RAPID DETECTION OF APOLIPOPROTEIN E GENOTYPES IN ALZHEIMER'S DISEASE USING POLYMERASE CHAIN REACTION-SINGLE STRAND CONFORMATION POLYMORPHISM

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Abstract. Apolipoprotein E (*APOE*) gene on chromosome 19q13.2 is encoded by three common alleles designated as ε2, ε3 and ε4. In Alzheimer's disease (AD) the ε4 allele is over-represented and is considered to be a major genetic risk factor. Several methods have been developed to determine *APOE* genotypes. Among them, polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) appears to be highly reliable. In this study, we improved the nonisotopic PCR-SSCP method for determining *APOE* genotypes in 42 cases of AD patients, 40 cases of non-AD dementia patients, and 49 cases of age-matched controls. DNA from the target sequence on *APOE* was amplified by PCR from peripheral blood genomic DNA. PCR products were electrophoresed in a non-denaturing polyacrylamide gel and visualized by silver staining. We found that the ε4 allele had a significantly high frequency of occurrence in AD patients (33.3%) compared with age-matched controls (13.3%) (χ^2 = 10.43, p = 0.001) and non-AD dementia (10%) (χ^2 = 13.02, p<0.001) whereas the ε3 allele was of high frequency in non-AD dementia (90%) compared with age-matched controls (85.7%) and AD patients (66.7%). *APOE* ε4 homozygotes were found only in AD groups. On the other hand, the ε2 allele was found only in an age-matched control. This study confirmed that the *APOE* ε4 allele is a risk factor in Thai AD subjects and that the PCR-SSCP method is a rapid and useful means of detecting the *APOE* genotype in AD.

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia and is a genetically heterogeneous neurodegenerative disorder that occurs in middle or late life characterized by global cognitive decline and distinct neuropathological hallmarks in the brain. Neuropathologically, the brains of AD patients contain abundant amounts of neurofibrillary tangles and β-amyloid in the form of senile plaques and blood vessel deposit (Hardy, 1997). It is now well-established that some cases of AD, mainly early-onset, show autosomal dominant inheritance patterns due to the presence of mutated genes, including those encoding amyloid precursor protein, presenilin 1 and presenilin 2. However, the late-onset disease, which is more challenging for genetic analysis, has been associated with the presence of apolipoprotein E type 4 (*APOE* ε4) (Price *et al*, 1998). The *APOE* gene encodes a 299 amino acid secreted protein

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(Blacker, 1998). There are three major isoforms of ApoE (E2, E3 and E4) that are three products of three allelic forms (ε2, ε3 and ε4) of this single gene locus. Three homozygous phenotypes (ApoE2/E2, E3/E3 and E4/E4) and three heterozygous phenotypes (ApoE3/2, E4/3 and E4/2) arise from the expression of any two of three alleles. The three isoforms differ by interchange of cysteine (Cys) and arginine (Arg) residues at positions 112 and 158 of the mature ApoE. ApoE2 has Cys residues in both of these positions, ApoE3 has Cys-112 and Arg-158, and ApoE4 has Arg in both positions. The presence of *APOE* ε4 allele has been identified as a major risk factor for both sporadic and late-onset AD (Corder *et al*, 1993; Saunders *et al*, 1993; Parker *et al*, 2005) accounting for about 50% of the genetic risk associated with the late-onset AD (Farrer *et al*, 1997). It is found in both men and women, but appears to be more predominant in women (Corder *et al*, 2004).

Several methods have been developed to determine *APOE* genotypes, *eg* polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Kontula *et al*, 1990; Hixson and Verneir, 1999; Senanarong *et al*, 2001) and polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) (Tsai *et al*, 1993; Wilton and Lim, 1995). The former is commonly used to detect the *APOE* genotype but it is rather time-consuming and there is an association with incomplete cleavage. The latter method does not require any restriction enzyme and can be used successfully to distinguish the three common *APOE* alleles that differ from each other by either one or two single-base substitutions. However, PCR-SSCP as previously reported is not very convenient, since it requires radiolabelled primers followed by autoradiography (Orita *et al*, 1989; Hayashi and Yandell, 1993). In this study we have developed a rapid and nonisotopic PCR-SSCP method for determining the distribution of *APOE* genotypes in Thai Alzheimer's patients.

