A Genomewide Screen for Late-Onset Alzheimer Disease in a Genetically Isolated Dutch Population

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Alzheimer disease (AD) is the most common cause of dementia. We conducted a genome screen of 103 patients with late-onset AD who were ascertained as part of the Genetic Research in Isolated Populations (GRIP) program that is conducted in a recently isolated population from the southwestern area of The Netherlands. All patients and their 170 closely related relatives were genotyped using 402 microsatellite markers. Extensive genealogy information was collected, which resulted in an extremely large and complex pedigree of 4,645 members. The pedigree was split into 35 subpedigrees, to reduce the computational burden of linkage analysis. Simulations aiming to evaluate the effect of pedigree splitting on false-positive probabilities showed that a LOD score of 3.64 corresponds to 5% genomewide type I error. Multipoint analysis revealed four significant and one suggestive linkage peaks. The strongest evidence of linkage was found for chromosome 1q21 (heterogeneity LOD [HLOD] = 5.20 at marker *D1S498*). Approximately 30 cM upstream of this locus, we found another peak at 1q25 (HLOD = 4.0 at marker *D1S218*). These two loci are in a previously established linkage region. We also confirmed the AD locus at 10q22-24 (HLOD = 4.15 at marker *D10S185*). There was significant evidence of linkage of AD to chromosome 3q22-24 (HLOD = 4.44 at marker *D3S1569*). For chromosome 11q24-25, there was suggestive evidence of linkage (HLOD = 3.29 at marker *D11S1320*). We next tested for association between cognitive function and 4,173 single-nucleotide polymorphisms in the linked regions in an independent sample consisting of 197 individuals from the GRIP region. After adjusting for multiple testing, we were able to detect significant associations for cognitive function in four of five AD-linked regions, including the new region on chromosome 3q22-24 and regions 1q25, 10q22-24, and 11q25. With use of cognitive function as an endophenotype of AD, our study indicates the that the *RGSL2, RALGPS2,* and *C1orf49* genes are the potential disease-causing genes at 1q25. Our analysis of chromosome 10q22-24 points to the *HTR7, MPHOSPH1,* and *CYP2C* cluster. This is the first genomewide screen that showed significant linkage to chromosome 3q23 markers. For this region, our analysis identified the *NMNAT3* and *CLSTN2* genes. Our findings confirm linkage to chromosome 11q25. We were unable to confirm *SORL1*; instead, our analysis points to the *OPCML* and *HNT* genes.

Alzheimer disease (AD) is a progressive neurodegenerative disorder that accounts for the vast majority of dementia. The population prevalence of the disease rises steeply with age from <2% at age 65 years to >35% after age 90 years.[1,2] Family history is an important indicator of risk of AD, and, in a large number of families, the disease segregates as an autosomal dominant trait. The heritability for AD was recently estimated to be 79%.[3] Several dominant mutations have been identified, including mutations in the presenilin 1 (*PSEN1* [MIM 104311]),[4] presenilin 2 (*PSEN2* [MIM 600759]),[5,6] and amyloid precursor protein (*APP* [MIM 104760]) genes.[7] A common polymorphism (ε4) in the gene encoding apolipoprotein E (*APOE* [MIM 107741]) increases susceptibility to both early- and late-onset AD.[8,9] These four genes together explain less than a quarter of the disease prevalence, indicating that additional genetic risk factors remain to be identified.[10,11] In addition to *APOE,* various candidate genes were reported to be associated with late-onset AD. In most cases, findings have not been consistently replicated.[12,13] A large meta-analysis of all genes studied so far pinpointed 13 potential AD-susceptibility genes: angiotensin I converting enzyme (*ACE* [MIM 106180]); cholinergic receptor, nicotinic, beta 2 (*CHRNB2* [MIM 118507]); cystatin C (*CST3* [MIM 604312]); estrogen receptor 1 (*ESR1* [MIM 133430]); glyceraldehyde-3-phosphate dehydrogenase, spermatogenic (*GAPDHS* [MIM 609169]); insulin-degrading enzyme (*IDE* [MIM 146680]); 5,10-methylenetetrahydrofolate reductase (*MTHFR* [MIM 607093]); nicastrin (*NCSTN* [MIM 605254]); prion protein (*PRNP* [MIM 176640]); *PSEN1*;

