Evidence for an Alzheimer Disease Susceptibility Locus on Chromosome 12 and for Further Locus Heterogeneity

Ekaterina Rogaeva, PhD; Smita Premkumar, PhD; Youqiang Song, PhD; Sandro Sorbi, MD; Nicholas Brindle, MB BCH, MRCP; Andrew Paterson, MD, FRCP; Ranjan Duara, MD; Georges Levesque, PhD; Gang Yu, PhD; Masaki Nishimura, MD, PhD; Masaki Ikeda, MD; Colleen O'Toole, BA; Toshitaka Kawarai, MD, PhD; Ricardo Jorge, MD, PhD; Daniel Vilarino, MD; Amalia C. Bruni, MD; Lindsay A. Farrer, PhD; Peter H. St. George-Hyslop, MD, FRCP

Context.—Alzheimer disease (AD) susceptibility genes have been identified on chromosomes 1, 14, 19, and 21, and a recent study has suggested a locus on chromosome 12.

Objective.—To confirm or refute the existence of a familial AD susceptibility locus on chromosome 12 in an independent sample of familial AD cases.

Design.—Retrospective cohort study. DNA data for 6 chromosome 12 genetic markers were evaluated using parametric lod score and nonparametric linkage methods and linkage heterogeneity tests. The latter include the admixture test of homogeneity in the total group of families and the predivided sample test in families stratified by the presence or absence of an apolipoprotein E (APOE) ε4 allele among affected members. Parametric analyses were repeated assuming autosomal dominant inheritance of AD and either age- and sex-dependent penetrance or zero penetrance for the analysis of unaffected relatives.

Setting.—Clinical populations in the continental United States, Canada, Argentina, and Italy.

Patients.—Fifty-three white families composed of multiple members affected with AD, from whom DNA samples were obtained from 173 patients with AD whose conditions were diagnosed using established criteria and from 146 nondemented relatives.

Main Outcome Measure.—Presence of an APOE ε4 allele among affected family members.

Results.—Using parametric methods, no evidence for linkage to the region spanned by the chromosome 12 markers could be detected if familial AD is assumed to arise from the same genetic locus in all 53 families. However, significant evidence for linkage was detected in the presence of locus heterogeneity using the admixture test (odds ratio, 15, 135:1). The estimated proportion of linked markers was 0.40 and 0.65, depending on the admixture test of homogeneity in the total group of families and the predivided sample test in families stratified by the presence or absence of an apolipoprotein E (APOE) ε4 allele among affected members. Parametric analyses were repeated assuming autosomal dominant inheritance of AD and either age- and sex-dependent penetrance or zero penetrance for the analysis of unaffected relatives.

Conclusions.—Our data provide independent confirmation of the existence of an AD susceptibility locus on chromosome 12 and suggest the existence of AD susceptibility genes on other chromosomes. Screening a larger set of families with additional chromosome markers will be necessary for identifying the chromosome 12 AD gene.

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comparatively rare causes that are inherited as classical autosomal dominant traits with age-dependent penetrance. A more common genetic risk factor for AD is the e4 allele of apolipoprotein E (APOE). Multiple studies have revealed that the e4 allele is disproportionately represented among patients with both late-onset and early-onset AD and that the e4 allele shows a dose-dependent relationship with increasing risk for AD and decreasing age at onset. Conversely, several studies have suggested that inheritance of the e2 allele may be protective. A recent meta-analysis of more than 14,000 patients with AD and controls demonstrated that the e4 allele represents a major risk factor for AD in both men and women from a large number of racial and ethnic groups across all ages between 40 and 90 years. The genetic risk of AD attributable to APOE e4 has been estimated to be 45% to 60%.

In another study, a genome-scan approach has generated preliminary evidence of a putative new locus for familial late-onset AD within an approximately 30-cM region on chromosome 12. In the current study, we reexamined the segregation of 6 polymorphic chromosome 12 markers from this region in an independent pedigree data set.

METHODS

Subjects

Fifty-three families consisting of multiple members affected with AD were recruited from clinics at the University of Toronto, Toronto, Ontario, University of Florence, Firenze, Italy, and other academic clinics in North America, South America, and Europe. (These pedigrees do not overlap with those reported previously to show linkage to chromosome 12.) Our data set included 173 patients with AD and 146 nonaffected at-risk relatives from whom DNA samples had been obtained (Table 1).

