Biomedical Research 14 (3) 223-231, 1993

PCR-SSCP SCREENING OF β-AMYLOID PRECURSOR PROTEIN MUTATIONS IN TWO JAPANESE PEDIGREES WITH FAMILIAL EARLY ONSET ALZHEIMER'S DISEASE

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ABSTRACT

Five different types of point mutation of the β-amyloid precursor gene (APP) have been reported to cosegregate with familial Alzheimer's disease (FAD) in each of examined pedigrees (Table 1). Here we report a screening result of the APP gene mutations in two Japanese pedigrees with FAD of an early onset type which have previously been reported (2, 3). Primer pairs corresponding respectively to each of 19 exons of the APP gene were designed. Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis was performed on genomic DNA of one affected member from each of these two pedigrees. In addition, a pair of primers was designed to assess specifically codon 717 of the APP gene even in the poorly-preserved sample of genomic DNA. PCR-SSCP analysis of all 19 exons of the APP gene of both patients did not show any mutations, but disclosed one polymorphism in the intron 9. Sequencing of exons 16 and 17 of the APP gene in both patients, where all reported pathogenic mutations are located, revealed normal sequences. The results support that the genetic defect causing FAD is heterogeneous and that most cases with FAD are apparently due to the gene-defect of other than the APP gene.

Alzheimer's disease (AD) is one of the major causes of dementia, and its progressive neurodegeneration is characterized by the histological findings of neurofibrillary tangles, extracellular amyloid plaques, and cerebrovascular amyloid deposits in the brain (12, 29). Although the etiology of sporadic cases of AD is unknown, an autosomal dominant mode of inheritance with age-dependent penetrance has been observed in pedigrees with familial Alzheimer's disease (FAD) (2, 3, 10, 20, 22, 23, 30, 33). These clinical data suggest that FAD is a genetic disorder caused by a single dominant gene defect, although the etiology of sporadic cases may be different.

In 1984, a peptide consisting of 39 to 43 amino acid residues, designated as β/A4 peptide or amyloid β-protein, was first isolated from cerebrovascular amyloid protein (4). Subsequently amyloid β-protein was suggested to be produced by proteolytic cleavage of a much larger precursor protein designated as β-amyloid precursor protein (APP) (11, 27). APP exists in three major isoforms of 695, 751 and 770 amino acid residues due to alternative splicing of its mRNA (11, 13, 21, 28). The mechanism of cleavage of the β/A4 peptide remains unknown. Because the gene encoding APP was mapped to the long arm of chromosome 21, where DNA markers with a demonstrable genetic linkage to FAD (D21S1/S11 and D21S16) reside, APP was considered as a possible candidate defective gene in some FAD pedigrees (25, 27). In 1991, Goate *et al.* (5) reported, for the first time, a FAD-associated mutation at codon 717 of APP. The same mutation was soon found in 3 Japanese FAD pedigrees (18, 33). By now, a total of seven different pathogenic mutations of the APP gene have been reported as summarized in Table 1.

In this study, we screened the APP gene mutations by the method of polymerase chain reaction-

Table 1 *Reported Pathogenic Mutations of the APP Gene*

Codon[1]	Amino acid substitution	Disease	Reference
717	Val→Ile	FAD	5, 18, 33
717	Val→Phe	FAD	17
717	Val→Gly	FAD	1
670 & 671	Lys→Asn Met→Leu	FAD	16
692	Ala→Gly	FAD (Presenile dementia and cerebral hemorrhage)	7[2]
693	Glu→Gln	HCHWA-D	15
713	Ala→Val	Schizophrenia	9

[1]Numbers of codons were designated according to the nomenclature for APP consisting of 770 amino acid residues (APP_{770}). [2]A pedigree was reported of which members suffered either from presenile dementia of Alzheimer type or from cerebral hemorrhage due to amyloid angiopathy. APP, β-amyloid precursor protein; FAD, familial Alzheimer's disease; HCHWA-D, hereditary cerebral hemorrhage with amyloidosis of Dutch type