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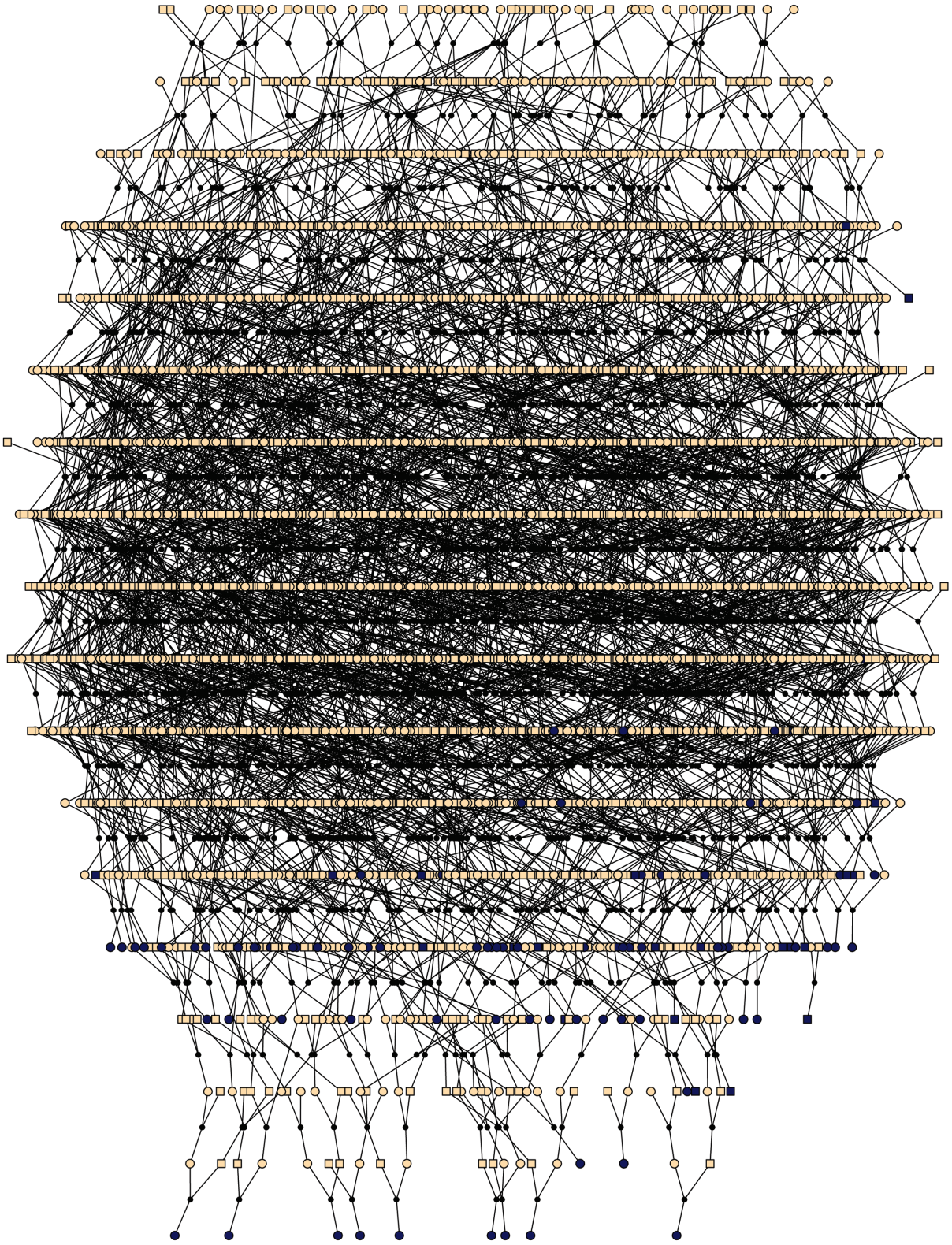


Figure 1. The entire pedigree with 4,645 members, including 103 patients with late-onset AD, from the GRIP population. Men are represented with squares and women with circles. Black dots represent marriage nodes. Affected individuals are represented in black. Unknown affection status is represented with yellow. For simplicity, unaffected relatives of the patients are not shown. This figure was drawn using Pedfiddler version 0.5.

Table 1. Genealogic Characteristics of 103 Patients with Late-Onset AD and Their Relatives

Characteristic	Value[a]
Complete genealogy:	
No. of family members	4,645
No. of generations	18
Average no. of consanguineous loops per patient	71.7 (0–677)
Average no. of meioses in a consanguineous loop	9.9 ± 1.2 (0–29)
Mean inbreeding coefficient × 100	.39 ± .73 (0–3.2)
Average no. of lines of descent between a pair of patients	141.7 (0–2,673)
Average no. of meioses separating a pair of patients	17.1 ± 1.6 (0–34)
Mean kinship coefficient × 100	.18 ± 1.06 (0–26.4)
After clustering patients into subpedigrees:	
No. of subpedigrees	35
No. of founders	564 (46.0)
No. of females	630 (51.3)
Mean pedigree size	29.6 (18–75)
Mean no. of generations	7.5 (6–10)
Mean no. of genotyped individuals per pedigree	7.8 (2–14)
Mean no. of patients per pedigree	2.9 (2–6)

[a] Values in parentheses indicate range or percentage.

transferrin (*TF* [MIM 190000]); transcription factor A, mitochondrial (*TFAM* [MIM 600438]); and tumor necrosis factor (*TNF* [MIM 191160]).[14] Furthermore, genome screens targeting AD loci have been conducted. As reviewed online by the Alzheimer Research Forum, the replicated regions from previous genome screens include 1p36, 1q21-31, 2p23-24, 4q35, 5p13-15, 6p21, 6q15-16, 6q25-27, 9p21-22, 10q21-22, 10q25, 12p11-12, 19q13, 21q21-22, and Xp11-21.[9,15–29] Several genes have been suggested to explain the linkage to chromosome 9, 10, 12, and 19, but, so far, these genes also remain to be confirmed. Finally, there is evidence of linkage to chromosome 11,[22] which was explained recently by the identification of *SORL1* (MIM 602005).[30]

Each of the established loci for AD (*APP, PSEN1, PSEN2,* and *APOE*) was initially localized by linkage analyses. However, pedigrees suitable for localizing genes have become scarce, particularly for late-onset forms of AD. Genetically isolated populations provide opportunities for linkage analysis. With use of genealogical records, extended pedigrees can be constructed. Furthermore, the complexity of disease may be reduced in terms of number of genes involved, particularly for rare Mendelian forms.[31,32] Linkage analysis of complex traits has been used successfully in Iceland for complex diseases such as type 2 diabetes and stroke,[33,34] whereas, for AD, genome screens have been conducted successfully with Caribbean Hispanics.[35] We have followed this approach in a genetically isolated community from the southwestern area of The Netherlands, as part of the Genetic Research in Isolated Populations (GRIP) program.[36] A total of 103 patients with late-onset AD were ascertained and were included in a large pedigree on the basis of genealogical records. In this study, we present a genomewide screen of these families. The linkage analysis was followed by an association study of cognitive function in a series of 197 unrelated and nondemented people from the GRIP region who were extensively characterized by a cognitive battery. To further investigate the evidence of linkage, the regions identified in the linkage study were characterized with a dense panel of SNPs. Decline in cognitive function, particularly mild cognitive impairment, is an early predictor of AD,[37–39] and the heritability of cognitive function is as high as 56%, which suggests that cognition is a valuable endophenotype.[40–42] Further, memory function was found to be an endophenotype for families multiply affected with AD.[43]

Material and Methods

Population and Genealogy

This study was performed within the framework of the previously described GRIP program.[32,44,45] The Medical Ethics Committee of

