Association of apolipoprotein E allele ε4 with late-onset familial and sporadic Alzheimer’s disease

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Article abstract—Apolipoprotein E, type ε4 allele (APOE ε4), is associated with late-onset familial Alzheimer’s disease (AD). There is high avidity and specific binding of amyloid β-peptide with the protein ApoE. To test the hypothesis that late-onset familial AD may represent the clustering of sporadic AD in families large enough to be studied, we extended the analyses of APOE alleles to several series of sporadic AD patients. APOE ε4 was significantly associated with a series of probable sporadic AD patients (0.36 ± 0.042, AD, versus 0.16 ± 0.027, controls [allele frequency estimate ± standard error], p = 0.00031). Spouse controls did not differ from CEPH grandparent controls from the Centre d’Etude du Polymorphisme Humain (CEPH) or from literature controls. A large combined series of autopsy-documented sporadic AD patients also demonstrated highly significant association with the APOE ε4 allele (0.40 ± 0.026, p ≤ 0.00001). These data support the involvement of ApoE ε4 in the pathogenesis of late-onset familial and sporadic AD. ApoE isoforms may play an important role in the metabolism of β-peptide, and APOE ε4 may operate as a susceptibility gene (risk factor) for the clinical expression of AD.

Alzheimer’s disease is the leading cause of dementia in the elderly. Most patients encountered in practice have no obvious family history and are classified as sporadic. However, genetic factors in early- and late-onset familial Alzheimer’s disease (FAD) are now well documented. Early genetic...
linkage studies suggested a locus for early-onset FAD on chromosome 21. Subsequently, investigators identified a rare mutation of the amyloid precursor protein (APP717Val→) on chromosome 21 in 11 early-onset FAD kindreds. Two groups described several other very rare mutations of the APP gene in families segregating dementia of the Alzheimer type, Schellenberg et al. and St. George-Hyslop et al. localized most of the early-onset FAD kindreds showing support for linkage to chromosome 21 to a region on chromosome 14q. Another study reported possible linkage of late-onset FAD to the proximal long arm of chromosome 19.

Several independent lines of evidence led us to examine apolipoprotein E (ApoE, protein; APOE, gene) in late-onset FAD. We observed several proteins in CSF that bound to immobilized amyloid β-peptide with high affinity. Microsequencing and Western blotting techniques identified ApoE as one of these proteins. ApoE was known to be localized to the region of chromosome 19, which in previous studies had shown possible linkage to late-onset FAD. Furthermore, antisera to ApoE stained senile plaques, neurofibrillary tangles, and cerebral vessel amyloid deposits in AD brains. In this communication, we present data supporting the association of the ε4 allele of APOE with late-onset sporadic AD, including clinical (probable) AD cases and autopsy-confirmed AD cases.

**Methods.** Families. Blood samples for the genomic DNA studies were obtained from families described previously. All sampled individuals diagnosed with probable AD were examined by a neurologist and associated diagnostic personnel of the Joseph and Kathleen Bryan Alzheimer's Disease Research Center (ADRC) Memory Disorders Clinic at Duke University, the Center for Neurodegenerative Diseases at the University of Toronto, or the Departments of Neurology at Massachusetts General Hospital and the Harvard Medical School. The clinical diagnosis was made according to the NINCDS-ADRDA criteria. The Duke University pedigrees were primarily late-onset AD families with an average age of 66.1 ± 10.3 years in the 35 families. Three of the families could be classified as early-onset (mean, <60 years) AD families. One family segregates with the APP717Val→Ile mutation, a second segregates with the APP717Val→Phe mutation, and the other is linked to chromosome 14 markers with a maximum lod score of 3.5. The Toronto pedigrees were classified as primarily early-onset families (13 of the 17 families). Five of the families were linked to chromosome 14 with lod scores greater than 3.0 in each family, and a sixth pedigree had the APP717Val→Ile mutation. The family and genotypic data were processed via the PEDIGENE system.