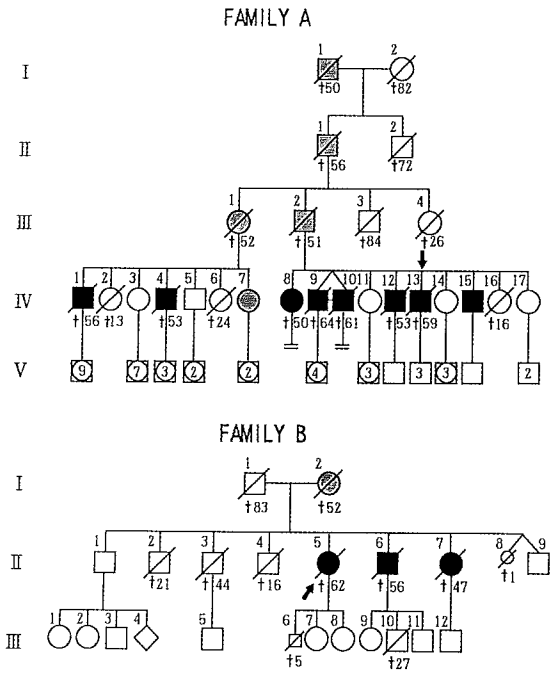

Fig. 1 Pedigrees of Families A and B. Symbols□and○ denote unaffected subjects,▨and◍denote subjects with suspected AD, and ■ and ● denote affected AD. A diagonal slash through a symbol indicates that the individual is deceased. Numbers below symbols indicate age at death. Numbers within the symbols indicate the number of offsprings. Arrows indicate the individuals whose genomic DNA was examined in the present study. Individuals marked as IV-9, 10 and 13 in family A, and II-5 and 6 in family B were confirmed as AD by autopsy findings.

single strand conformation polymorphism (PCR-SSCP) in two patients in two different pedigrees with early onset FAD (2, 3). Here we report the absence of mutations in the APP gene in these families, and one polymorphism of the APP gene in intron 9.

MATERIALS AND METHODS

Two Families Examined in This Study

Two unrelated families consisting of multiple affected individuals of FAD were examined in this study. In these families, both sexes were equally affected with about 50% incidence of appearance of AD in siblings. These suggest an autosomal dominant mode of inheritance of FAD with high penetrance (Fig. 1) (2, 3).

The number of affected members in the first family, abbreviated as Family A, is the largest among those Japanese families with FAD reported so far. In this study, the genomic DNA of an affected member designated as IV-13 in Fig. 1, and hereafter abbreviated as A-IV-13, was analyzed. His onset of AD was at the age of 46 years. He and his elder twin brothers (A-IV-9 and A-IV-10) fulfilled the clinical criteria for AD when they were admitted to the hospital of the University of Tokushima, School of Medicine. Brain samples of this patient and his twin brothers obtained at autopsy showed a typical histological appearance of AD.

In regard to the second family, designed as Family B, the genomic DNA of an affected member

