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The apolipoprotein E genotyping using the PCR-RFLP was useful to linkage analysis of Alzheimer's disease families

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Abbreviations: AD, Alzheimer's disease; apo E, apolipoprotein E; RFLP, restriction fragment length polymorphism

Abstract

Apolipoprotein E (apo E) has three common alleles (apo ε2, ε3 and ε4) that code for three major isoforms E2, E3 and E4. The isoforms differ from each other by a single amino acid substitutions at two positions and also differ in their binding affinity for the apo E receptors. Moreover, recently a strong association between the apo ε4 allele and late-onset Alzheimer disease (AD) was demonstrated. In this study, were analyzed the apo E genotypes using the Hhal digestion of PCR amplified samples, and the apo ε4 allele frequency from 70 AD patients and 106 normal population in Korea. The results suggested that the frequency of ε4 allele among the AD patients (35.7%) was 3 times higher than that among the control population (13.7%). The data, which are in agreement with recent reports, suggests that the apo ε4 allele is associated with AD in Korea.

Keywords : Alzheimer's disease (AD), apolipoprotein E genotype, polymerase chain reaction-restriction fragment length polymorphism

Introduction

Alzheimer's disease (AD) appears to abnormal aggregation of insoluble β-amyloid peptide in senile plaques and cerebral vessels (Hilbich *et al*., 1991), and to degenerative disorder of central nervous system which causes serious mental deterioration, dementia and death (Flier and Underhill., 1991; Marder *et al.,* 1994). Although the pathogenesis of amyloid deposition in AD is not completely elucidated, recent studies suggest that the apolipoprotein (apo) E4 isoform plays a key role in amyloid formation or in the pathogenesis of AD (Small *et al*., 1995). Apo E is expressed in the liver and many peripheral tissues, where it appears to participate in redistribution of lipids. In the nervous system, apo E participates in the growth and repair of injured neurons, and redistribution of cholesterol (Handlemann *et al.,* 1992). Apo E has genetic polymorphism by three alleles, apo ε2, ε3 and ε4, which are coding for three isoforms E2, E3 and E4 respectively (Figure 1). This polymorphism leads to the presence of six different types in the human population; three homozygotes (ε2/2, ε3/3 and ε4/4) and three heterozygotes (ε2/3, ε2/4 and ε3/4) (Hansen *et al.,* 1994; Richard *et al.,* 1994).

Recently, many studies demonstrated an association between the apo E4 allele and late-onset AD. Thus apo E genotyping could be an important tool for identifying individual with increased risk for developing AD (Strittmatter *et al.,* 1993; Appel *et al*., 1995; Corder *et al.,* 1995a,b; Jarvik *et al.,* 1995). In this study, we have developed the method of apo E genotyping using PCR-restriction fragment length polymorphism (PCR-RFLP) and analyzed the genotype frequencies of 70 AD patients and 106 normal control subjects in Korea.

Materials and Methods

Subjects

The blood samples of 70 AD patients for this study were selected from Kang Nam Hospital and Seoul National Mental Hospital. Diagnosis of dementia was based on diagnostic and stastical manual of mental disorders (American Psychiatric Association 1994, 4th edition), and established by neuropsychologist. Selected mean age of AD patients was 79 years, and mean age of estimated onset AD was after 65 years old. The 106 control samples were selected from unrelated sex and age in the normal population from Seoul Clinical Laboratories.

PCR amplification

Genomic DNA was isolated using the method of Walsh *et al*. (1991), and the extracted DNA was used as the template for PCR. The PCR mixture consisted 150 ng of genomic DNA, 2 μl of 10× reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.25 mM $MgCl_2$, 0.1% Tween), 5 pmol of each primer 1 (5'-ATAAATATAAAATATAAATAACAGAATTC GCCCCGGCCTGGTACAC-3') and primer 2 (5'-TAAGCTTG

GCACGGCTGTCCAAAGA-3'), 200 μM of each dNTPs and 0.5 unit *Taq* DNA polymerase (Advanced Biotechnologies Co., Leatherland, KT, UK) in a final volume of 20 μl. PCR was performed for 30 cycles of denaturation at 94˚C for 1 min, annealing at 60˚C for 1.5 min and extension at 72˚C for 1 min in 9600 thermal cycler (Perkin-Elmer Co., Norwalk, CT, USA). A final extension step of 72˚C for 5 min was also included. The PCR products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Restriction analysis of amplified DNA

For the restricted patterns from each apo ε alleles, nucleotide sequence analysis was performed using the PC/GENE computer program (Intelligenetics Inc., Mountain View, CA, USA). Five microliter of the PCR product was digested with 10 unit *Hha*I (New England Biolab. Co., Beverly, MA, USA) for 4 h. The digested fragments were separated by electrophoresis on a 12% polyacrylamide gel at 100 V for 3 h. After electrophoresis the gel was stained with ethidium bromide, and the polymorphic patterns were analyzed.