In these families, the diagnosis of AD was made by a qualified specialist using established diagnostic criteria. DNA from at least 1 affected member of each family was screened for mutations in exon 16 or 17 of βAPP, for mutations in the open reading frame of PS1, and for at least the asparagine 141 isoleucine (Asn141Ile) and methionine 219 valine (Met219Val) mutations in PS2 using methods previously described. No defects in these genes were found in any of these DNA specimens.

Genotype Analysis With Chromosome 12 Markers

Genomic DNA was prepared from buffy-coat leukocytes or from Epstein-Barr-transformed lymphoblast cultures as previously described. One hundred nanograms of genomic DNA from each available family member was amplified by radiolabeled polymerase chain reaction (PCR) as previously described, using the oligonucleotide primers and the PCR conditions recommended for the D12S358, D12S73, D12S1057, D12S1042, D12S1090, and D12S96 in the Collaborative Human Linkage Centre database (available at http://www.chlc.org). The radiolabeled PCR products were resolved on 6% denaturing polyacrylamide gels, which were blotted to Whatman filters and exposed to autoradiographic film, and the genotype of each individual was inferred from the resultant autoradiographic band pattern. To ensure consistency of allele scoring, all PCR products for members of each family were run on the same gel, and each gel contained several CEPH standards for cross-comparison between different gels.

Statistical Analysis

The hypothesis for the existence of an AD gene on chromosome 12 was evaluated by a multilocus linkage approach using parametric (lod score) and nonparametric methods. Parametric methods require specification of a genetic model and may therefore be less powerful for detection of linkage than nonparametric methods (eg, affected sib-pair or affected relative approaches) when assumptions about the mode of inheritance or penetrance are inaccurate. However, parametric lod score analysis facilitates the evaluation of hypotheses of heterogeneity without precluding the families by some a priori criterion, such as onset age or APOE genotype. Furthermore, lod score methods can be effective even when the gene frequency and penetrance are incorrectly specified as long as assumptions about dominance are correct, especially in a hypothesis-driven linkage replication study.

In the parametric analyses, AD was modeled as an autosomal dominant trait with a mutant allele frequency of 0.001. One set of analyses allowed for the possibility that unaffected children of AD patients may harbor a mutant gene but have not yet manifested the disease because of their age and sex. In this approach, age- and sex-dependent penetrance was defined as a step function based on 17 age intervals derived from censored data distributions for early-onset (mean family onset age, ≤65 years) and late-onset (mean family onset age >65 years) families. Because the age correction function may not account for nonpenetrance in some persons, a second set of analyses was carried out by assigning a constant low penetrance (0.02) to all unaffected at-risk individuals. This represents a conservative “affected only” analysis in which unaffected individuals provide minimal information with regard to the disease, but are important for linkage phase determination with respect to marker data.

We also applied the nonparametric linkage (NPL) approach of Kruglyak et al, which considers the proportion of marker alleles identical by descent among affected relatives. The computed score, z, which captures the allele sharing among all permutations of pairs in a set of affected relatives, follows a normal distribution with a mean of 0 and a variance of 1 under the null hypothesis of no linkage. The z scores were combined across pedigrees by taking a linear combination, where m is the number of pedigrees, z denotes the normalized score for the ith pedigree, and the yγi are weighting factors. In the absence of criteria for choosing an optimal scheme for defining γi, we assigned equal weight to each pedigree such that γi = 1/m.

Support for linkage (ie, lod score) in various intervals of the linkage map of chromosome 12 was evaluated by multilocus linkage analysis using the GENEHUNTER program. These multilocus lod scores, also known as location scores, were calculated from simultaneous consideration of the disease locus and all 6 marker loci. Marker order and allele frequencies were obtained from the Collaborative Human Linkage Center database. The following genetic map was used: pter — D12S358 — (18.5 cM) — D12S873 — (2.9 cM) — D12S1057 — (3.2 cM) — D12S1042 — (21.7 cM) — D12S1090 — (20.7 cM) — D12S96 — qter.