Table 2 *List of Primers*

Exon (size[1])	Sense primer	Antisense primer
1 (266)	5′-CAGTTTCCTCGGCAGCGGT-3′	5′-TCTTGGGGGGTATCGCGTC-3′
2 (275)	5′-TTCCTCCAAGCCTCTGCCT-3′	5′-TGCATGTGATCCAACGTGAAT-3′
3 (291)	5′-TTAGATGCTTGTAAATGCCAG-3′	5′-AGTCTGTGTATGTGACCTAAC-3′
4 (170)	5′-AGATGGAATGACGGGAAGT-3′	5′-AAGAATTTATGGGCTGGTAC-3′
5 (283)	5′-ATCTACTCTAGCCACTCACTG-3′	5′-TACAAGATGTTTCAGCATCTCTG-3′
6 (295)	5′-GACTTTTTCTGTTTGCCTTCAC-3′	5′-TTTCCAACTCTGGTATTACGCT-3′
7 (252)	5′-TGAACAGAGAGACAGTGCCT-3′	5′-AGGCTCGAAGAAGGGTCCA-3′
8 (143)	5′-CACCATCATTCCCATGTTTCTC-3′	5′-TTCCCTCAGGTGAATGACAAC-3′
9 (230)	5′-CTATTAAACGAGTGGATTATTCTG-3′	5′-CTGAATGATGGAAGAGCAGACT-3′
10 (172)	5′-GTCTGTATTCAAAGGATGAACT-3′	5′-TGGATGTGATGTTTGGTAGGA-3′
11 (217)	5′-CCCATGGACATATGTGTTTATGAT-3′	5′-AGTGTGAACTCGGCTGCAGC-3′
12 (218)	5′-TCGCCAATGGAAGAAGCAGT-3′	5′-AAGATGATTATACCCCACGCT-3′
13 (165)	5′-GTGACCTGGAGTGTCATCCT-3′	5′-CATGTCCATGTGCAGCATCA-3′
13a (128)	5′-TGTCCCAGCACCATTGTTGA-3′	5′-GTTTCCCAATCTGGTCTTGAA-3′
14 (276)	5′-GTTACTCACCAAAGAGATGG-3′	5′-TGGCTCAGGGGACTCTTAC-3′
15 (149)	5′-TTGGGAGCCACGACTTACCA-3′	5′-GCACAACGTCAAGCGGTTCT-3′
16 (180)	5′-TAATTGGTTGTCCTGCATAC-3′	5′-CAAACAGTAGTGGAAAGAGG-3′
17 (293)	5′-CCTCATCCAAATGTCCCCTG-3′	5′-CCACTTGGAAACATGCAGTC-3′
18 (203)	5′-ACTGCTTCTCCATGTTCACC-3′	5′-GTCCAACTTCAGAGGCTGCT-3′
C717[2] (56)	5′-CGGTGTTGTCATAGCGACAG-3′	5′-GTTTCTTCTTCAGCATCACCA-3′

[1]Size of an amplified fragment is shown in base pairs. [2]This primer pair of C717 is specifically to assess codon 717 of the APP gene in a damaged DNA sample.

designed as II-5 in Fig. 1, and hereafter abbreviated as B-II-5, was analyzed. Her onset of AD was at the age of 49 years. Her mother, brother and sister also suffered from AD by the age of as early as 50 years. AD of this patient was clinically diagnosed at the age of 52 years by the symptoms of progressive dementia and general cerebral atrophy revealed by a computerized tomography (CT) scan. Her brain obtained at autopsy also showed typical histological characteristics of AD.

DNA Samples

High molecular weight DNA was prepared by proteinase K digestion and phenol/chloroform extraction (26) from the brain tissues of patients A-IV-13 and B-II-5, which had been stored at −80°C. Genomic DNA was isolated from formalin-fixed paraffin-embedded liver tissues of the patients A-IV-9 and A-IV-10 by a standard method (31). Briefly, a tissue section in 10 μm thickness was deparaffinized by washing twice in xylene, twice in 100% ethanol, and finally dried under reduced pressure. Then the tissue was treated with proteinase K (200 μg/ml) in 100 μl of digestion buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, and 0.5% Tween 20) at 37°C overnight. After inactivating proteinase K by incubating at 95°C for 10 min, the sample mixture was used as a DNA sample in PCR analysis. The DNA samples prepared from peripheral white blood cells of unrelated individuals were used as controls.

