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ORIGINAL ARTICLE

Covariate analysis of late-onset Alzheimer disease refines the chromosome 12 locus

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Alzheimer disease (AD) is a progressive neurodegenerative disorder of later life with a complex etiology and a strong genetic component. Several genomic screens have suggested that a region between chromosome 12p13 and 12q22 contains at least one additional locus underlying the susceptibility of AD. However, localization of this locus has been difficult. We performed a 5 cM microsatellite marker screen across 74 cM on chromosome 12 with 15 markers in 585 multiplex families consisting of 994 affected sibpairs and 213 other affected relative pairs. Analyses across the entire data set did not reveal significant evidence of linkage. However, suggestive linkage was observed in several subsets. In the 91 families where no affected individuals carry an ApoE ε4 allele, an HLOD score of 1.55 was generated at D12S1042. We further examined the linkage data considering the proposed linkages to chromosome 9 (D9S741) and chromosome 10 (α-catenin gene). There was a modest ($P = 0.20$) increase in the LOD score for D12S368 (MLOD = 1.70) when using the D9S741 LOD scores as a covariate and a highly significant ($P < 0.001$) increase in the MLOD score (4.19) for D12S1701 in autopsy-confirmed families ($n = 228$) when using α-catenin LOD scores as a covariate. In both cases, families with no evidence of linkage to D9S741 or α-catenin demonstrated most of the evidence of linkage to chromosome 12, suggesting locus heterogeneity. Taken together, our data suggest that the 16 cM region between D12S1042 and D12S368 should be the subject of further detailed genomic efforts for the disease.
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Introduction

Alzheimer disease (AD) is a devastating neurodegenerative disorder of late life with complex inheritance. It is the leading cause of dementia in the elderly and the most common form of dementia occurring after the age of 40. There are over 4.5 million individuals with AD in the US.[1] This number is projected to quadruple over the next 50 years as the population ages. Clinically, AD is slowly progressive, resulting in memory loss and alterations of higher intellectual function and cognitive abilities.[2] Pathologically AD is characterized by neurofibrillary tangles in the neurons of the cerebral cortex and hippocampus and the deposition of amyloid within senile plaques and cerebral blood vessels.[3]

Increasing evidence demonstrates that the etiology of AD is a complex web of genetic and environmental factors. A rare Mendelian subform with early onset exhibits autosomal dominant inheritance and both locus and allelic heterogeneity. This form can be caused by any of over 120 mutations in three genes encoding β-amyloid precursor protein (APP), presenilin 1, and presenilin 2.[4–7] These mutations all affect APP metabolism such that more Aβ42 peptide, found in senile plaques, is produced.

In contrast, late-onset Alzheimer disease (LOAD) shows familial clustering, but does not demonstrate a clear Mendelian mode of inheritance. The only locus universally accepted as an important risk factor for LOAD is the apolipoprotein E (ApoE) gene on chromosome 19.[8,9] However, more than 50 percent of AD cases do not carry an ApoE ε4 allele, suggesting that other risk factors exist. Efforts to identify these additional loci have largely taken two approaches: whole-genome scans for linkage in multiplex families, and association tests of candidate genes in case-control samples. Numerous studies have tested over 150 functional candidate genes and over 70 such genes have reported positive associations in at least one study, including α-antichymotrypsin, low-density lipoprotein receptor-related protein (LRP),

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presenilin-1 (PS1), ubiquitin, the human leukocyte antigen (HLA) complex, mitochondrial mutations, and angiotensin converting enzyme (ACE).[10] However, none of these additional loci have been consistently replicated in multiple study populations.

Various genome screens have implicated numerous chromosomes (e.g. 2, 9, 10, 12, and 15) as potential locations of additional AD loci.[11–13] It is likely that AD susceptibility is the result of multiple genes acting either independently or interactively in their contribution to overall risk.[11,12,14–16] One of the most consistently identified regions is between chromosome 12p13 and 12q22.[17] However, the implicated region is still broad and provides an impractically large area for a molecular genetic search. To determine the most likely location of an AD susceptibility gene, we performed a 5 cM microsatellite marker screen across 74 cM on chromosome 12 for LOAD with 15 markers in 585 multiplex families. We hypothesized that at least some of the difficulty in localization results from locus heterogeneity. We therefore used autopsy confirmation, ApoE genotypes, and chromosome 9 and chromosome 10 linkage data to define more homogeneous subgroups. Our data suggest that the region likely to harbor an AD gene is a 16 cM interval, which should be the subject of further detailed genomic efforts for the disease.