To test for genetic heterogeneity, we used 2 different statistical tests. First, we used the predivided sample test. This test makes the assumption that a genetic condition can be divided a priori into 2 different classes based on an external criterion. We used the criterion of

<table>
<thead>
<tr>
<th>Sample Characteristics</th>
<th>No.</th>
</tr>
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<tbody>
<tr>
<td>Total subjects</td>
<td>173</td>
</tr>
<tr>
<td>Patients with Alzheimer disease</td>
<td>146</td>
</tr>
<tr>
<td>Patients per family, mean (range)</td>
<td>3.3 (1-6)</td>
</tr>
<tr>
<td>At-risk persons per family, mean</td>
<td>2.7 (0-19)</td>
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<tr>
<td>Sibship size*</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>41</td>
</tr>
<tr>
<td>2</td>
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<tr>
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<tr>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
</tr>
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<td>Sibships with an affected parent</td>
<td>10</td>
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<tr>
<td>Extended kindreds</td>
<td>22</td>
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</table>

*Affected sampled sibs only.
APOE e4 status to divide the families into 2 classes. Families were considered e4-positive (e4+) if at least 75% of the genotyped affected members had at least 1 e4 allele, and e4-negative (e4−) otherwise. In our sample of 53 families, 30 were e4+ and 23 were e4−. Among the e4+ families, 4 families had 1 patient with AD each lacking an e4 allele and would therefore have been classified as e4− using the criteria for the APOE split used by Pericak-Vance et al.15

We also assessed the possibility of linkage in the presence of heterogeneity using Smith’s admixture test implemented in the HOMOG program.25,26 This test does not require assumptions about differences between linked and unlinked families and evaluates evidence for linkage and heterogeneity simultaneously. Comparisons of the null hypothesis of no linkage (H0), the alternate hypothesis of linkage homogeneity (H1), and the hypothesis of linkage with heterogeneity (H2: α=1), for the location (lod) scores computed from the parametric analyses were carried out as likelihood ratio tests with P values calculated from the asymptotic χ² distribution with 1 or 2 df. To test the hypothesis of linkage homogeneity (ie, heterogeneity given linkage), we compared H2 with H0. Since this test assumes linkage, we also tested the hypothesis of linkage and heterogeneity (ie, linkage given heterogeneity) by comparing H2 with H1. However, this test attempts to declare linkage while allowing for heterogeneity as a sort of nuisance parameter, thus leading to a nonconservative test for linkage. Moreover, the recombination frequency parameter, θ, disappears under H2, because α = 0 in this hypothesis, which leads to a problem with the asymptotic distribution of the likelihood ratio having 1 parameter under H0 and 2 parameters under H2. Therefore, it has been recommended that the χ² statistic should not be applied in this situation but rather that the criterion of a likelihood ratio test exceeds the threshold for suggestive linkage. However, because for replication studies such as this a P value of .01 is needed for an interval-wide significance of 5%,2 the likelihood of achieving the observed score at random in this limited genomic region is small.) Similar results were obtained in the subset of e4− families. Evidence for linkage in the subset of e4− families at D12S96 did not reach statistical significance (P = .08).

RESULTS

Patients with AD had a mean (SD) age at onset of 70.5 (10.7) years (range, 33-89 years). The mean (SD) censoring age of the unaffected relatives was 48.8 (16.7) years (range, 15-80 years). On average, each family contained approximately 3.3 affected and 2.7 at-risk members from whom DNA samples were obtained. More than half (62/173) of the patients with AD were members of sibships having 3 or more affected members from whom DNA was collected. In more than 40% of the families, DNA samples were collected from affected members from outside the nuclear family of the proband. In 3 families, DNA was studied from affected members in 3 generations.

When the data were analyzed by parametric methods assuming that the cause of AD in all 55 pedigrees arose from a single homogeneous locus, there was no evidence for linkage to the region spanned by the chromosome 12 markers, regardless of whether an age- and sex-dependent penetrance (Figure 1) or an affecteds only model (Figure 2) was used. When the families were predisposed according to their predominant APOE e4 status and analyzed using the reduced penetrance model, e4+ and e4− families were heterogeneous with respect to linkage to chromosome 12 (χ² = 6.03, P = .02), with suggestive evidence for linkage (maximum lod, score 2.0) at D12S586 in the subset of e4+ families (Figure 1). However, this result is inconclusive because linkage was not demonstrated statistically in either group of families.