PCR-SSCP Analysis

PCR-SSCP analysis was performed by the method of Orita *et al.* (19) with a slight modification. Pairs of intronic primers were designed based on sequences reported by Lemaire *et al.* (14) and Yoshikai *et al.* (32) (Table 2). Five microliters of the PCR mixture contained 50 ng of genomic DNA as a template, 5 pmol each of primers, 0.125 nmol each of dNTPs, 0.25 μl of [α-^{32}P]dCTP (110 TBq/mmol; 370 MBq/ml, Amersham, Buckinghamshire, U.K.), and 0.25 units of Taq DNA polymerase (Promega, Madison, U.S.A.) in the buffer supplied by Promega. Thirty cycles of the reaction at 94, 55 and 72°C for 1 min each were carried out in a thermal cycler (Astek PC-700, Fukuoka, Japan). For the PCR reaction of exon 1, the initial

denaturing of template DNA by heating at 95°C for 9 min was followed by 40 cycles of the reaction at 95, 58 and 72°C for 1, 1, and 2 min, respectively. The resultant PCR product in 5 μl was then mixed with 245 μl of a formamide dye mixture (95% formamide, 20 mM EDTA, 0.05% xylene cyanol, and 0.05% bromophenol blue), heated at 90°C for 3 min, and applied (1.5 μl/lane) to 6% polyacrylamide gels containing 45 mM Tris-borate (pH 8.3) and 4 mM EDTA. All of the PCR products were analyzed by PCR-SSCP in three different glycerol concentrations of either 0, 5 or 10%, respectively. Electrophoresis was performed at 20 W for 3 to 6 h with cooling by a fan at room temperature. The gel was dried on filter paper and exposed to an X-ray film with an intensifying screen overnight at −80°C.

DNA Sequencing

DNA fragments of 172 bp, 180 bp and 293 bp in size were amplified using genomic DNA of the patient A-IV-13 or B-II-5 as a template and pairs of specific primers to amplify exons 10, 16 and 17, respectively. Each of amplified fragments was cloned into the *Sma*I site of pUC19, and JM 109 competent *E. coli* cells were transformed with these recombinant pUC19. The plasmid DNA was prepared by a small-scale boiling method (8). Nucleotide sequences were determined by fluorescence-based dideoxy sequencing method with an automated DNA sequencer (model 373A, Applied Biosystems, Foster, CA, U.S.A.).

Assessment of the APP717 Val→Ile Mutation

A pair of primers was designed specifically for assessing the APP717 mutation of Val→Ile in the damaged genomic DNA isolated from acid formalin-fixed paraffin-embedded tissue sections (Table 2). A DNA fragment of 56 bp encompassing the codon 717 was amplified by two repetitive PCRs. In the first PCR, 30 cycles of the reaction at 94, 55 and 72°C for 1 min each were carried out using the genomic DNA of the patient A-IV-9 or A-IV-10 isolated from paraffin sections as a template and C717, a pair of specifically designed primers to detect the codon 717 mutation. The resultant PCR product was diluted 20 times with water and used as a template in the second PCR, which was performed by 35 cycles of the reaction using the same C717 as a pair of primers and the same conditions as used for the first PCR. Aliquot of the amplified DNA was electrophoresed directly or electrophoresed after digestion with *Bcl*I or *Mbo*I on a 10% polyacrylamide gel. DNA fragments were visualized with ethidium bromide staining.

RESULTS

PCR-SSCP Analysis

The electrophoresis of the amplified double-stranded DNA, by PCR with primers listed in Table 1 and each of genomic DNAs obtained from A-IV-13 or B-II-5 as a template, showed the same migration pattern as that of control DNA used as a template (Fig. 2). By PCR-SSCP analyses in three different glycerol concentrations in the polyacrylamide gel, an altered migration pattern was observed only when the amplified DNA of A-IV-13 with primers encompassing exon 10 was analyzed (Fig. 3). No altered migration patterns were detected in the remaining 18 exons of this patient nor all exons of the patient B-II-5. To determine the mutated sequence in the patient A-IV-13, the amplified DNA fragment with primers encompassing exon 10, which includes exon 10 and a part of intron 9, was cloned into pUC19. Out of six determined sequences, three consistently revealed a single nucleotide substitution from T to C in intron 9 at 31 bp in the upstream from its 3′-end, while other three clones did not show any nucleotide substitutions (Fig. 4). No nucleotide substitution was found in determined sequences of exon 10 in all six clones. These results indicate that this nucleotide substitution in intron 9 is a polymorphism and that the patient A-IV-13 was heterozygous for this polymorphic site.