Materials and methods

Families

We used a total of 585 multiplex families with LOAD defined as having a family mean age at onset (AAO) ⩾60 years (Table 1). Family data were ascertained by the following centers: the NCRAD repository at Indiana University (NCRAD); the Collaborative Alzheimer Project (CAP), including Duke and Vanderbilt Universities and University of California at Los Angeles; and the National Institute of Mental Health repository (NIMH). The 585 multiplex families include 994 affected sibpairs and 213 other affected relative pairs. In all data sets, affected individuals were classified as probable or possible AD in accordance with NINCDS-ADRDA clinical diagnostic criteria.[18] There were 228 families in the data set with at least one individual diagnosed with AD by autopsy.

Table 1 Alzheimer disease study population

	Multiplex families (n = 585)				*Autopsy-confirmed families (n = 228)*			
	Total	*NCRAD*	*CAP*	*NIMH*	*Total*	*NCRAD*	*CAP*	*NIMH*
Multiplex families	585	129	84	372	228	71	26	131
Affected individuals	1435	334	249	852	608	187	93	328
Unaffected individuals	810	192	286	332	340	91	94	155
Affected sibpairs (ASP)	994	264	190	540	488	161	87	240
Affected relative pairs (ARP)	213	40	111	62	113	23	53	37

DNA analysis

Genomic DNA was obtained from the repositories (NIMH, NCRAD) or extracted from whole blood (CAP) by use of the Puregene system (Gentra Systems, Minneapolis, MN, USA). Microsatellite markers were selected on the basis of heterozygosity, ease of genotyping, and location. In total, 15 markers at ∼5 cM intervals were selected and genotyped in all 585 multiplex families (Table 2). Marker order was determined using genetic linkage reference maps.[19] Where order was undetermined, the human genome sequence was used to determine order (http://www.ncbi.nih.gov). Genotyping of microsatellite markers was performed by SybrGold® (Molecular Probes, Eugene, OR, USA) staining and Hitachi Biosystems FMBIOII laser scanning (Brisbane, CA, USA). SNP genotyping (for the α-catenin gene) was performed using Assays-On-Demand™ or Assays-by-Design™ with the ABI7900HT TaqMan system (Applied Biosystems, Foster City, CA, USA). Laboratory personnel were blinded to pedigree structure, affection status, and location of quality control (QC) samples. Systematic genotyping errors were minimized by use of a system of QC checks with duplicated samples.[20]

Table 2 Markers used for investigation of chromosome 12

Marker	*Map location[a] (cM)*	*Physical location[b] (Mb)*
D12S1623	17	6.7920
D12S391	28	12.3412
D12S1303	33	15.5249
D12S373	35	16.9063
D12S1057	45	24.5684
D12S1042	51	27.5386
D12S1090	57	41.1965
D12S1701	62	46.2085
D12S368	67	50.9177
D12S398	67	51.4834
D12S1632	72	54.7017
D12S75	79	67.7842
D12S1722	85	69.2991
D12S92	87	72.4202
D12S326	91	76.4763

[a]The map location is from deCODE map.
[b]The map location is from Ensembl, build 35.

Duplicate QC samples were placed both within and across 96-well plates and equivalent genotypes were required for all QC samples to ensure accurate genotyping. Hardy–Weinberg calculations were performed for each marker and Mendelian inconsistencies were identified using PedCheck.[21] Suspect genotypes were re-read and/or re-run. All microsatellite markers were required to have >90% of possible genotypes. Unlikely double recombinations within the region were identified using SIMWALK v.2.0.[21] If apparent double recombinations were confirmed in multiple readings, the marker data remained in the data set.