DNA sequencing

The apo E types analyzed by PCR-RFLP were confirmed using the direct sequencing. The PCR products were purified by DNA purification system (Wizard PCR preps, Promega, USA), and sequenced by ABI 310 genetic analyzer (PE Applied Biosystems., Norwalk, CT, USA) following the manufacture's instruction. The sequences were compared with the previously published report (Appel *et al*., 1995).

Results

PCR amplification and restriction analysis of apo E gene

Apo E gene had three alleles which contain two polymorphic *Hha*I sites on codon 112 and codon 158 (Figure 1). Figure 2 showed the restricted patterns of three homozygous and three heterozygous types. The apo ε2 allele was characterized by the presence of the 104 and 91 bp bands, apo ε3 by the 91, 53 and 48 bp bands, and apo ε4 by the 72, 53 and 48 bp bands. Each of the heterozygous types was showed by mixed bands of homozygotes. As shown in Figure 3 the partial nucleotide sequences of the apo ε3/3 and apo ε3/4, were most frequently found alleles in normal and AD patients. They were confimed by direct sequencing, and the heterozygote generated two peaks at the same base position.

The apo E genotype frequencies for AD patients and control group

The apo E genotype frequencies for AD patients and control subjects were compared. As shown in Table 1, allelic frequences of AD patients were 44.3% for ε3/4, 25.7% for ε3/3, 12.9% for ε2/3, 10.0% for ε4/4, 7.0% for ε2/4 and 0% for ε2/2. The apo E allele frequencies were 0.100 for ε2, 0.543 for ε3 and 0.357 for ε4. The apo E genotype frequencies for control subjects were 59.4% for ε3/3, 19.8 % for ε3/4, 13.2% for ε2/3, 3.8% for ε2/4 and 1.9% for ε2/2 and ε4/4, and allele frequencies were 0.763 for ε3, 0.137 for ε4 and 0.100 for ε2. A comparison of the frequencies for apoE genotypes among the AD and control subjects demonstrated a significant difference between the two groups (P= 0.0001). Apo ε3/4 was the most common genotype in the AD patients (44.3%),

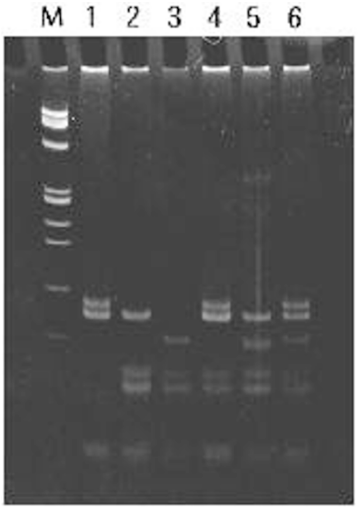

Figure 2. Six RFLP types of apo E gene digested by *Hha*I. After amplification of apoE genomic DNA by PCR, the amplified products were completely digested by *Hha*I and analyzed on the 12% polyacrylamide gels. M, *PhiX174/HaeIII* molecular weight marker; lane 1, apo ε2/2; lane 2, apo ε3/3; lane 3, apo ε4/4; lane 4, apo ε2/3; lane 5, apo ε3/4 and lane 6, apo ε2/4.

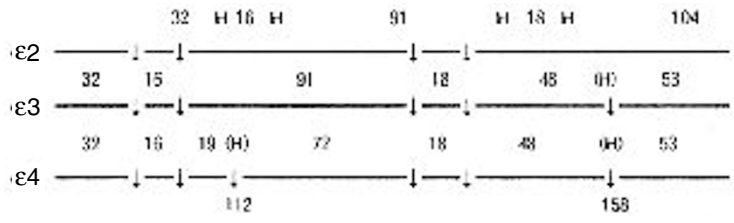

Figure 1. Restriction map of three principal alleles, ε2, ε3 and ε4 of apo E gene. The map was drawn based on the published sequence. The polymorphic regions are codon 112 and 158. H and (H) mean cutting sites and variant sites of *Hha*I, respectively. Numbers between cutting sites indicate fragment size in base pairs.