Assessment of Exons 16 and 17 of the APP Gene by Sequencing

DNA fragments encompassing exon 16 or 17, respectively, were amplified using genomic DNA from the patient A-IV-13 or B-II-5 as a template, and cloned into pUC19. Five clones per each exon were sequenced, and no nucleotide substitution was found in any of the examined clones.

Assessment of the APP717 Val→Ile Mutation in the Genomic DNA Isolated from Formalin-Fixed Paraffin Sections

The patients A-IV-9 and A-IV-10 are a very rare twin case with FAD. Unfortunately, only formalin-fixed paraffin-embedded tissues obtained at

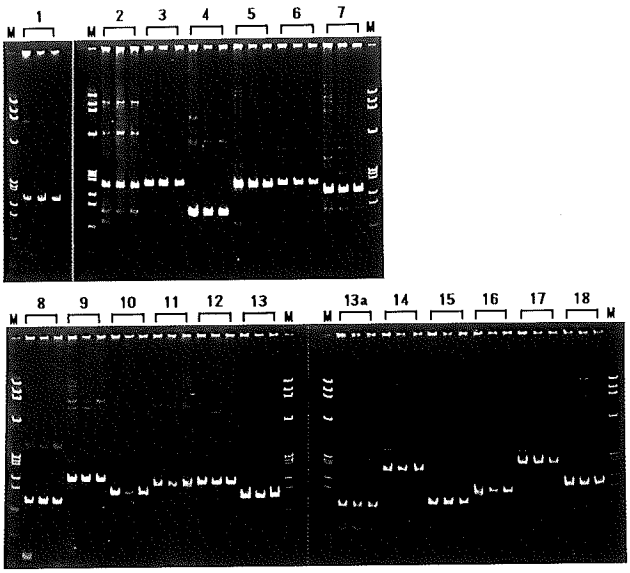

Fig. 2 Polyacrylamide gel electrophoresis of PCR products visualized with ethidium bromide staining. Numbers above brackets indicate numbers of amplified exons of the APP gene using the primers listed in Table 2. The left, middle and right lanes under each bracket show PCR products amplified using genomic DNA of the control, the patient A-IV-13 and the patient B-II-5 as a template, respectively. Electrophoresis was performed in 8% polyacrylamide gels. M, DNA size markers of *Hae*III-digested ϕx174

Fig. 3 PCR-SSCP analysis of the APP gene in two families with FAD. DNA was PCR-amplified with the primers for exon 10. Electrophoresis was performed in a 6% polyacrylamide gel containing 10% glycerol. An arrowhead indicates the band with altered migration relative to control. C, control; 1, patient A-IV-13; 2, patient B-II-5

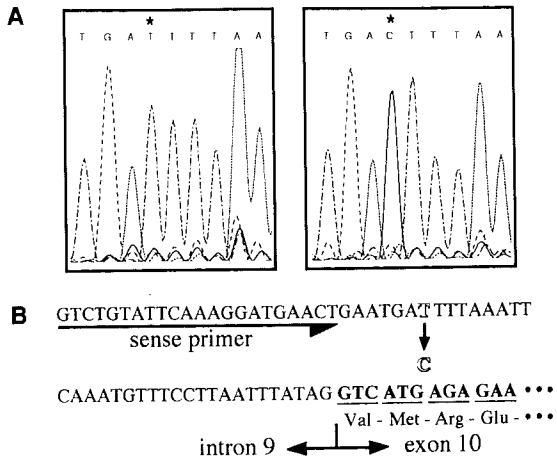

Fig. 4 Fluorescence-based DNA sequence of exon 10 and a part of intron 9. A: The previously-reported and newly-found polymorphic sequence of intron 9 are shown in the left and right panel, respectively. Asterisks denote the location of this polymorphic site. B: Diagramatic presentation of the location of the new polymorphism of C instead of T in intron 9.