Linkage analysis
Two-point heterogeneity LOD score (HLOD) analyses were computed using FASTLINK and HOMOG[22–24] As the mode of inheritance for AD is unknown, affected-only parametric analyses were performed using both autosomal dominant and autosomal recessive models with disease allele frequencies of 0.001 and 0.20, respectively, to model the susceptibility allele. Marker allele frequencies were obtained from the data set by counting all independent chromosomes. Multipoint model-free testing was conducted with ALLEGRO.[25] ALLEGRO uses ARP data to compute an allele-sharing LOD* score, based on an exponential model that uses the S_{pairs} scoring function as recommended by McPeek.[26] Due to computational limitations of the ALLEGRO software, some large pedigrees were trimmed to conduct the analysis. The standards for suggestive scores in the follow-up analyses were a LOD score ⩾1.0 or a P-value ⩽0.05.

Ordered subset analysis
Ordered subset analysis (OSA) was used to test for the presence of heterogeneity.[27] OSA rank orders families by a continuous covariate (such as LOD scores at other locus) and then identifies the subset with maximum evidence for linkage to a particular map of markers. This approach identifies a set of families in which the LOD score in a particular region is higher than in the overall data set. The statistical significance of the increased evidence for linkage relative to evidence for linkage in the entire sample is assessed via random permutation of the order of inclusion of the families. Continuous covariates (LOD score for the SNP in α-catenin and the microsatellite marker D9S741) were used to rank order families to test for a subset generating a significantly increased LOD score relative to the overall sample.

Results

Overall analysis
We performed two-point linkage analysis under both dominant and recessive models. No LOD score met our initial criterion for interest (LOD score ⩾1.0) across all analyses (Table 3). The multipoint analysis also failed to show linkage between markers and the disease (data not shown). Stronger evidence of linkage was seen in the autopsy-confirmed subset (n = 228), where a maximal HLOD score of 1.28 was generated under the dominant model at marker D12S368 (Table 3).

Table 3 Two-point linkage analysis HLOD scores for overall and autopsy-confirmed dataset

Marker	*Overall data set (n = 585)*		*Autopsy-confirmed subset (n = 228)*	
	Dominant model	*Recessive model*	*Dominant model*	*Recessive model*
D12S1623	0.13	0.13	0.61	0.62
D12S391	0.13	0.00	0.07	0.00
D12S1303	0.00	0.00	0.00	0.00
D12S373	0.00	0.00	0.00	0.00
D12S1057	0.13	0.00	0.67	0.13
D12S1042	0.16	0.18	0.19	0.06
D12S1090	0.00	0.32	0.16	0.62
D12S1701	0.00	0.00	0.10	0.31
D12S368	0.00	0.00	**1.28**	**1.22**
D12S398	0.00	0.00	0.00	0.00
D12S1632	0.00	0.00	0.04	0.00
D12S75	0.24	0.14	0.12	0.24
D12S1722	0.00	0.00	0.07	0.00
D12S92	0.00	0.00	0.01	0.00
D12S326	0.14	0.20	0.02	0.12

LOD scores higher than 1.0 are shown in bold.

Controlling for ApoE
Taking into account the known ApoE ε4 association, the stratification by ApoE ε4 status generated an HLOD score of 1.55 at marker D12S1042 in the 91 families where no affected individuals have an ApoE ε4 allele. The maximal HLOD in the 317 ApoE ε4 positive families was 0.68 at marker D12S1090 under the dominant model (data not shown). We also applied the OSA method to analyze both the overall and autopsy-confirmed subsets using the proportion of ApoE ε4 alleles in affected individuals. In the overall data set, D12S1042 generated the highest two-point LOD score of 1.45 (P = 0.16) with ∼17% of families contributing to the score when ordering families by the proportion of ApoE ε4 alleles from low to high (data not shown). In the autopsy-confirmed data set, the highest two-point LOD score was generated at D12S1090 (1.88) in an OSA subset identified when ordering families by the proportion of ApoE ε4 alleles from high to low. This increase was significant (P = 0.04) and the subset represented ∼10% of the autopsy-confirmed families (Figure 1).