Table 1. The frequencies of apoE alleles and genotypes in AD patients and control subjects

	apo ε allele frequencies %			apo ε genotypes % (n)					
	ε2	ε3	ε4	ε2/2	ε2/3	ε2/4	ε3/3	ε3/4	ε4/4
AD patients (n = 70)	10.0	54.3	35.7	0.0 (0)	12.9 (9)	7.0 (5)	25.7 (18)	44.3 (31)	10.0 (7)
Control subjects (n = 106)	10.0	76.3	13.7	1.9 (2)	13.2 (14)	3.8 (4)	59.4 (63)	19.8 (21)	1.9 (2)

whereas apo ε3/3 was in the control subjects (59.4%). The ε4 allele frequencies were also significantly different between AD patients (0.357) and control subjects (0.137) ($P < 0.0001$) (Table 1).

Discussions

The association of the apo ε4 allele has recently been reported for both late-onset familial AD (Saunder *et al.,* 1993; Kosumen *et al*., 1995) and sporadic AD (Strittmatter *et al*., 1993; Corder *et al*., 1994; Tsai *et al*., 1994).

Initially, apo E polymorphism was determined through phenotyping by means of isoelectric focusing technique which allowed the detection of charge variations consequent upon the minor sequence difference between the principal isoform (Utermann *et al*., 1977). But the isoelectric focusing technique requires a large volume of sample, time-consuming and expensive. To avoid these problems, apo E genotyping based on PCR has been developed and several different approaches have been proposed; use of allele-specific oligonucleotide (ASO) (Weisgraber *et al*., 1988), amplification refractory mutation system (ARMS) (Wehhon *et al.,* 1991), single strand confomation polymorphism (SSCP)-sequencing (Aozaki *et al.,* 1994) and RFLP (James *et al*., 1990). The SSCP-sequencing technique is most accurate and can detect rare mutations, but tends to time-consuming and inappropriate for routine test. In contrast RFLP is a simple, accurate and rapid method, and useful to analyze the allelic variation of apo E (James *et a*l., 1990).

In the present study, we investigated the apo ε allele frequency of AD patients and normal subjects by PCR-RFLP. Approximately 35.7% of the AD patients had at least one apo ε4, and apo ε4/4 allele may account for 10.0% of the AD case. Our result was similiar to previously published reports, which showed a significantly higher apo ε4 allele frequency in the AD group compared to control group (Michael *et al.,* 1994; Tsai *et al.,*1994; Kosunen *et al.,*1995). The apo ε allele frequency was also dependent on the ethnic and genetic background of the population being examined, therefore control group determination is also very crucial. The apo ε allele frequencies of control subject in Korean population were also similiar to previously published Japanese data (Tsukamoto *et al*., 1993).

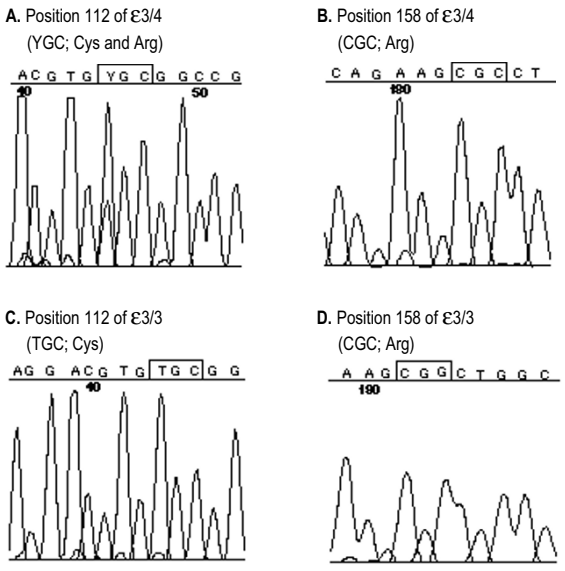

Figure 3. DNA sequence analysis at position 112 and 158 of ε3/4 (A and B) and apo ε3/3 (C and D). Apo ε3/4 showed both of arginine (CGC) and cysteine (TGC) at position 112 by two peaks, and same arginine (CGC) at position 158. Apo ε3/3 showed cysteine (TGC) at position 112 and arginine (CGC) at position 158. Amplified products were purified and sequenced using ABI 310 genetic analyzer. All DNA sequences were confirmed by reading antisense strands.

Apo ε4 might be an important factor for the detection of development of AD. Moreover apo ε4 gene could be considered as susceptibility gene of AD. All persons who had apo ε4 gene were not patients and some people may get the disease without having the apo ε4, therefore it is not absolute factor to diagnose the AD (Boyles *et al*., 1995; Corder *et al*., 1995).

In conclusion, we suggested that the apo ε genotyping using the PCR-RFLP was useful for linkage analysis of AD families. Further studies are necessary for broad range of AD group, accurate differentiation according to sex and age, precise onset age and family history.

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