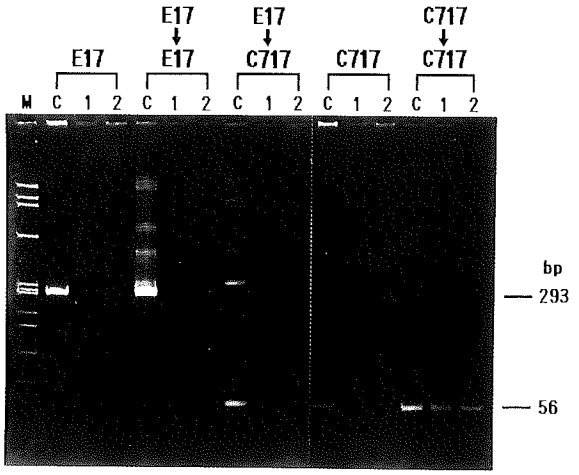

Fig. 5 Polyacrylamide gel electrophoresis of PCR products visualized with ethidium bromide staining. DNA isolated from formalin-fixed paraffin-embedded tissues of the FAD-affected twin brothers (A-IV-9 and -10) were used as templates. E17 or C717 denotes a pair of primers used in PCR. PCR products of either the first PCR or two repetitive or nested PCRs were electrophoresed. The number and order of repeated PCR are shown by the abbreviated name of primers and arrows above each bracket. E17, one PCR using the primers for exon 17; E17→E17, two repetitive PCRs using the primers for exon 17 twice; E17→C717, two nested PCRs using the primers for exon 17 in the first PCR and the primers for codon 717 in the second PCR; C717, one PCR using the primers for codon 717; C717→C717, two repetitive PCRs using the primers for codon 717 twice. In each of the second PCR, the 20 times-diluted product of the first PCR was used as a template. Electophoresis was performed in 10% acrylamide gels. C, control; 1, patient A-IV-9; 2, patient A-IV-10; M, DNA size markers of *Hae*III-digested ϕx174

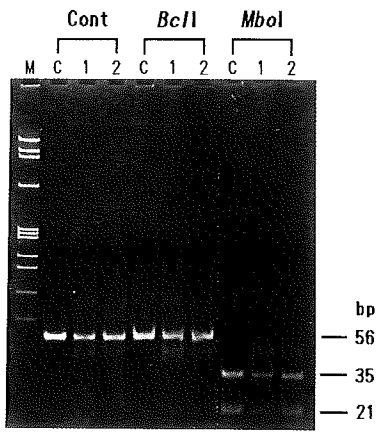

Fig. 6 *Bcl*I or *Mbo*I digests of repetitive PCR products using the C717 primer pair encompassing codon 717 of the APP gene from the FAD-affected twin brothers. The PCR products were undigested or digested with enzymes at 37°C overnight and electrophoresed in a 10% polyacrylamide gel. DNA was visualized with ethidium bromide staining. Cont, undigested PCR products; *Bcl*I, *Bcl*I digests; *Mbo*I, *Mbo*I digests. C, control; 1, patient A-IV-9; 2, patient A-IV-10; M, DNA size markers of *Hae*III-digested ϕx174

autopsy were available as the source of their genomic DNAs. We tried to amplify exons 16 and 17 using their genomic DNAs isolated from the paraffin-embedded tissues, but no amplified fragments were observed even after two repetitive or nested PCRs. Their genomic DNAs were apparently depurinated and degraded by fixation with acid-formalin and did not serve as templates to be amplified by the used primers in PCR for both exons. Therefore, we designed a pair of primers to amplify a very short DNA fragment of 56 bp in the total length including a stretch of 15 bp after subtracting the length of two primers. This 15 bp stretch encompasses the codon 717 of the APP gene, the mutation spot in FAD (29). Although the amplified DNA was not observed after the first PCR reaction with the C717 primer pair, the 56 bp DNA fragment was amplified and became visible by ethidium bromide staining after the second repetitive PCR reactions using the same C717 primer pair and the diluted PCR product of the first PCR as a template (Fig. 5). The nested PCRs using the primers of exon 17 in the first PCR and the primers of C717 in the second PCR did not amplify the same 56 bp DNA fragment. *Mbo*I digestion (restriction site: 5′GATC3′) of the 56 bp amplified DNA fragments from the control and those two patients created the similar two bands of 35 and 21 bp as expected, while *Bcl*I digestion (restriction site: 5′TGATCA3′) did not create new bands from any of these DNAs (Fig. 6). These results provide evidence for the absence of APP717 Val (GTC)→Ile (ATC) mutation, which creates a new restriction site for *Bcl*I, in either genomic DNA of the twin brothers with FAD.