Genomic DNA from some patients diagnosed as having sporadic cases of probable AD at Duke, Toronto, and Boston have been banked over the past 6 years. Sporadic AD patients were defined as those without a known family history of AD or dementia. The sporadic, probable AD patients represented all the banked DNAs in the Toronto and Duke banks as of November 1992 except for an ongoing prospective series begun in August 1992 at the Bryan ADRC Memory Disorders Clinic. The DNA from those individuals had been collected randomly prior to any interest in APOE isotyping. Not all sporadic AD patients evaluated in these clinics were banked routinely. The diagnosis of probable AD in this group can be expected to be in the 80% to 90% accuracy range that is observed in most specialized AD clinics. Brain DNA was obtained from autopsy-confirmed, white cases of AD that had been banked in the Kathleen Bryan Brain Bank at Duke, the University of Toronto, and the Harvard Medical School. Six black or Native American autopsied subjects were eliminated from the series of sporadic AD autopsies because the association analyses are sensitive to the control group.

Two sets of controls were used in the study. The first set was 91 unrelated grandparents from the Centre d'Etude du Polymorphism Humain (CEPH) reference families. These families were collected for human gene mapping and are characterized by having grandparents, parents, and many grandchildren available for DNA mapping. The grandparents represent a random group of white, aged controls of European and American background, similar to the late-onset FAD families and the autopsy-confirmed sporadic AD population. Twenty-one white spouses of patients participating in an ongoing prospective analysis of probable AD patients and spouses were also used as a control group.

Genomic DNA. High-molecular-weight DNA was obtained from transformed lymphoblasts according to a method described elsewhere or to the Genepure 341 nuclease acid extractor's supplied protocol (Applied Biosystems). Genomic DNA from brain tissue was isolated by pulverizing approximately 300 mg of frozen brain tissue under liquid nitrogen, adding 4 ml of lysis buffer (Applied Biosystems) and 1 mg of proteinase K (Applied Biosystems), and gently rocking overnight at 37 °C before extracting on the Genepure 341.

Amplification and restriction isotyping of APOE. Genomic DNA was amplified by polymerase chain reaction (PCR) in a Techne MW-2 thermocycler using HI-TEMP 96-well plates (Techne) and the primers described by Wenham et al. The PCR protocol was based on those described by Vanhalm et al. and Hixson and Vernier. Each amplification reaction contained 20 ng genomic DNA, 1.0 pmol/μl of each primer, 10% dimethylsulfoxide (Sigma), 200 μM each dNTP (Pharmacia), 1.0 μCi (alpha-32P) dCTP (800 Ci/mol in 10 mM Tricine; NEN Research Products), 0.05 U/μl Tag DNA polymerase, and supplied 1× buffer (Boehringer Mannheim) in a final volume of 15 μl. An initial denaturation at 94 °C for 5 minutes was followed by 35 cycles of annealing at 65 °C for 0.5 minutes, extension at 70 °C for 1.5 minutes, and denaturation at 94 °C for 0.5 minutes, and a final extension at 70 °C for 10 minutes. After amplification, 5 U of Hha I (Gibco) was directly added to each well, and the plates were incubated at least 3 hours at 37 °C. Fifteen μl of 2× type III stop dye was added to each well, and 3 μl of each reaction was loaded on a 6% nondenaturing polyacrylamide gel (0.4 mm thick × 43 cm long) and electrophoresed for 1 hour under constant current (45 mA). After electrophoresis, the gel was transferred to Whatman 3M chromatography paper, dried, and autoradiographed for 1 hour using Kodak XAR-5 film. Each autoradiograph was read independently by two different observers.