Table 2. Age-Dependent Liability Classes and Penetrances

Liability Class	Age (years)	Population Prevalence[a]	Penetrance	No. of Patients	No. of Unaffected Relatives
1	<65	<.02	.00	0	129
2	65–69	.02	.09	4	6
3	70–74	.05	.23	22	11
4	75–79	.09	.46	32	14
5	80–84	.23	.99	30	8
6	85–89	.35	.99	24	1
7	⩾90	>.35	.99	0	1

[a] Obtained from the Rotterdam Study.[1]

Table 3. LOD Scores and Corresponding Genomewide Type I Error Rates

LOD	HLOD	Type I Error Rate (%)
4.08	4.08	1
3.64	3.64	5
3.24	3.24	10
2.11	2.11	50

NOTE.—Values are based on 100 simulations.

the Erasmus Medical Center approved study protocol. The GRIP population is a genetically isolated community in the southwestern area of The Netherlands. Fewer than 400 individuals were present in the region in the middle of the 18th century. Considerable population growth occurred in 1850–1900, as was the case in many European populations. An estimated 20,000 descendants of the population are now scattered over eight adjacent communities. There was minimal immigration. The genealogical database currently contains information about 107,091 people spanning 23 generations. Residents in the GRIP area are generally related via multiple lines of descent and are inbred via multiple consanguineous loops.[45]

Patients with AD were traced through general practitioners, neurologists, and nursing-home physicians. Data relevant for the diagnosis of AD were collected by a research physician, and the diagnosis of AD was verified by two independent neurologists with criteria of the National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer's Disease and Related Disorders Association.[46] Data about the presence of AD, parkinsonism, essential tremor, and dementia were collected for first-, second-, and third-degree relatives by means of a family-history questionnaire. First-degree relatives also underwent a brief neurological examination. All patients and their relatives who were invited to participate in our study provided informed consent. A total of 112 probable patients with late-onset AD (age at onset $\geq$65 years, mean age [$\pm$SD] at onset 75 $\pm$ 5.3 years) and 170 unaffected first-degree relatives (mean age 63.5 $\pm$ 13.1 years; range 40–102 years) were ascertained.

Tracing the genealogy of the 112 probable patients with late-onset AD, we were able to include 103 patients in a single pedigree containing 4,645 individuals in 18 generations, as depicted in figure 1. The other nine patients were singletons and therefore were not included in the linkage analysis. This large pedigree showed multiple, distant lines of descent and consanguineous loops (table 1). The average kinship coefficient among patients was 0.0018. This value is between a third cousin once removed and a fourth cousin. Using such a pedigree in linkage analysis is computationally impossible. A common approach to reduce the computational complexity is to split the large pedigree into smaller and computable units. For this purpose, we used a kinship clustering method that is similar to the maximal-cliques-partitioning method proposed by Falchi et al,[47] and we added a restriction that the resulting subpedigrees have no more than 35 bits, where the bit size is twice the number of founders minus the number of nonfounders. Our software for splitting large pedigrees, PedCut, is available, free of charge, at the MGA Web site.

We further studied a series of 197 individuals who were not related withini 5 generations and were not related to the patients with AD. The average age of these people was 31.2 $\pm$ 6.4 years; 51% were female. These individuals were evaluated with use of an extensive cognitive battery.[48] In brief, the selection of tests included the 15-word test, the color word card of the Stroop Color Word test, part B of the Trail making test (TMTB), and the verbal fluency test. These tests were selected to target early cognitive problems related to AD. From the 15-word test, we derived three scores for further analysis—that is, learning (or working memory), delayed recall, and recognition. The verbal fluency test consists of two subdomains: semantic fluency and phonological fluency. The performance of each individual on each test was scored quantitatively. Power calculation showed that this sample has 80% power to detect a SNP explaining 4% of phenotypic variance with an α of .05.

Genotyping

For all patients and their 170 first-degree relatives, DNA was extracted from peripheral leukocytes following a standard protocol.[49] Elsewhere, mutations in the *APP, PSEN1,* and *PSEN2* genes were excluded as AD-causing genes.[36] The *APOE* genotype was determined in all DNA samples by use of TaqMan allelic discrimination technology on an ABI Prism 7900HT Sequence Detection System with SDS version 2.1 (Applied Biosystems). Patients and their first-degree relatives underwent a full genome screen in two sequential experiments. Both screens were conducted using the same set of microsatellite markers, evenly spaced by ~10 cM (ABI Prism Linkage Mapping Set MD-10 v. 2 and v. 2.5 [Applied Biosystems]). PCRs were performed according to the manufacturer's specified conditions. PCR products were separately pooled and analyzed on ABI377 and ABI3100 automated sequencers (Applied Biosystems). Because the genome scan had been performed with different sequencing devices, the genetic data had to be merged. The genotypic data was pooled using Pool_STR-1.1, on the basis of the allele lengths and allele frequencies observed in each group.[50] Two independent technicians read the results from the sequencers, and a third reader resolved the discordant results. Only the markers with a discordance proportion <5% were selected for further analysis (N = 402). Genotyping errors leading to Mendelian inconsistencies were detected using PedCheck (Statgen).[51] Unlikely double-recombination events were detected using Merlin.[52] Definitive genotyping errors and unlikely genotypes were rechecked using the data from the laboratory. Regions linked to late-onset AD were later fine typed by placing 45 additional microsatellite markers between those from the initial set, at a distance of 1–5 cM.

SNPs in the linkage regions were selected from the 250K Nsp array of the GeneChip Human Mapping 500K Array Set (Affymetrix). Genomic DNA was extracted from whole-blood samples drawn at the baseline examination, with use of the salting-out method.[49] The 250K Nsp array from Affymetrix was used to determine genotypes. The chips were run and analyzed according to the manufacturer's protocols. A total of 4,173 SNPs were selected for the association test on the basis of the following criteria:

The figure is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Figure 2. The 35 subpedigrees obtained by applying a kinship-partitioning algorithm to the entire pedigree. The legend is available in its entirety in the online edition of *The American Journal of Human Genetics*.