MATERIALS AND METHODS

Subjects

Subjects were patients from the Department of Neurology, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok, Thailand. Written informed consent was obtained from all patients or their primary caregivers. All subjects were diagnosed according to the criteria of the Diagnostic and Statistical Manual of Mental Disorders, fourth edition, DSM-IV by American Psychiatric Association, 1994 (Boller and Traykov, 1999). One hundred and thirty-one of subjects were obtained in our study. They were classified into 3 groups: 42 Alzheimer's patients, 40 non-AD demented patients, and 49 age-matched controls. DNA was extracted from peripheral blood leukocytes using the method of Blin and Stafford (1976).

PCR-SSCP analysis

Oligonucleotide primers for amplification of *APOE* gene were synthesized by ABI Applied Biosystems (Frederick, MD). The sequences were 5′ GGA CAA CTG AGC CCG GTG GCG G 3′ (sense) and 5′ GGA TGG CGC TGA GGC CGC GCT C 3′ (antisense) (Tsai *et al*, 1993). This set encompasses the sequence from nucleotides 3649-3943 (codons 80-178) and should generate a 295-bp product. The PCR reaction mixture (25 μl) contained 50-100 ng of genomic DNA, 2.5 units of *Taq* polymerase (Pharmacia Biotech, USA), 1x thermophillic-magnesium free buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1% Triton X-100, 50 % glycerol), 2.0 mM $MgCl_2$, 0.2 mM each deoxyribonucleotide triphosphates and 1.0 μM of each primer. The reactions were performed with a Perkin- Elmer 9600 thermal cycler. Amplification cycles were as follows: 30 cycles of denaturation at 95ºC for 1 minute, annealing at 65ºC for 30 seconds, and extension at 70ºC for 75 seconds. This was followed by one cycle of denaturation at 95ºC for 1 minute, primer annealing

Table 1
APOE genotypes and allele frequencies in Alzheimer's disease (AD), non-AD dementia (non-AD) and age-matched control subjects.

Subject (n)	Median onset age (range)	*APOE* genotype						*APOE* allele frequency (%)		
		ε2/ε2	ε2/ε3	ε2/ε4	ε3/ε3	ε3/ε4	ε4/ε4	ε2	ε3	ε4
AD (42) M (18) : F (24)	73 (51-93)	0	0	0	19	18	5	0	66.7	33.3
Non-AD (40) M (12) : F (28)	72 (53-93)	0	0	0	32	8	0	0	90	10
Control (49) M (14): F (35)	70 (54-81)	0	1	0	35	13	0	1	85.7	13.3

M, male; F, female ; n, numbers of cases.

at 65ºC for 30 seconds and primer extension at 70ºC for 7 minutes.

SSCP was carried out with a Hoefer MiniVE vertical electrophoresis system. Two μl of PCR-amplified DNA product was mixed with 2 μl of formamide-loading dye solution. The mixture was denatured at 95ºC for 5 minutes and cooled rapidly at 4ºC before loading onto a 12% non-denaturing polyacrylamide gel (0.1x10x10 cm) in 1xTBE buffer (pH 8.0). Electrophoresis was carried out at 25ºC, 90 V for 4 hours. The gel was stained using the silver staining method (Bassam *et al*, 1991).

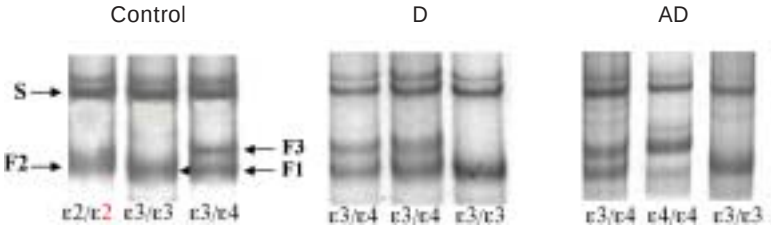

Fig 1–APOE genotype determinations using PCR-SSCP analysis from Alzheimer's patients (AD), non-AD demented patients (D) and age-matched controls (C). Arrows mark the bands representing the major conformers for the three alleles. S, major slow-moving bang; F1, F2, F3, major fast-moving bands for ε3, ε2 and ε4, respectively. Lane 1 (Control), homozygous ε2/ε2 ; lane 2, homozygous ε3/ε3; and lane 3, heterozygous ε3/ε4. Briefly, the amplification cycles were as follows: 30 cycles of denaturation, annealing and extension. The PCR-amplified DNA product was electrophoresed at 25ºC, 90 V for 4 hours. The gel was stained using the silver staining method.