Controlling for linkage on chromosomes 9 and 10
The α-catenin gene on chromosome 10 has been suggested to influence Aβ42,[28] and to contribute to the previously reported linkage for plasma Aβ42 in LOAD families.[29] D9S741 also demonstrates linkage

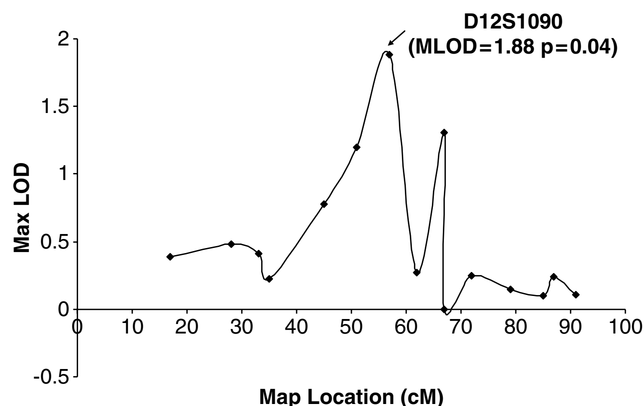

Figure 1 Two-point OSA LOD scores for autopsy-confirmed families using the proportion of ApoE $\varepsilon 4$ alleles (ranked from high to low) as a covariate.

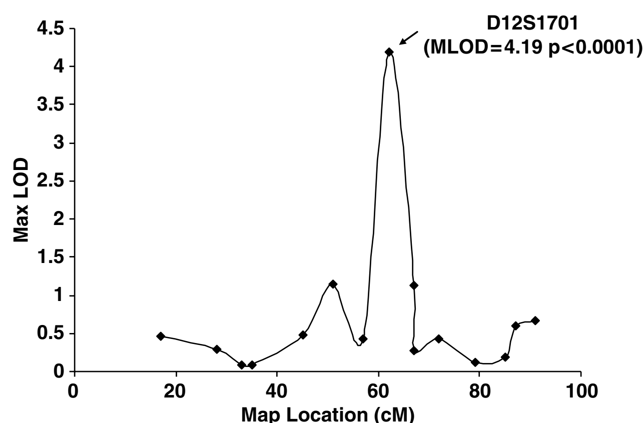

Figure 2 Two-point OSA LOD scores for autopsy-confirmed families using α-catenin LOD scores (ranked from low to high) as a covariate.

with AD.[30] We applied the OSA method to analyze data using LOD scores for α-catenin (SNP RS7070570) and D9S741 as covariates. The highest two-point LOD score was generated at D12S1701 (HLOD = 4.19) when the autopsy-confirmed families were ranked using LOD scores for the α-catenin gene (low to high) (Figure 2). OSA defined a subset of 45 families representing ~21% of the autopsy-confirmed families. This increase was highly significant ($P < 0.0001$).

Interestingly, marker D12S368 (~5 cM away from D12S1701) generated a maximum LOD score of 1.70 ($P = 0.20$) in the autopsy-confirmed families using the LOD score of D9S741 (low to high) as a covariate (Figure 3). The subset included 179 families (88% of all autopsy-confirmed families). When using the sum of LOD scores of D9S741 and α-catenin (low to high) to rank order autopsy-confirmed families, marker D12S368 generated a maximum LOD score 2.51 (Figure 4). This increase was not quite significant ($P = 0.06$). The subset included 187 families (92% of the autopsy-confirmed families).

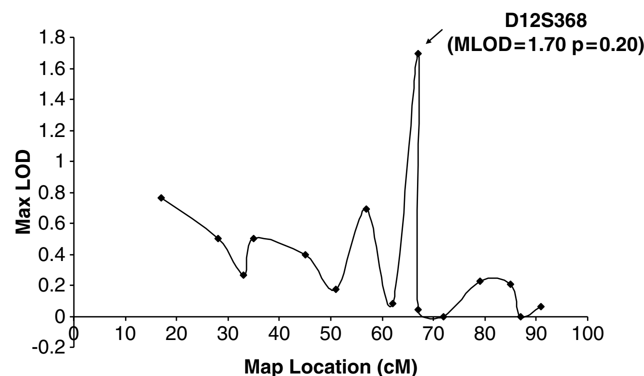

Figure 3 Two-point OSA LOD scores for autopsy-confirmed families using D9S741 LOD scores (ranked from low to high) as a covariate.