DISCUSSION

Most cases of AD are sporadic, and its molecular etiology still remains unknown (12, 29). Although FAD is a rare hereditary disorder, elucidation of the involved gene and its correlation with the clinical picture is essential to understand the mechanism of

not only FAD but also sporadic AD. Five different types of missense mutations found in FAD pedigrees were restricted to exons 16 or 17 of the APP gene. They are now known to be pathogenic mutations based on its cosegregation with the AD phenotype (1, 5, 7, 16–18, 33). Because of the rarity of FAD and of the importance of determining the etiologic gene defect in FAD, we extensively screened all APP exons for mutations by PCR-SSCP. In addition, we sequenced exons 16 and 17 of the APP gene of these patients. Consequently, neither the reported types of missense mutations nor any other new mutations were detected in the APP gene of both patients. Because the sensitivity of PCR-SSCP analysis to detect mutations in DNA fragments of 100- to 300-bp, under three different conditions used in the present study, is very high (6), the chance of overlooking unknown pathogenic mutations should be negligibly low.

The pair of primers for the codon 717 which we designed specifically to assess the APP717 mutation in the damaged genomic DNA isolated from acid formalin-fixed paraffin-embedded tissues should be useful for further retrospective study, because only this type of tissues are left available in a large number of deceased familial or sporadic AD cases. Fifteen base pairs including four codons, encompassing the codon 717 of the APP gene from poorly preserved genomic DNA, can be assessed using this pair of primers.

Recent studies revealed that the APP mutations may account for a small portion of FAD (10, 30), i.e. 8 of 135 FAD pedigrees (30). Genetic linkage studies showed that there may be another gene defect on chromosome 21 with a more centromeric location relative to the APP gene (29). Moreover, some pedigrees with the late onset FAD were reported to be associated with markers on chromosome 19 (20). Evidence for the presence of a major early onset FAD locus on the long arm of chromosome 14 was reported quite recently by several groups (22, 23). Thus the gene defect in FAD is regarded not to be restricted to the APP gene but to be heterogeneous (24), as was further suggested in two FAD pedigrees in our present study. In addition, it is important to test whether FAD patients in the present families show genetic linkage to markers on chromosome 14 or 19 by collecting a sufficient number of DNA samples from family members.

In summary, we have ruled out the presence of APP gene mutations in two unrelated pedigrees with an early onset FADs. The effort to disclose etiologic mutations in FAD should include further analysis of the APP gene in other pedigrees of FAD using not only peripheral white blood cells but also paraffin-embedded tissues as a source of genomic DNA, and linkage study with chromosome 14 or 19 in FAD families.

We appreciate Dr Akira Doi at Kawamura Hospital, Tokushima, Japan and Dr Kenzo Fujimoto at Aisato Hospital, Tokushima, Japan for clinically taking care of FAD patients, Dr Hideo Susaki at Tokushima Municipal Sonose Hospital, Tokushima, Japan and Dr Tadashi Hasegawa at The First Department of Pathology, The University of Tokushima, Tokushima, Japan, for having made the necessary arrangement and autopsy of the patient B-II-5. This study was supported in part by an Otsuka Pharmaceutical Factory grant for Otsuka Department of Clinical and Molecular Nutrition, School of Medicine, The University of Tokushima.

Received 29 March 1993; and accepted 19 April 1993

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