The figure is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Figure 3. Multipoint LOD and HLOD scores for each autosome in the genome screen of late-onset AD. The legend is available in its entirety in the online edition of *The American Journal of Human Genetics*.

(1) position within the regions that show significant or suggestive evidence of linkage after fine mapping, (2) minor-allele frequency ⩾2.5%, (3) *P* value for Hardy-Weinberg equilibrium test ⩾.01, and (4) call rate ⩾95%.

Statistical Analysis

In linkage analysis, we assumed a dominant model of inheritance with age-dependent penetrance. Seven liability classes were defined on the basis of age (in years): <65, 65–69, 70–74, 75–79, 80–84, 85–90, and >90. For each age group *j*, age-dependent population prevalence P_j was obtained from the Rotterdam Study.[1] The disease-gene penetrance, f_j, of the *j*th age group can be estimated as

$$f_j = \frac{\text{PAF} \times P_j}{q^2 + 2q(1-q)} ,$$

where PAF is the population-attributable fraction—that is, the proportion of the population prevalence that can be explained by the studied gene (10% assumed)—and q is the disease-allele frequency (1% assumed). The estimated penetrance for each defined age group is shown in table 2. Marker-allele frequencies were estimated on the basis of 144 chromosomes from unaffected elderly GRIP population members (age at last examination ⩾65 years). For small pedigrees (bits ⩾18), we used the exact calculation of multilocus likelihood, the Lander-Green algorithm implemented in GENEHUNTER 2.0.[53] For larger pedigrees, we used Markov chain–Monte Carlo estimation methods implemented in SIMWALK 2.91 (Statgen). Overall LOD scores and heterogeneity LOD (HLOD) scores were computed by combining results per family with use of standard formulas, such as

$$\text{HLOD} = \log_{10}(\max \text{LR}) ,$$

where maxLR is maximized with respect to α, the proportion of the linked families, yielding maximum-likelihood estimate $\hat{\alpha}$,

$$\max \text{LR} = \prod_{i=1}^{n} (\hat{\alpha} LR_i + 1 - \hat{\alpha}) .$$

Haplotypes were reconstructed on the basis of the genotypes of patients, spouses of patients, and their offspring, with the Merlin package.[52] These families are further expanded to depict the haplotype sharing of other patients who are relatively closely related to the patients in the families with high LOD scores but who were assigned to different families in the pedigree-splitting procedure.

Breaking pedigrees may increase the possibility of spurious linkage findings.[54] Therefore, we estimated the threshold for statistical significance by use of simulations. To evaluate genomewide type I error, we simulated our genome scan 100 times. We used the complete pedigree, including all 4,645 members, for marker simulation. Unlinked markers were dropped in the complete pedigree. Number of markers, intermarker distances, and marker-allele frequencies were simulated according to the typed marker set. We performed linkage analysis using the split subpedigrees. Disease-allele frequency, liability classes, genetic model, and penetrances were the same as those we used later in the actual linkage analysis. Genotypes of untyped individuals were set to "missing." For each genome screen, the highest HLOD score was stored. Cumulative density function of the obtained 100 maximum HLOD scores approximates the distribution of the genomewide type I error rates. Our simulations showed that an HLOD score of 3.64 corresponds to a genomewide type I error rate of 5% and that an HLOD of 2.11 corresponds to a genomewide type I error of 50% (table 3).

We used linear regression to test for association between a single SNP with a single cognitive trait. In accordance with Affymetrix annotation, SNP genotypes were coded as 0 = AA, 1 = AB, and 2 = BB, where A represents the allele in lower alphabetical order and B represents the other allele. Thus, in the case of a C→T change where T is the minor allele, C is coded as the A allele and T as the B allele, whereas, for a T→C change where C is the minor allele, A denotes the C allele and B denotes the T allele. In the model, we adjusted for age, sex, intelligence, and highest education. Because a causal SNP (or a SNP in linkage disequilibrium [LD] with the causal SNP) is likely to be associated with multiple cognitive domains, we used the Fisher product method for combining the findings of all cognitive tests.[55] Because the SNPs that are in LD and cognitive traits are also correlated, we used a permutation method to evaluate significance level for each SNP empirically (500,000 replicates). To break the associations between the markers and traits while keeping the correlations between traits and the LD pattern between markers, we permuted the vectors of individuals' traits (scores of cognitive tests and covariates) among individuals, without replacement. For each permutation,

Table 4. Regions with Genomewide Empirically Significant or Suggestive Linkage after Fine Mapping

Chromosome and Marker	Position (cM)	LOD	HLOD	α	Region(s) Identified Elsewhere[a]
1A:					
D1S498	164	**5.1**	**5.2**	.9	A,[25] D,[26] F,[56] and G[22]
D1S305	167	**4.5**	**4.5**	1.0	
1B:					
D1S218	201	2.6	**4.0**	.6	A,[25] D,[26] F,[56] and G[22]
D1S366	208	2.7	3.5	.6	
3:					
D3S1549	151	2.8	3.6	.6	B[57]
D3S1569	158	**4.3**	**4.4**	.8	
10:					
D10S1686	105	**3.7**	**3.7**	1.0	C,[18] E,[19] F,[56] G,[22] H,[27] and I[24]
D10S185	116	**4.2**	**4.2**	1.0	
11[b]:					
D11S4151	127	.3	2.8	.4	G[22]
D11S4131	138	1.3	3.1	.5	
D11S1320	142	1.6	3.3	.6	
D11S968	148	.3	2.0	.5	

NOTE.—Significant values are shown in bold.

[a] Overlaps with regions reported with suggestive linkage or significant association in previous genome screens. Note that region B was screened for only two chromosomes.