By contrast, NPL analysis of the entire data set revealed significant evidence for linkage in the region between D12S358 and D12S373 and in the region between D12S1090 and D12S96 (Figure 3). The strongest evidence was obtained precisely at D12S96 (P < .001). (Based on the recommendations of Lander and Kruglyak20 for significance of linkage results obtained in a genome scan, this result exceeds the threshold for suggestive linkage. However, because for replication studies such as this a P value of .01 is needed for an interval-wide significance of 5%,2 the likelihood of achieving the observed score at random in this limited genomic region is small.) Similar results were obtained in the subset of e4+ families. Evidence for linkage in the subset of e4− families at D12S96 did not reach statistical significance (P = .08).

To more fully explore the possibility of linkage in the presence of heterogeneity, the parametric lod scores from all families in our data set were evaluated using the admixture test.25,26 Despite significant evidence for heterogeneity of lod scores in all strata of families analyzed under the affected persons only model, linkage to chromosome 12 could not be demonstrated conclusively because none of the odd ratios (ORs) were greater than 2000:1 (Table 2). However, when the overall data set was examined without prior stratification under the model of age- and sex-dependent penetrance, there was significant evidence (P < .001) for the existence of both a subset of pedigrees with linkage to chromosome 12 and a subset of pedigrees un-
linked to these markers. The associated OR of 15 (135:1 favoring linkage), which is equivalent to a lod score of 4.2, exceeds the suggested threshold of 2.0001. Significant evidence for linkage in the presence of heterogeneity was also obtained in the subset of e+ families (OR, 9120:1) but not in the subset of e− families. These results are still statistically significant after applying the Bonferroni correction to adjust for the multiple models and strata being tested.

**COMMENT**

Our data provide independent confirmation of the existence of an AD susceptibility locus on chromosome 12. Moreover, our data suggest that other familial AD susceptibility loci may exist in addition to the present locus on chromosome 12 and the known mutations/polymorphisms within the open reading frames of BAPP, APOE, PS1, and PS2.

Our results are based on parametric lod score analysis and NPL analysis of multiple linked markers. Using the parametric approach, evidence for linkage to chromosome 12 was strongest in the analyses including data from asymptomatic at-risk family members. Similar trends were evident in the relatively conservative affecteds only analyses but were not significant, probably because of reduced power. We elected to use parametric methods because they permit assessment of linkage heterogeneity without dividing families a priori on the basis of a measurable characteristic. This aspect is an important consideration for designing searches of additional AD loci.

It is noteworthy that we detected linkage to chromosome 12 in the presence of heterogeneity using NPL analysis. In fact, both this approach and the heterogeneity analysis of the parametric lod scores indicated the strongest evidence for linkage near D12S96, although this location was not statistically better than other locations. The NPL approach (Figure 3) yielded significant evidence for linkage, whereas results were unequivocally negative using the affecteds only approach (Figure 2). Given the absence of parental genotype data in all but a few cases, both approaches are essentially assessing the degree of sharing alleles identical by descent among affected relatives in this situation. One notable difference between the 2 approaches is the relative contribution of individual pedigrees to the summary linkage statistic. In the parametric approach, lod scores are dependent on the number of informative meiotic events and summed across pedigrees. The summary statistic for the NPL analysis was weighted by the number of pedigrees. Therefore, families with a large number of recombinants (or a low degree of allele sharing) are more likely to overshadow the evidence from other families without recombinants (or a high degree of allele sharing) in a lod score analysis compared with the NPL approach. Our study suggests that NPL tests can viably detect linkage in the presence of moderate heterogeneity because there is still an excess of allele sharing if some pedigrees contain an excess and allele sharing is random in others; however, parametric methods are still necessary to quantify heterogeneity without using potentially flawed a priori criteria.

