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Abstract

Familial Alzheimer's disease (FAD) has been shown to be associated with three distinct point mutations within the same codon of the amyloid precursor protein (APP) gene. The mutation identified in the Indiana kindred is a G→T transversion at the first position of the codon for amino acid 717, resulting in a substitution of phenylalanine for valine in the APP protein. Screening of persons at risk for the APP Phe-717 mutation using a variation of the polymerase chain reaction identified nine positives among 34 tested. In addition, DNA from 145 FAD subjects were tested for the three known APP 717 mutations.

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Alzheimer's disease, a form of amyloidosis with deposition localised to the central nervous system, is characterised by a progressive dementia. Both sporadic and hereditary forms, with autosomal dominant inheritance, show the characteristic neuropathological findings of senile plaques in the cerebral cortex, neurofibrillary tangles, and congophilic angiopathy of the leptomeningeal vessels. The amyloid forming protein found in fibrils isolated from the blood vessels and senile plaques has been identified as a 39 to 43 amino acid segment of the amyloid precursor protein (APP).[1] Three separate missense mutations within the same codon of the APP gene have recently been reported in patients from several different families with hereditary Alzheimer's disease.[2–4] The first of these to be described is a single base change that produces a substitution of isoleucine for valine at amino acid 717 of the APP protein (APP-770 transcript). Several subsequent reports have shown that this mutation is rare among FAD patients.[5] A T→G transversion in the position corresponding to the second base of codon 717 giving a Val to Gly substitution in APP has also been reported.[3] There have been no reports as yet of any attempt to determine the frequency of this mutation in FAD kindreds. The valine to phenylalanine change found in the Indiana kindred results from a G→T transversion in the first position of the codon for amino acid 717 of the APP cDNA sequence. We have conducted extensive screening of subjects with FAD in order to determine the frequency of these three mutations and describe a rapid, non-isotopic testing method for the APP Phe-717 and APP Gly-717 mutations based on the polymerase chain reaction.

Materials and methods

DNA ISOLATION

Total genomic DNA was isolated from either peripheral blood leucocytes[6] or formalin fixed tissue sections,[7] as described previously, from 34 members of the Indiana FAD kindred. Genomic DNA from an additional 123 FAD patients was obtained from transformed cell lines stored by the Alzheimer's Disease Research Centers' National Cell Bank. Each of these samples is from an affected member of a distinct FAD family with at least two affected members. Another 25 samples were kindly provided by Duke University, representing another 22 FAD families (22 affected, three unaffected).

APP PHE-717 SCREENING

Enzymatic amplification was performed in 50 µl reactions containing 1 × PCR buffer (10 mmol/l Tris-HCl (pH 8·3 at 25°), 50 mmol/l KCl, and 3 mmol/l $MgCl_2$), 200 µmol/l each dNTP (Pharmacia), 0·25 U Amplitaq DNA polymerase (Perkin-Elmer Cetus), 0·5 µg DNA, and 25 pmol each primers APPEX17-3′ and APP Phe-717 PCR-IMRA. Primer APPEX17-3′ (5′-CCTCCACCACACCATGA-3′) anneals to a region near the 3′ end of exon 17 of the APP-770 transcript. Primer APP Phe-717 PCR-IMRA (5′-GGCGGTGTTGTCATAGCTACATATAT-3′) was designed to anneal immediately 5′ to the site of the G→T mutation and contains a single mismatch near its 3′ end. Amplification of the variant allele with these primers produces a *Bgl*II site (AGATCT) in the resulting 91 base pair PCR product, while the PCR product derived from the normal allele using the same set of primers contains no *Bgl*II site (fig 1). Amplification was performed using a Perkin-Elmer Thermal Cycler for 35 cycles consisting of denaturing at 94° for one minute, primer annealing at 60° for one minute, and extending at 72° for one minute. Following amplification, the reactions were extracted with 100 µl Sevag and a 10 µl aliquot was electrophoresed on a 4% composite (3% FMC NuSieve/1% Bethesda Research Labs) agarose gel for one hour at 80 V, stained with 1 µg/ml ethidium bromide, and photographed on a UV light source to ensure successful amplification. Additional 10 µl aliquots of reactions containing a predominant band of the expected size (91 bp) were digested with 14 U *Bgl*II (United States Biochemicals) in 1 × supplied HIGH buffer at 37°. After at least one hour of digestion, the digests were electrophoresed on a 4%

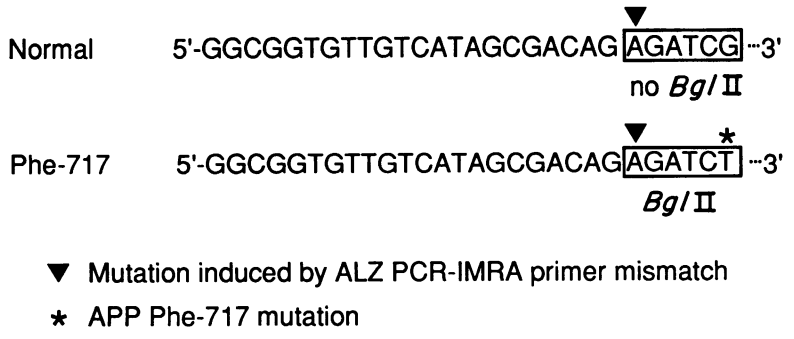

Figure 1 Scheme of PCR-IMRA testing method. A partial sequence of the 5' end of PCR products from the normal and mutant alleles is shown. The box encloses the induced restriction site. Both the induced and Phe-717 mutations are indicated.

composite agarose gel at 80 V for 90 minutes, stained with ethidium bromide and photographed as described above. *Bgl*II digestion of PCR products containing the Phe-717 mutation results in a 68 base pair digestion product, while PCR products from the normal allele remain undigested (fig 2).