[b] Included to confirm a recent report about the *SORL1* gene.[30]

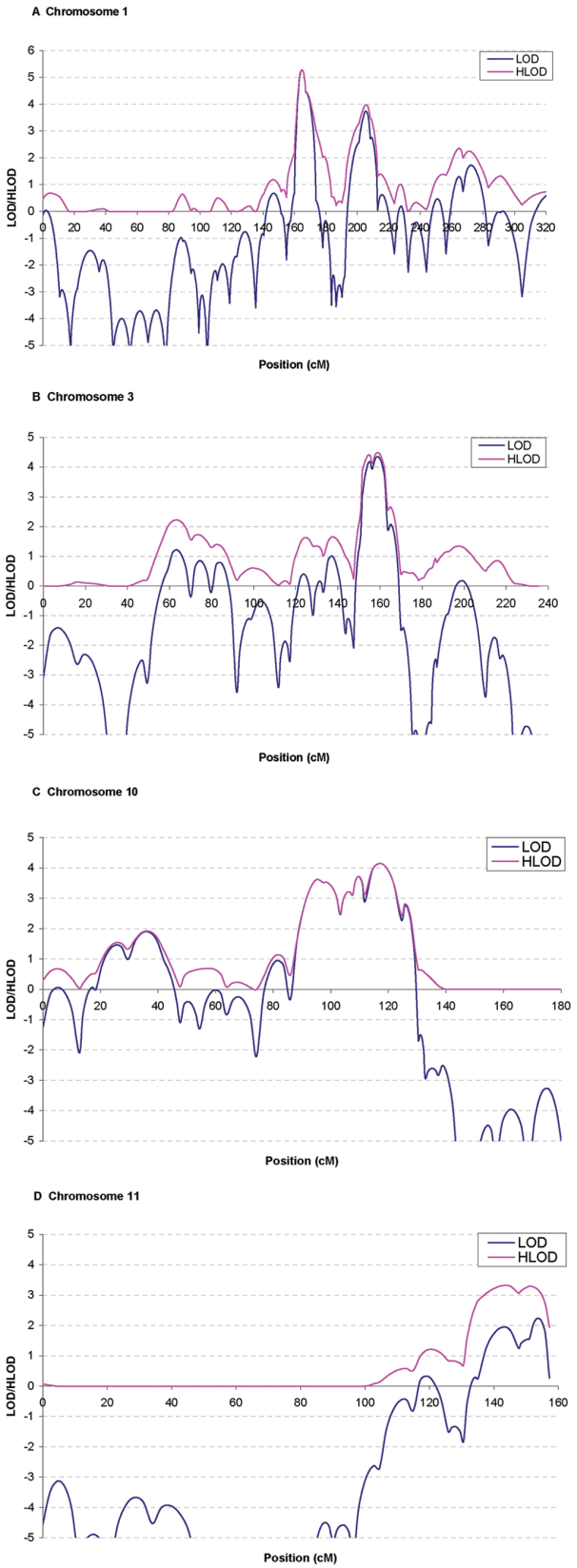
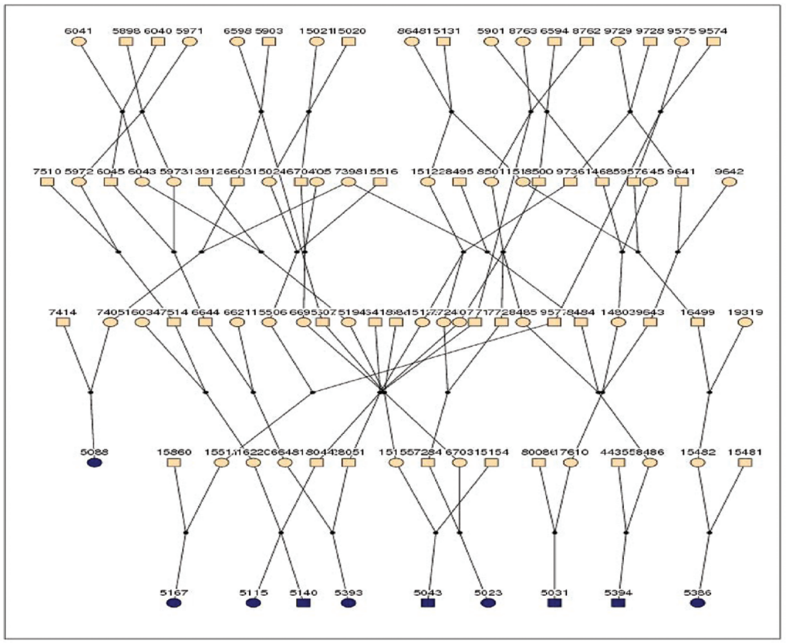


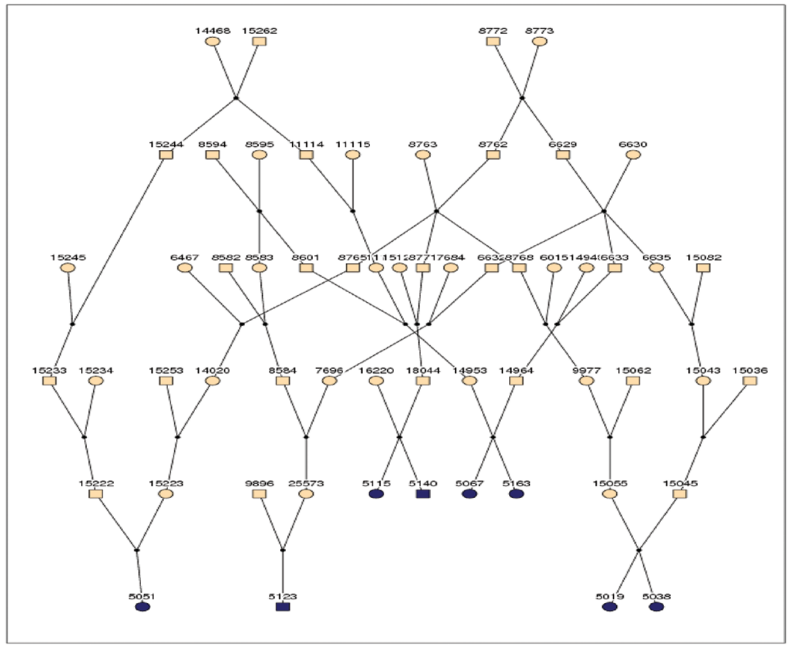
Figure 4. Multipoint LOD (*blue*) and HLOD (*pink*) scores for chromosomes 1, 3, 10, and 11 in the genome screen of late-onset AD after fine typing. Marker locations are given in Kosambi centimorgans.