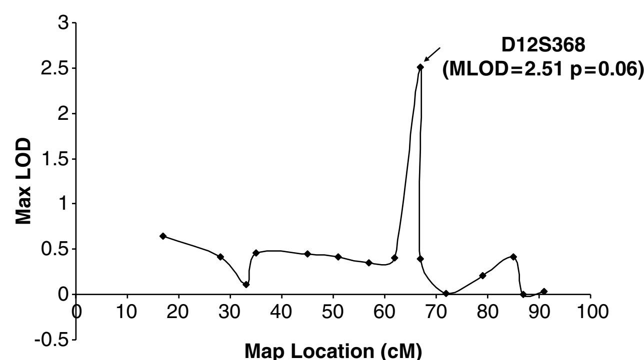

Figure 4 Two-point OSA LOD scores for autopsy-confirmed families using the sum of D9S741 and α-catenin LOD scores (ranked from low to high) as a covariate.

Discussion

Multiple genomic screens have found evidence of linkage to the region between chromosome 12p13 and 12q22. However, localization of this signal has been difficult. We used an expanded data set to examine this region in more detail under the hypothesis that locus heterogeneity might be obscuring the chromosome 12 linkage signal.

The known linkage to ApoE and association with the ApoE $\varepsilon 4$ allele[9] is one potential source of heterogeneity. We observed a nominally significant increase in the LOD score at D12S1042 when the data set was stratified by ApoE $\varepsilon 4$ status (Figure 1), consistent with our previous report.[31] While such a stratification approach is simple to apply, it leads to smaller subsets (in our case 91 families, ~13% of the entire data set) and subsequent loss of power.

An alternative approach to deal with heterogeneity is the OSA[27]. OSA rank orders families by a continuous covariate to identify the subset with maximum evidence for linkage to a particular map of markers.

Our OSA analyses using ApoE $\varepsilon 4$ as a covariate did not substantially increase evidence for linkage from the original subset analysis. However, using the

linkage data on chromosome 10 gave a maximum LOD score of 4.19 at D12S1701 using the OSA method. This increase in the LOD score is highly statistically significant ($P<0.0001$). We used the LOD score for an SNP in the α-catenin gene, as it is the strongest linkage signals we have on chromosome 10 (Martin *et al.*, in press). Our previous analysis of chromosome 12 data[31] also found a maximum LOD score of 1.10 at D12S1701 in the ApoE-negative subset. The rank ordering from low to high scores, which gives families with negative α-catenin LOD scores more weight, suggests that the locus on chromosome 12 acts independently of a locus on chromosome 10. The optimal subset included 45 families (21%) that all generated negative LOD scores at the α-catenin locus.

We previously reported a strong linkage signal (LOD = 4.31) on chromosome 9p at D9S741.[30] In the current data set, the OSA analysis generated an MLOD of 1.70 at D12S368 ($P=0.20$) when D9S741 LOD scores were used as a covariate and the families were ordered from low to high. Chromosome 9 linkage data did not increase the LOD score as significantly as chromosome 10 linkage data did although we ordered our families from low to high by the covariate LOD scores in both cases.

There are multiple possible reasons for the difference between the chromosome 10 and chromosome 9 results. These include different levels of locus heterogeneity for the chromosomes 9 and 10 loci, and a different level of informativeness for the markers on each chromosome. In addition, it is not yet clear how to use OSA analysis to account for multiple covariates simultaneously. One approximation is to use the sum of LOD scores of D9S741 and α-catenin from low to high as a covariate. When we applied this approach, the MLOD was 2.51 at D12S368 with a suggestive *P*-value ($P=0.06$), only 5 cM from D12S1701. We do not know the interaction between loci on chromosome 9 and 10. There could be additive, multiplicative and epistasis effects. The analysis could depend on different models to use covariates.

In conclusion, our analyses suggest that there is substantial locus heterogeneity remaining in LOAD. The linkage signal on chromosome 12 is strengthened in families without ApoE ε4 with a peak at approximately 51–57 cM and in families with no evidence of linkage to chromosome 9 and, most strongly, no evidence of linkage to chromosome 10 with a peak at 62–67 cM. Thus, despite the large initial size of the data set, by the time ApoE and linkage to chromosome 9 and/or 10 is accounted for, the effective data set is quite small, making further localization through linkage difficult. The 16 cM region between D12S1042 and D12S368 is most likely to harbor this gene and thus should be the subject of further detailed genomic efforts for the disease.

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Electronic-database information

http://www.ncbi.nih.gov
http://www.ensembl.org

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