group	28 (38.9)
E 3/3	28 (38.9)
E4 group	38 (52.8)
E 3/4	34 (47.2)
E 4/4	4 (5.6)

[a] To evaluate the effect of apoE genotype, 72 subjects were categorized into three groups: apoE2/2 and 2/3 (E2 group), apoE3/3 (E3 group), apoE4/3 and 4/4 (E4 group).

3. Relationship between apoE genotype and other parameter

No statistical differences were shown to exist in mean age, blood pressure, serum levels of fasting glucose, cholesterol, triglycerides, HDL-cholesterol, LDL-cholesterol, and hs-CRP levels among the apoE genotypes. In contrast, BMI shows significant different among the apoE genotypes ($P<0.05$) (Table 3).

DISCUSSION

Methods were developed for apoE typing at the DNA level using genomic DNA for Southern blots with allele-specific oligonucleoitde (ASO) probes that span positions 112 and 158 (Funke et al., 1986; Smeets et al., 1988). The sensitivity of this method was increased by using the PCY (Saiki et al., 1985) to amplify apoE sequences for dot-blots with ASO probes (Emi et al., 1988). In addition, automated sequence analysis of PCR products has been used for typing common and rare apoE isoforms (Emi et al., 1988).

Restriction isotyping may provide a simpler alternative to these methods by enabling detection of substitutions using restriction enzymes that cut more frequently (4 bp recognition sites) and survey a large proportion of a given sequence for cleavage sites. Restriction enzymes produce a large number of small DNA fragments that have not been suitable for Southern blot analyses of complex genomes. In addition, in vitro amplified DNA is not methylated, allowing the use of

Table 3. Relationship between apoE genotype and other parameter

Characteristics	E2 (N=6)	E3 (N=28)	E4 (N=38)	*P*-value
Age (years)	58.7±5.9	55.0±5.3	56.6±6.3	0.304
BMI[a] (Kg/m^2)	27.1±3.3	24.1±2.6	23.9±2.9	0.037
Systolic BP[b] (mmHg)	135.3±6.7	129.2±20.4	127.6±14.8	0.572
Diastolic BP[c] (mmHg)	79.8±1.8	79.0±12.0	76.2±10.0	0.491
Fasting glucose (mg/dl)	90.5±6.0	89.6±12.2	92.5±9.3	0.531
Cholesterol (mg/dl)	185.7±25.5	202.3±30	202.0±45.6	0.612
Triglycerides (mg/dl)	138.2±87.7	131.2±70.3	119.3±71.3	0.729
HDL-cholesterol[d] (mg/dl)	51.0±13.0	49.9±10.1	55.1±15.5	0.298
LDL-cholesterol[e] (mg/dl)	107.0±31.6	126.1±28.8	125.1±43.7	0.514
HS-CRP[f] (mg/dL)	0.24±0.2	0.64±1.6	0.31±1.2	0.586

Data are shown as means ± the standard deviation. *P*-values are calculated by ANOVA.
[a] Body mass index. [b] Systolic blood pressure. [c] Diastolic blood pressure.
[d] High density of lipoprotein cholesterol. [e] Low density of lipoprotein cholesterol. [f] High-sensitivity C-reactive protein.

a wide variety of restriction enzymes. For example, methylation may have prevented *Hha*I cleavage of genomic DNA for Southern blots in a previously study of apoE (Wallis et al., 1983). For these reasons, restriction isotyping may be useful as a general approach where substitutions alter cleavage sites that distinguish particular isoform genotypes, or for detection of unknown variants in surveys with many different enzymes.

After amplification of apoE sequences that encompass amino acid positions 112 and 158, we simply digest the PCR products with *Hha*I and separate the resulting digestion fragments by electrophoresis on 4% agarose gel. Because the nucleotide substitutions that result in arg-cys interchanges at positions 112 and 158 also alter *Hha*I cleavage sites (Wallis et al., 1983), each genotype can be distinguished by unique combinations of *Hha*I fragment sizes in all homozygotic and heterozygotic combinations.

In this study, significant differences in lipid profile between apoE genotypes have not been observed. McCarron MO et al. (1999) indicated that the effect of this allele on CVD risk is independent of effects on plasma levels at least in men. There was no significant difference in mean serum adjusted HDL-cholesterol levels between the three apoE genotypes (Sheehan D et al., 2000). Uteman et al. (1984) reported that E4 allele is frequently observed in patients with high plasma cholesterol levels.

The variation at the apoE gene locus has been shown to affect levels of total cholesterol and LDL cholesterol in the general population. Many studies assessing the role of apoE genetics on plasma lipids have shown that the presence of the ε4 allele is associated with elevations in LDL cholesterol, while the presence of ε2 is associated with decreased levels of LDL cholesterol (Davignon et al., 1988). It has been suggested that the presence of the ε2 allele results in decreased LDL cholesterol because of delayed clearance of chylomicron remnants by the liver and upregulation of LDL receptor activity, while the ε4 allele results in elevated levels of LDL cholesterol because of enhanced uptake of chylomicron remnants and down-regulation of the LDL receptor. A meta analysis by Dallongeville et al. indicated that subjects with the ε2 and ε4 alleles had higher triglyceride levels than subjects with the ε3 allele (Dallongeville et al., 1992). These data provide evidence suggesting that the apoE gene is a protypical susceptibility gene.

The authors are aware of some of the limitations of our study. Despite the small size of the study population, we believe this study provides useful data on apoE genotypes in the Korean population. However further study is needed in patients with several diseases.

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