Statistical analyses

Statistical analysis was performed using SPSS for Windows, version 9. Pearson chi-square (χ^2) test was used to analyze differences in the frequencies of *APOE* genotypes in AD patients, non-AD demented patients and age-matched controls.

RESULTS

Clinical characteristics including age and sex distribution of patients with AD, non-AD dementia and aged-matched controls are summarized in Table 1. The mean age of all groups does not differ significantly. Eighty percent of patients had onset of the disease after the age of 60. The PCR-SSCP gel system provided results within 8 hours. The *APOE* polymorphism patterns of PCR products were silver-stained on 12% non-denaturing polyacrylamide gel. The DNA fragments showed different migration patterns among the alleles coded by ε2, ε3 and ε4 (Fig 1) as previously described by Tsai *et al* (1993). The fragment

coded by ε3 allele moved fastest, followed by that of ε2 and the DNA of ε4 allele. We detected that the *APOE* ε3/ε3 was the most common genotype in all groups followed by ε3/ε4, ε4/ε4 and ε2/ε3, respectively (Table 1). The frequency of *APOE* ε4 allele was significantly higher in AD patients (33.3%) than in age-matched controls (13.3%) (χ^2 = 10.43; p = 0.001) and in non-AD dementia (10%) (χ^2 = 13.02; p < 0.001) which was mainly due to an increase of *APOE* ε4 homozygotes. The frequency of *APOE* ε3 was significantly lower in AD patients (66.7%) than in age-matched controls (85.7%) (χ^2 = 10.68; p = 0.002) and in non-AD dementia (90%) (χ^2 = 14.53; p < 0.001).

DISCUSSION

Our study showed significant differences in *APOE* ε3 and ε4 allele frequencies between AD and other groups including non-AD dementia and age-matched controls. The *APOE* ε4 allele frequency in the AD group and in the control group was similar to those of published clinical series (Gomez-Isla *et al*, 1996; Saunders *et al*, 1996) and showed the expected increase in ε4 allele frequency associated with AD. In addition, homozygosity for the ε4 was found only in patients with AD. Furthermore, the ε4 allele distribution in the control group showed no differences from that of the non-AD dementia. This raises the possibility that unrecognized cases of AD exist among the non-AD dementia. Our findings confirmed that *APOE* ε4 allele is a genetic risk factor for AD which is consistent with previous studies (Senanarong *et al*, 2001; Liu *et al*, 1995; Fabian *et al*, 1996; Kakulas and van Bockxmeer, 1996; Kuo *et al*, 2003). We observed a protective effect of the ε2 allele but it was limited to the ε2/ε3 genotype which was detected only in the control group. These data confirm previous reports (Saunders *et al*, 1996; Farrer *et al*, 1997) showing that the ε2/ε3 genotype is associated with a lower risk of AD. It is noteworthy that none of the subjects are homozygous for the ε2 allele. Similarly, Farrer *et al* (1997) reported that the influence of the rare ε2/ε2 genotype on AD risk could not be discerned even in a very large sample.

We demonstrated the feasibility of using PCR-SSCP to distinguish the genotypes of *APOE* polymorphism. This method avoids the need for restriction enzyme digestion and possible problems with incomplete cleavage (Tsai *et al*, 1993; Aozaki *et al*, 1994; Wilton and Lim, 1995) Moreover, the *APOE* genotype in DNA extracted from the blood can be identified within 8 hours. Our method is faster than PCR-RFLP, which needs at least 6 hours for the restriction-enzyme digestion step (Zivelin *et al*, 1997; Riemenschneider *et al*, 2002).

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