**Statistical analysis.** We estimated allele frequencies for the control and AD groups by counting alleles and calculating sample proportions. Allele frequency esti-
Figure. Example of APOE alleles from 12 different people. The three major alleles of APOE differ by single nucleotide substitutions within the amino acid codons at positions 112 (Cys→Arg; e3→e4) and 158 (Arg→Cys; e3→e2). These sequence differences can be demonstrated by using PCR to amplify DNA obtained from blood samples. After the alleles are amplified from blood DNA, a restriction enzyme can cut the PCR product DNA so that the substitutions can be recognized on an autoradiogram following gel electrophoresis. This enzyme, HhaI, cuts the PCR product of e3 to generate 91-bp and 48-bp fragments (lane 1, e3 homozygote). The fragment sizes in base pairs (bp) are shown to the left. Fragments of 72-bp and 48-bp are produced in e4 homozygotes (lane 6). The rare allele, e2, produces a 91-bp and 83-bp doublet, and is illustrated in heterozygotes in lanes 8 and 12. The presence of the 83-bp fragment makes e2 recognition easier. The absence of the 72-bp fragment in lane 12 distinguishes the e2/e3 genotype from the e2/e4 genotype in lane 8. In this assay, the 244-bp PCR fragment also yields constant, smaller HhaI cleavage fragments that are irrelevant to the genotyping and are not illustrated.

Table. APOE €4 allele frequency estimates

<table>
<thead>
<tr>
<th>Population†</th>
<th>€4 allele*</th>
<th>Z†</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal controls</td>
<td>0.16 ± 0.027</td>
<td>0.027</td>
<td>0.000017</td>
</tr>
<tr>
<td>CEPH (182)</td>
<td>0.10 ± 0.046</td>
<td>0.59</td>
<td>0.56</td>
</tr>
<tr>
<td>Spouses§ (42)</td>
<td>0.14 ± 0.008</td>
<td>0.71</td>
<td>0.48</td>
</tr>
<tr>
<td>Menzel� (2,000)</td>
<td>0.42 ± 0.058</td>
<td>4.30</td>
<td>0.000017</td>
</tr>
<tr>
<td>Alzheimer’s LOAD‡ (72)</td>
<td>0.19 ± 0.069</td>
<td>0.40</td>
<td>0.69</td>
</tr>
<tr>
<td>Clinical sporadic AD** (138)</td>
<td>0.36 ± 0.042</td>
<td>4.17</td>
<td>0.00031</td>
</tr>
<tr>
<td>Autopsy sporadic AD†† (352)</td>
<td>0.40 ± 0.026</td>
<td>6.49</td>
<td>&lt;0.00001</td>
</tr>
</tbody>
</table>

* Number of chromosomes counted is presented in parentheses; allele frequency estimates ± SE.
† Z values are versus the CEPH control group.
‡ Ninety-one unrelated grandparents from the Centre d’Etude du Polymorphisme Humain (CEPH).
§ Spouse controls in the Bryan ADRC Memory Disorders Clinic, Duke University.
¶ One randomly selected affected individual from each of 32 Duke and four Toronto/Boston late-onset AD families.
# One randomly selected affected individual from each of 13 Toronto/Boston and three Duke early-onset AD families.
** Thirty-nine Duke and 30 Toronto/Boston sporadic (probable) AD patients.
†† One hundred forty-three Duke and 33 Toronto/Boston autopsy-confirmed sporadic AD subjects.

Results. The figure illustrates an autoradiograph from individuals with several different APOE genotypes. These restriction isotyping methods have been used in many laboratories for several years, especially in studies relating to atherosclerosis and heart disease.24,25

The table illustrates the APOE €4 allele frequency estimates in three control populations: (1) 91 grandparents from the CEPH reference families, (2) 21 spouses from an ongoing prospective study examining consecutive sporadic, probable AD patients, and (3) a representative control series from a similar population in the literature.32 Also illustrated are the APOE €4 allele frequency estimates for several different Alzheimer’s disease
groups: (1) one randomly selected affected individual in the combined Duke and Toronto/Boston late-onset FAD series; (2) one randomly selected affected individual in the combined Toronto/Boston and Duke early-onset FAD families; (3) banked DNA samples from sporadic patients carrying the diagnosis of probable AD from the Duke and Toronto/Boston clinics; and (4) DNA from 176 autopsy-confirmed sporadic AD patients from Duke and Toronto/Boston.