A Haplotype of chromosome 1q21



Markers	5088*		5167		5115		5140		5393*		5043		5023*		5031		5394*		5386	
D1S2746	(1)	(3)	2	1	7	1	7	1	1	3	1	8	7	6	1	1	7	7	7	3
D1S252	2	3	1	4	1	1	1	1	1	2	1	4	1	1	1	1	1	4	1	6
D1S514	(6)	(4)	6	1	6	4	6	7	6	5	6	5	6	4	6	1	6	5	6	3
D1S498	1	9	1	4	1	10	1	6	1	5	1	4	1	2	1	6	1	4	3	9
D1S2635	3	1	3	1	3	3	3	6	3	6	3	4	(3)	(6)	3	3	(3)	(6)	(3)	(1)
D1S484	3	3	3	2	3	3	3	3	3	1	3	1	3	2	3	1	1	3	1	2

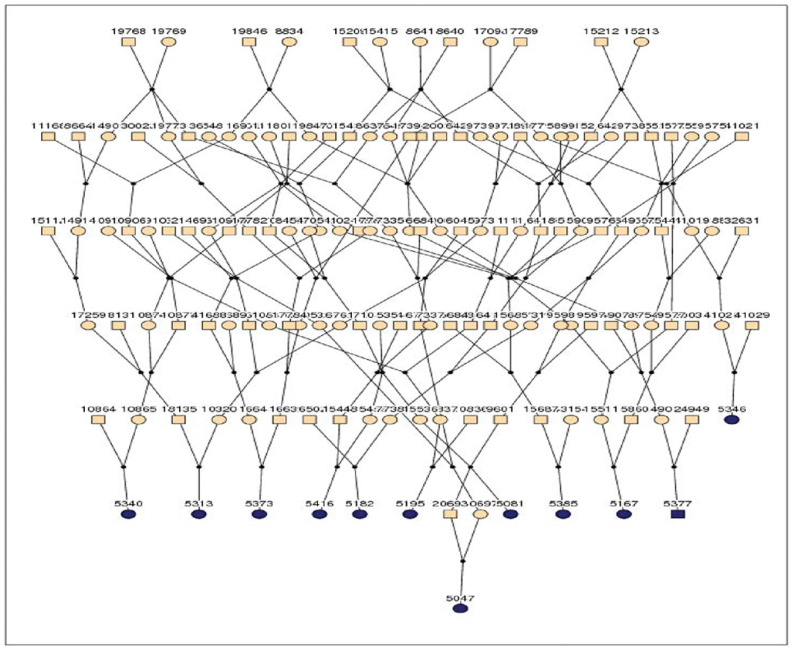
B Haplotype of chromosome 1q25



Markers	5051*		5123		5115*		5140*		5067*		5163*		5019		5038	
D1S2799	3	8	3	4	3	1	4	1	7	1	7	1	1	2	1	2
D1S218	1	1	1	5	5	1	5	1	1	1	1	1	1	2	1	2
D1S466	5	1	1	3	3	1	(1)	(1)	1	1	1	1	1	1	1	1
D1S238	1	2	1	7	2	2	7	2	4	1	4	1	2	1	2	1

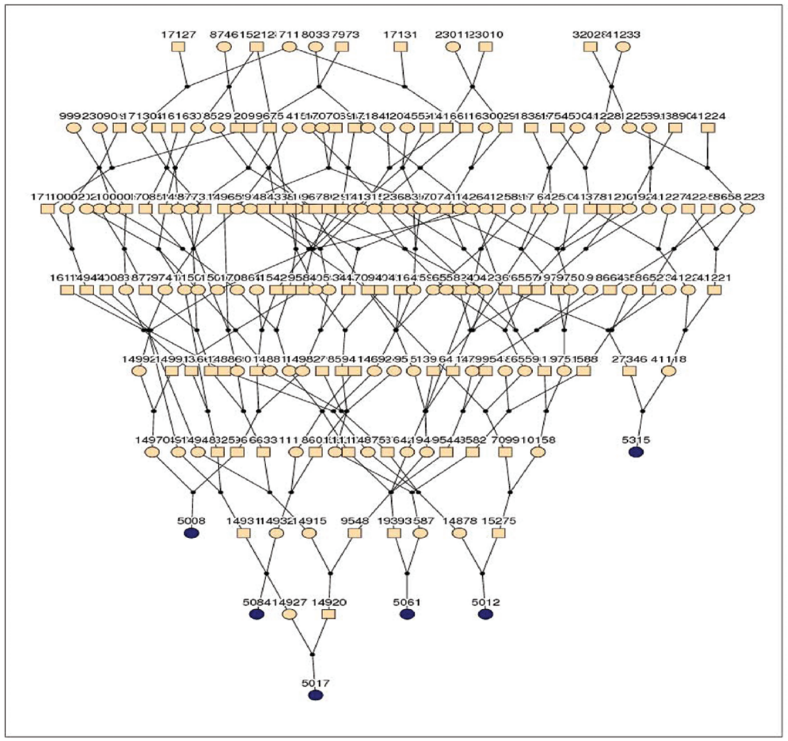
Figure 5. Haplotypes of chromosomes 1, 3, and 11 segregating with AD-affected families from the GRIP population. Panels A, B, C, and D are from subpedigrees 1, 3, 9, and 4, respectively. These families are expanded to depict the haplotype sharing of other patients who are relatively closely related to the patients but were assigned to different families in the pedigree-splitting procedure. Patients with asterisks (*) were from the corresponding subpedigrees; patients without asterisks were assigned to different families. Alleles in parentheses are inferred. Pink bars depict the segment that is shared between the patients.