APP ILE-717 SCREENING

Enzymatic amplification was performed as described above using primers APPIN16-5' (5'-CCAAATGTCCCCGTCATTTAA-3') and APPIN17-3' (5'-CTCTCATAGTCTTAA-TTCCCAC-3'), which yield a 305 base pair PCR product containing all of exon 17 of the APP gene. The conditions for amplification were identical to those used in the APP Phe-717 PCR-IMRA technique described previously, except the annealing temperature was 58°. A 10 µl aliquot of each amplification reaction was digested at 37° with 10 U *Bcl*I (New England Biolabs) in 1 × supplied buffer NEB3. After one hour, the digests were electrophoresed, stained, and photographed as described above. PCR products containing the Ile-717 mutation will yield two digestion products of 188 and 117 base pairs, while normal PCR products show no digestion (fig 2).

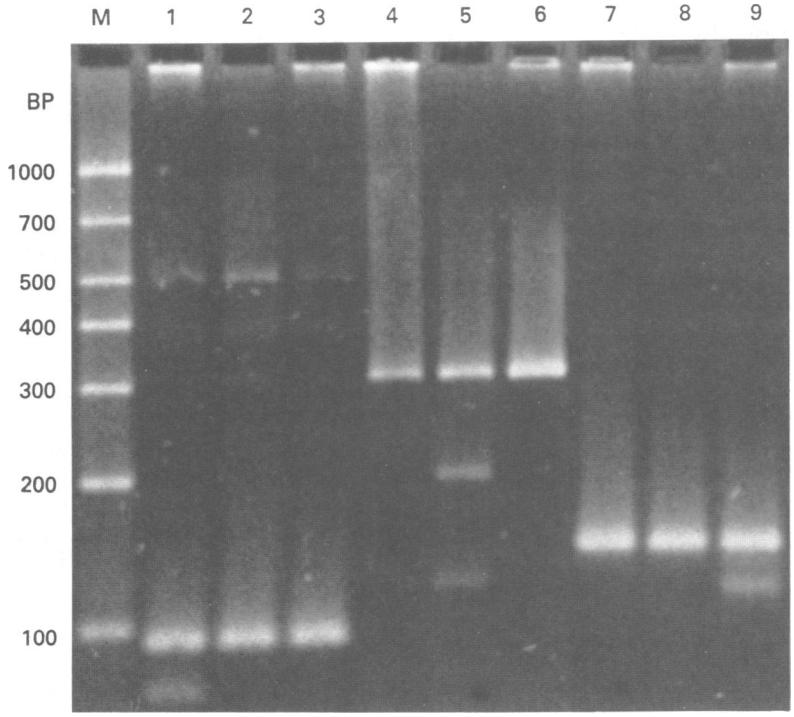

Figure 2 4% composite agarose gel of digested PCR products from an APP Phe-717 positive control (lanes 1, 4, and 7), an APP Ile-717 positive control (lanes 2, 5, and 8), and an APP Gly-717 positive control (lanes 3, 6, and 9). Each positive control was tested for, from left to right, the Phe-717 mutation by PCR-IMRA, the Ile-717 mutation by RFLP, and the Gly-717 mutation by PCR-IMRA. Lane M contains a 100 bp ladder (BioMarker Low from BioVentures, Inc).

APP GLY-717 SCREENING

Primers APPIN17-3' and APP Gly-717 PCR-IMRA (5'-GGCGGTGTTGTCATAGCG-ACAGTGACCG-3') were used to perform amplification under the conditions previously described, except the annealing step was 62° for 30 seconds. This set of primers yields a 140 base pair product, with a unique *Msp*I site introduced by the APP Gly-717 PCR-IMRA primer in the PCR product from the Gly-717 allele. The resulting PCR products were digested with 10 U *Msp*I (Bethesda Research Laboratories) in supplied buffer REact 1 for one hour at 37°, electrophoresed, stained, and photographed as above. *Msp*I digestion of PCR products from the Gly-717 allele generates a 114 base pair digestion product, but no digestion of normal PCR products occurs (fig 2).

Results

The detection method used for screening at risk subjects in this study, which we have termed polymerase chain reaction-induced mutation restriction analysis (PCR-IMRA), was initially tested for accuracy and reliability using DNA from patients known to be heterozygous for the APP Phe-717 mutation by direct sequencing. Once the technique had been shown to detect reproducibly the mutant allele in all tested positive controls, it was used to screen an additional 17 members of the Indiana FAD kindred (34 total). Of those tested, a total of nine subjects heterozygous for the Phe-717 mutation were identified.