As previously reported,\(^{13}\) the \(e^4\) allele frequency of the randomly selected affected patients in the predominantly late-onset FAD families was significantly different from that of the CEPH controls: 0.50 \(\pm\) 0.06 versus 0.16 \(\pm\) 0.027 (allele frequency estimate \(\pm\) SE), \(Z = 2.44, p = 0.014\). Likewise, the combined Duke and Toronto/Boston late-onset FAD series presented here is significantly different from the CEPH controls (\(p = 0.000017\)). The \(e^4\) frequency of the combined early-onset FAD series from Toronto/Boston and Duke (0.19 \(\pm\) 0.069) did not differ significantly (\(p = 0.69\)) from the frequency in the CEPH controls. Statistical analyses demonstrated highly significant differences in the \(e^4\) allele frequencies in both sporadic (probable) AD (\(p = 0.00031\)) and autopsy-confirmed sporadic AD (\(p < 0.000001\)) when compared with the CEPH controls.

We also examined the association of the 3.5-kb Taq I restriction fragment length polymorphism of APOCII with FAD in the same randomly chosen affected individuals from the Duke pedigrees who previously were shown to have a significantly higher frequency of the \(e^4\) allele.\(^{13}\) We did not find a statistically significant association of the 3.5-kb allele with FAD in these families (allele frequency = 0.52 \(\pm\) 0.069; \(Z = 1.46, p = 0.14\)) when compared with the frequency in a literature control population (0.41 \(\pm\) 0.03).\(^{26,39}\)

**Discussion.** We previously demonstrated a statistically significant association between the APOE \(e^4\) allele and late-onset FAD\(^{13}\) and have now extended this association to sporadic AD. The initial data were derived from a randomly selected affected individual in each of 30 families with FAD;\(^{13}\) that series contained late-onset FAD families used for association/linkage studies of chromosome 19 markers as well as two early-onset FAD families in our series. One of the early-onset FAD families had been linked to chromosome 14.\(^{6,11}\) The other was reported in the original description of the APP717Val\(\rightarrow\)Ile mutant.\(^2\)

Several points are illustrated in the table. First, the CEPH grandparent control group reflects the APOE \(e^4\) allele frequency observed in other published populations of the same race and ethnicity.\(^{3,34-38}\) Second, the \(e^4\) allele frequency in the ongoing series of spouse controls from the Bryan ADRC Clinic is not significantly different from that of the CEPH controls even though the number of chromosomes counted is relatively small. There was a highly significant association of APOE \(e^4\) in each of the late-onset AD groups, including the combined Duke/Toronto/Boston families, the clinical sporadic AD patients, and the combined sporadic AD autopsy groups. In contrast, there was no significant difference from controls in the combined early-onset FAD groups.

The Toronto/Boston series of FAD families represents a primarily early-onset group that has been robustly linked to a locus on chromosome 14.\(^{11}\) The early-onset families linked to chromosome 14 are not associated with an increase of the \(e^4\) allele, which suggests a genetic locus on chromosome 14 that is sufficient for expression of the AD phenotype at an early age and that segregates as a typical autosomal dominant trait. Schellenberg et al\(^{28}\) reported an association of an APOCII 3.5-kb Taq I polymorphism with predominantly early-onset FAD families. APOCII is also on chromosome 19,\(^{14}\) within 50 kb of APOE.\(^{37}\) We found no significant association of this polymorphism with our predominantly late-onset FAD families. The significance of the Schellenberg et al\(^{28}\) data in their families remains to be interpreted.

Published data using the APM method of linkage analysis found a highly significant association of multiple contiguous chromosome 19q markers with late-onset FAD.\(^{12}\) However, it could not be determined at that time whether these results indicated true linkage or association; APOE data were not included in the original analyses. Standard likelihood analysis of these data, assuming a single-gene autosomal dominant model of inheritance, was suggestive but could not firmly establish linkage to this region. APM analysis of APOE and the FAD in this communication also resulted in highly significant results (\(p < 0.001\)) for linkage/association between APOE and AD. Lod score analysis, although suggestive, is not significant (\(Z = 2.4\) at a maximum recombination fraction of 0.10).