C Haplotype of chromosome 3q23



Markers	5340		5313*		5373		5416*		5182*		5195		5047*		5081*		5385		5167*		5377		5346	
D3S3514	2	3	2	9	4	4	2	2	2	5	2	1	2	2	2	2	2	2	2	1	7	2	2	1
D3S1292	2	9	2	4	5	2	2	2	2	9	2	1	2	2	2	2	2	6	2	1	2	2	2	1
D3S1549	5	2	5	3	5	5	5	2	5	3	5	3	5	2	5	5	5	5	5	8	5	1	5	4
D3S1569	3	1	3	1	3	1	3	2	3	3	3	2	2	6	3	1	3	1	3	1	3	4	3	3
D3S3626	2	8	2	1	3	2	7	3	3	1	2	2	3	3	2	2	2	2	2	8	2	1	2	2

D Haplotype of chromosome 11q24



Markers	5008*		5084*		5017*		5061		5012*		5315	
D11S925	1	2	8	6	8	3	3	9	8	1	11	2
D11S1328	5	5	3	5	3	5	5	5	3	1	9	3
D11S4151	5	2	2	5	2	2	2	3	2	3	2	4
D11S4131	6	3	6	7	6	3	6	3	6	7	6	3
D11S1320	1	1	1	1	1	1	1	2	1	3	1	3
D11S968	7	1	5	5	5	1	3	1	1	1	5	3

Table 5. Select SNPs in the Regions Linked to AD

	Start				End				No. of SNPs	
Chromosome	Marker	Marshfield (cM)	SNP	Physical Position (bp)	Marker	Marshfield (cM)	SNP	Physical Position (bp)	Initial	After QC[a]
1q21	*D1S514*	152.5	*rs2790308*	141501392	*D1S2635*	165.6	*rs16827466*	156462773	828	585
1q25	*D1S218*	191.5	*rs17838246*	171261041	*D1S466*	198.5	*rs16860717*	179972352	750	584
3q23	*D3S1549*	151.5	*rs7632392*	139168654	*D3S3626*	164.3	*rs10513332*	149350824	954	769
10q22-24	*D10S580*	96.7	*rs7101263*	77715269	*D10S205*	125.4	*rs12765878*	105659612	2,518	2,006
11q25	*D11S4131*	138.6	*rs1526562*	131160829	*D11S968*	147.8	*rs7936592*	133620325	260	229

[a] QC = quality control.

we tested for association between SNPs in each region and cognitive traits, and we derived corresponding Fisher products. The cumulative density function of all Fisher products for each region empirically approximates the regionwide type I error rate. Therefore, the empirical *P* value for each SNP can be defined as the probability of observing an equal or smaller regionwide Fisher product by chance.

Results

For linkage analysis, we clustered all patients and 170 first-degree relatives into 35 subpedigrees (fig. 2). During pedigree splitting, distant ancestors who have no phenotypic and genotypic information and do not contribute to linkage information were discarded. The resultant subpedigrees contained a total of 1,227 individuals. The characteristics of the subpedigrees are shown in table 1.

Multipoint LOD and HLOD score plots for the initial scan are shown in figure 3. A total of eight regions showed suggestive linkage (LOD or HLOD > 2.11), of which the chromosome 1 region exceeded the threshold of 3.64 (LOD = 4.1 at marker *D1S484*). These eight regions were fine typed with 45 additional markers and include chromosome 1 (14 additional markers), chromosome 3 (10 additional markers), chromosome 5 (2 additional markers), chromosome 6 (5 additional markers), chromosome 7 (3 additional markers), chromosome 10 (6 additional markers), chromosome 11 (2 additional markers), and chromosome 18 (3 additional markers).

Table 4 shows the regions for which the evidence of linkage remained significant or suggestive (LOD > 2.11) after fine mapping. AD remained linked to three known regions, two on chromosome 1 (fig. 4*A*) and one on chromosome 10 (fig. 4*C*). The maximum HLOD at 1q21 was 5.2 at *D1S498*. This is the highest peak over the genome. The maximum HLOD at 1q25 was 4.0 at *D1S218* and 4.2 at *D10S185*. References for the previously identified regions are shown in table 4. In addition to the known regions, we found genomewide significant evidence of linkage of AD to a region on chromosome 3 that spanned 18 cM from *D3S3514* to *D3S3626* and reached a maximum HLOD of 4.4 at *D3S1569* (fig. 4*B*). This is the second highest peak over the genome. In table 4, we also included chromosome 11, in which a new gene (*SORL1*) responsible for AD was recently reported.[30] There is suggestive evidence of linkage of AD to chromosome 11 (HLOD = 3.3 at *D11S1320*) (fig. 4*D*), which overlaps with a region reported earlier. On chromosome 11, the HLOD at the position of *SORL1* (118 cM) is 1.1.

Haplotype analysis showed that the two linkage peaks on chromosome 1q21 and 1q25 are explained by different haplotypes segregating in different families. On chromosome 1q21, we identified a 15-cM region shared by four patients in family 1 and six other closely related patients who were assigned to different pedigrees for computational reasons in the process of pedigree splitting (fig. 5*A*). The 21-cM haplotype of 1q25 segregates in family 3 (four patients) and is shared by four other closely related patients who were assigned to different subpedigrees (fig. 5*B*). Six patients from family 9 and six closely related patients carry the haplotype of chromosome 3q23 (18 cM), as shown in figure 5*C*. The linkage of AD to the region on chromosome 10 was based on moderate contributions from multiple families with different haplotypes. There is not a single haplotype segregating in this region (data not shown). For chromosome 11q24, which showed suggestive linkage, we observed a single haplotype (3.4 cM) shared by four patients from family 4 and two additional closely related patients (fig. 5*D*).