The current data set, although confirming linkage to the general region of chromosome 12 containing the markers D12S558 to D12S96, does not provide the precise localization necessary for positional cloning strategies (the 95% confidence interval includes the entire 67-cM interval between D12S558 and D12S96). This data set also does not allow a precise estimate of the true proportion of pedigrees linked to chromosome 12 (varies between 0.4 and 0.65 depending on the stratum and penetrance assumptions). These ambiguities arise from the limited informativeness of many families with the current markers and from the inability to define an a priori linked subgroup of families. Evidence for linkage to chromosome 12 in the previous report was derived almost entirely from the subset of e+ families. However, our data raise the possibility that e− status may not be an accurate predictor of chromosome 12 linkage status. This conclusion would not be changed by reclassifying as e− families the 4 families in our data set labeled as e+ that had several e+ patients as well as 1 patient with e+e− genotype. A reasonable strategy for both refining the estimate of linked pedigrees and for narrowing the minimal cosegregating region will be to investigate the segregation of the numerous additional markers available from this region of chromosome 12 to increase the informativeness of these pedigrees.

All 4 of the previously characterized AD susceptibility genes appear to have some functional relationship to the pathologic processing of βAPP either through increasing production of Aβ peptides or through sequestration of Aβ peptides in the extracellular space. It is unclear whether these biochemical effects are central to the neurodegenerative process or are simply a secondary epiphenomenon arising from another more important biochemical pathway leading to neurodegeneration. However, bearing this caveat in mind, a potentially useful strategy will be to examine candidate genes on chromosome 12 that might relate to βAPP processing. Several such genes have been mapped in close physical proximity to the polymorphic markers used in this study. One of these genes, low-density lipoprotein receptor-related protein (LRP1), maps distal to D12S1090, near D12S96. This gene, which is expressed in hippocampal neurons (a prominent site of AD pathology), is a plausible candidate gene for familial AD because it is receptor for APOE and a putative receptor for clearance of extracellular βAPP. Nevertheless, recent genetic association studies have yielded conflicting results. Thus, 3 analyses suggest associations between AD and different alleles of LRP1 whereas a fourth analysis, conducted in the data set showing evidence of linkage to chromosome 12, failed to detect an association between AD and LRP1. It is evident that direct nucleotide sequencing of the opening reading frame of this gene (and also of any other candidate gene) in affected individuals of families with a high posterior probability of linkage of chromosome 12 and no recombination events at the candidate gene locus.

In agreement with the previous study, families with a high posterior probability of linkage to chromosome 12 are characterized by an age of onset between 60 and 80 years and multiple affected members in several generations. This observation has 2 implications. First, it suggests that AD caused by a mutation or variant at the chromosome 12 locus may be transmitted as an auto-

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**Table 2.—Linkage and Heterogeneity Test Results**

<table>
<thead>
<tr>
<th>Genetic Model</th>
<th>Family Group</th>
<th>Test of H₂ vs H₁</th>
<th>Test of H₁ vs H₀</th>
<th>Maximum Likelihood Estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>χ²</td>
<td>P</td>
<td>α (95% CI)</td>
</tr>
<tr>
<td>Age- and sex-dependent Total</td>
<td>15135:1</td>
<td>12.93</td>
<td>&lt;.001</td>
<td>.55 (.25-.85)</td>
</tr>
<tr>
<td>e+</td>
<td>9120:1</td>
<td>8.85</td>
<td>.002</td>
<td>.65 (.25-.95)</td>
</tr>
<tr>
<td>e−</td>
<td>20:1</td>
<td>2.98</td>
<td>.04</td>
<td>.65 (.05-.10)</td>
</tr>
<tr>
<td>Affected persons only Total</td>
<td>631:1</td>
<td>12.92</td>
<td>&lt;.001</td>
<td>.40 (.15-.75)</td>
</tr>
<tr>
<td>e+</td>
<td>1202:1</td>
<td>13.88</td>
<td>&lt;.001</td>
<td>.55 (.15-.85)</td>
</tr>
<tr>
<td>e−</td>
<td>23:1</td>
<td>6.30</td>
<td>.006</td>
<td>.55 (.05-.95)</td>
</tr>
</tbody>
</table>

*Hypothesis: H₂ indicates linkage and heterogeneity; H₁, linkage and homogeneity; and H₀, no linkage. †indicates proportion of linked families; η, chromosomal map position; and CI, confidence interval."
somal dominant trait with incomplete but age-dependent penetrance. Second, our results suggest that the examination of simple sib-pair family structures may not be an efficient strategy for isolation of the chromosome 12 gene either because the current markers are not sufficiently close to the actual chromosome 12 locus to allow detection of linkage in sib-pair families or because other loci may be more important in the genesis of AD in these types of families.

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References