Further screening was conducted on 123 DNA samples obtained from the Alzheimer's Disease Research Centers' National Cell Bank and 25 samples from Duke University. None of these showed the presence of the Phe-717 allele. In addition, 100 unrelated, non-AD controls were tested by PCR-IMRA, as reported previously.[4] None of the 200 control alleles screened was positive for the APP Phe-717 mutation.

The 123 samples from the Alzheimer's Disease Cell Bank and all 34 members of the Indiana FAD kindred were also screened for the APP Ile-717 mutation, using conventional RFLP analysis, and the APP Gly-717 mutation, using an additional PCR-IMRA test. One patient from the Alzheimer's Disease Cell Bank was found to be heterozygous for the Ile-717 mutation, while the remainder showed neither mutation (data summarised in the table).

The 25 samples from Duke University were tested for the APP Ile-717 mutation by RFLP analysis. Two samples were heterozygous for the APP Ile-717 allele. Direct sequencing of exon 17 of the APP gene was used to determine that none was positive for the APP Gly-717 allele.

*Summary of APP screening results. Number of patients screened for each mutation is indicated, with the number of positive results in parentheses. *Denotes members of original Duke University APP Ile-717 kindred, as described in the text. All positive results indicated represent subjects heterozygous for the mutant allele.*

Source of sample	Mutations		
	APP Phe-717	APP Ile-717	APP Gly-717
Alzheimer's Disease Research Centers' National Cell Bank	123 (0)	123 (1*)	123 (0)
Indiana FAD kindred	34 (9)	34 (0)	34 (0)
Duke University	25 (0)	25 (2*)	25 (0)
Unrelated, non-FAD controls	100 (0)	Not tested	Not tested

Discussion

The Phe-717 mutation in an Indiana family was first detected by direct genomic sequencing.[4] Additional family members were identified by this method to establish direct association of the mutation and FAD. In order to conduct large scale screening, such as population studies, a faster, simpler, and more reliable technique was needed. Screening for the APP Ile-717 and Gly-717 mutations can be performed by conventional RFLP analysis, since these mutations produce new *Bcl*I and *Sfa*NI restriction sites, respectively. Unfortunately, the Phe-717 mutation does not alter the restriction pattern of the APP gene, so it was necessary to use the PCR-IMRA technique to induce a new restriction site. Use of this rapid, non-isotopic approach has greatly increased the ease of screening for this particular mutation.

Testing of members of the Indiana FAD kindred by PCR-IMRA confirmed the earlier sequencing results that detected five APP Phe-717 carriers among 19 subjects tested. In addition, four more APP Phe-717 carriers were identified during the screening of 15 other family members. These results confirmed the association of the mutation with clinical disease and allowed the identification of presymptomatic gene carriers, providing subjects for studies looking for early markers of FAD.

Extensive screening of DNA samples obtained from the Alzheimer's Disease Cell Bank and Duke University failed to identify any Phe-717 carriers among an additional 144 unrelated FAD patients (123 from AD cell bank and 21 from Duke University). Thus, although it clearly appears to be involved in the disease process in the Indiana kindred, the Phe-717 mutation seems to be extremely rare among FAD patients as a group. These results agree with earlier reports which indicated that the Ile-717 mutation was also rare among unrelated FAD patients, although additional kindreds have been found in Europe and Japan.[8–10] By using the PCR-IMRA method reported here, investigators from around the world, including Europe and Japan, will easily be able to screen a large number of additional FAD patients for the Phe-717 allele.

Screening of the 123 Alzheimer's Disease Cell Bank samples, 34 members of the Indiana FAD kindred, and the 25 samples from Duke University was also performed for the other two reported APP mutations. Conventional RFLP analysis was used to screen for the Ile-717 allele, but the excessive cost of the restriction enzyme used to detect the Gly-717 allele by similar means prompted the devising of an alternative means of detection. The PCR-IMRA technique was used, owing to its accuracy and ease, to create a unique *Msp*I site in the PCR product from the variant Gly-717 APP allele. The use of this considerably less expensive restriction enzyme greatly reduced the overall cost of screening a large number of subjects.

Only one of the AD cell bank DNA samples tested showed the presence of the Ile-717 APP mutation. Further analysis showed this patient was actually a previously untested member of the original FAD kindred studied at Duke University in which the Ile-717 mutation was discovered. Two other members of this kindred were identified among the 25 DNA samples provided by Duke University. The Gly-717 mutation was not detected in any of the patients screened. Continued efforts are under way to extend the screening process for all three of the reported APP mutations.

Our previous data suggest that the Phe-717 mutation is involved in the pathogenesis of FAD in the Indiana kindred. This mutation, however, appears to be very rare, since we did not find it in a large number of unrelated FAD subjects. With availability of the PCR-IMRA we describe here, other investigators can pursue more extensive world wide testing. Even so, present studies suggest that mutations at codon 717 of the APP gene are a rare cause of Alzheimer's disease.[11 12]

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