Clinicians routinely accept the contributions of multiple factors in the development of a disease. Words such as “susceptibility” and “predisposition” are used to describe why some individuals develop disease when others do not. In the field of atherosclerosis, the contribution of multiple loci to the development of a particular phenotype, such as coronary artery disease, is well defined, and atherosclerosis accepted as a complex genetic disease.\(^38\) In fact, locus heterogeneity of FAD is now well documented.\(^{2,10-12}\) Our data support the view that late-onset AD can also be viewed as a complex genetic disease, clustered in families that segregate the APOE \(e^4\) allele. The extension of the data from the late-onset FAD families to the population of apparently sporadic AD patients suggests an important functional role for the \(e^4\) allele in the pathogenesis of AD. The \(e^4\) allele may be viewed as a susceptibility gene or risk factor for AD that can be tested in properly constructed epidemiologic studies.

Multiple genes can apparently contribute to sim-
ilar phenotypic expression in AD. Some, like APOE e4, may contribute to increased susceptibility, while others may be sufficient to express the disease. In the presence of the APP mutations, the rate of pathogenesis of the disease in these families is apparently increased, leading to an earlier age of onset. In these families, the disease segregates as a classic autosomal dominant trait. The finding of linkage for early-onset FAD on chromosome 14q will presumably lead to the identification of another locus sufficient to accelerate the rate of disease expression. We continue to screen the late-onset FAD pedigrees for other associations and expect that other susceptibility loci will be identified.

Association of late-onset FAD with a contiguous series of polymorphic markers on chromosome 19 suggested that evaluation of potential candidate genes from this region might be a productive research strategy. Antisera to ApoE demonstrate the presence of ApoE in plaques, neurofibrillary tangles, and vascular amyloid in AD. ApoE immunoreactivity is also present in the plaques of Creutzfeldt-Jakob disease, scrapie, Down's syndrome, and other cerebral and systemic amyloidoses, and ApoE fragments are associated with human amyloid A protein. These data suggest a broad role for ApoE in diseases in which amyloids are deposited. With the recent finding that amyloid β-peptide may be a normal cellular product, the metabolism of normally produced β-peptide becomes important. The deposition of β-amyloid in AD can be viewed as a pathologic sign that is used to define AD. Thus, faulty mechanisms of clearance or sequestration of β-amyloid may occur during the development of AD. Studies with purified ApoE-E3/3 and ApoE-E4/4 have demonstrated a difference in the rate of binding to amyloid β-peptide. Bridging the gap between genetic data and relevant pathogenetic interactions may dissect the interactions that can be targeted for rational therapeutic interventions.

Late-onset FAD can be recognized only in families that are large enough to ascertain and test. In contrast, the diagnosis of “sporadic AD” is used when there is no family history. This distinction is confounded by family size, age of onset of disease, and age at death of family members. If, in fact, discrimination between late-onset FAD and sporadic AD is a function of family size and longevity, then data developed in the late-onset FAD subgroup should be applicable to sporadic AD patients. The association with APOE e4 supports this view and provides a rationale for using the late-onset FAD pedigrees to search for other associated genetic regions.

ApoE is the major apolipoprotein in brain. While its functional role with lipid transport is well described, we have proposed a broader role for ApoE in the metabolism, transport, and targeting of amyloid β-peptide. Differential metabolism of ApoE-E3 and ApoE-E4 isoforms may lead to differences in β-peptide sequestration, particularly in the brain parenchyma and cerebral blood vessels. Studies of the specific molecular interactions of amyloid β-peptide with ApoE-E3/3 and ApoE-E4/4 isoforms are currently in progress. The combined weight of linkage/association, immunohistochemistry, and binding data in late-onset FAD and sporadic AD, as well as the published immunochemical studies in scrapie and Creutzfeldt-Jakob disease, should provide an impetus to examine the functional roles of APOE isoforms in late-onset AD.

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