Next, we tested for association between cognitive function and a set of 4,173 SNPs within regions 1q21, 1q25, 3q23, 10q22-24, and 11q25, using an independent sample consisting of 197 individuals from the GRIP population (table 5). All of the linked regions except 1q21 contain at least one SNP showing significant association with use of an empirical *P* value of .05 (table 6). Statistically, the most significant SNP is *rs7071717* at 10q23, both for the nominal *P* value in a single test (*P* = .000005, by Stroop test) and for the empirical Fisher product (*P* = .002), which combines the results of cognitive tests and adjust for multiple testing. This SNP, together with *rs17129662* and *rs11185978,* is in a range of 80 kb and shows evidence of association with the Stroop test, TMTB, and semantic (except *rs17129662*) and phonological fluency, all of which are subdomains of executive function. These three SNPs are 2–80 kb downstream of the *MPHOSPH1* gene encoding M phase phosphoprotein 1 and ~760 kb upstream of the 5-hydroxytryptamine receptor 7 (*HTR7* [MIM 182137]) gene. Another SNP, *rs4110517* at 10q23, showed associa-

Table 6. SNPs Significantly Associated with Various Aspects of Cognitive Function in 197 Unrelated Individuals

Linked Region and SNP[a]	Marshfield (cM)[b]	Physical Position[c] (bp)	Memory			Executive Function			Verbal Fluency		All Tests Combined[d]	Empirical P[e]	Gene(s)[f]	SNP Position
			Learning	Delay	Recognition	Stroop	TMTB	Block	Semantic	Phonological				
1q25:														
rs17361286	193.9	176788539	.68	.56	.91	.44	**.0003**	.51	.96	.64	1.2×10^{-5}	**.04**	*C1orf49* and *RALGPS2*	Intergenic
rs2493864	194.0	176811626	.76	.63	.96	.46	**.0003**	.49	.99	.62	1.7×10^{-5}	**.04**	*C1orf49* and *RALGPS2*	Intergenic
rs2584820	197.6	180705709	.20	.19	.76	**.0001**	.89	.98	.07	**.03**	5.9×10^{-9}	**.02**	*RGSL2*	Intron 4
3q23:														
rs952797	152.6	141005823	.06	.47	.57	**.05**	.89	**.0002**	.82	.24	2.8×10^{-8}	**.04**	*NMNAT3* and *CLSTN2*	Intergenic
10q23:														
rs17129662	111.8	91730916	.23	.55	.48	**.00003**	**.00235**	.84	.10	**.002**	7.0×10^{-13}	**.02**	*MPHOSPH1* and *HTR7*	Intergenic
rs11185978	111.8	91778768	.36	.60	.60	**.00002**	**.01**	.61	**.05**	**.001**	8.9×10^{-13}	**.01**	*MPHOSPH1* and *HTR7*	Intergenic
rs7071717	111.8	91808267	.30	.73	.67	**.000005**	**.01**	.90	**.04**	**.01**	2.9×10^{-12}	**.002**	*MPHOSPH1* and *HTR7*	Intergenic
rs4110517	116.8	96640318	.10	.09	.94	.18	.24	.87	**.00003**	**.04**	4.7×10^{-10}	**.02**	*CYP2C19* and *CYP2C9*	Intergenic
11q25:														
rs1629316	139.7	131440484	.07	.21	.41	.94	**.0007**	.10	.35	**.05**	8.0×10^{-9}	**.05**	*HNT*	Intron 1
rs1547897	139.7	131451988	.06	.16	.39	1.00	**.0006**	.16	.29	**.05**	4.8×10^{-9}	**.04**	*HNT*	Intron 1
rs11222931	139.8	131483366	.07	.15	.33	.87	**.0007**	.16	.27	**.05**	4.5×10^{-9}	**.04**	*HNT*	Intron 1
rs11222932	139.8	131483471	.07	.15	.34	.86	**.0006**	.18	.30	**.05**	4.6×10^{-9}	**.04**	*HNT*	Intron 1
rs11223225	142.7	132198420	**.03**	**.0004**	.26	**.02**	.09	.06	.47	.26	3.2×10^{-11}	**.03**	*OPCML*	Intron 1

NOTE.—Significant values are shown in bold.

[a] All SNPs with empirical P values $\leq .05$ are shown.

[b] According to the Marshfield map.

[c] According to the National Center for Biotechnology Information build 35 reference map.

[d] Fisher product over all tests.

[e] The probability of observing an equal or smaller Fisher product by chance per region, based on permutation test of 500,000 replicates.

[f] For intergenic SNPs, only the closest neighbor genes are listed.

tion with semantic ($P = .00003$) and phonological ($P = .04$) fluency (empirical $P = .02$). This SNP is 37.6 kb downstream of the *CYP2C19* (MIM 124020) gene and 48.1 kb upstream of the *CYP2C9* (MIM 601130) gene. At 1q25, the SNP *rs2584820* was associated with the Stroop test ($P = .0001$) and phonological fluency ($P = .03$), with an empirical P value of .04. This SNP is in intron 4 of the regulator of G-protein signaling like 2 (*RGSL2*) gene. Two other SNPs in this region showed association with the TMTB ($P = .0003$; empirical $P = .04$). They are 4 kb downstream of the *C1orf49* gene and 149 kb upstream of the Ral GEF with PH domain and SH3 binding motif 2 (*RALGPS2*) gene. At 3q23, the SNP *rs952797* was associated with the Stroop test ($P = .0001$), the Block test ($P = .0002$), and learning ($P = .06$). When all tests were evaluated simultaneously, the association was significant (empirical $P = .04$). This SNP is 126 kb downstream of the gene encoding nicotinamide nucleotide adenylyltransferase 3 (*NMNAT3* [MIM 608702]) and 131 kb upstream of the gene encoding calsyntein 2 (*CLSTN2*). SNP *rs11223225* (C→T) at 11q25 showed a consistent allelic effect across key cognitive domains for AD, including learning, delayed recall, and concept shifting (Stroop and TMTB), where the minor allele of this SNP is associated with poorer performance on delayed recall ($P = .0004$), learning ($P = .03$), the Stroop test ($P = .02$), TMTB ($P = .09$), and the Block test ($P = .07$). When the effect of various tests was combined, the overall empirical P value was .03. This SNP is in intron 1 of the gene encoding opioid binding protein/cell adhesion molecule-like (*OPCML* [MIM 600632]). Four close SNPs—*rs1629316, rs1547897, rs1122931,* and *rs11222932*—at 11q25 were associated with TMTB and phonological fluency. These SNPs are in intron 1 of the gene encoding neurotrimin (*HNT* [MIM 607938]). The *OPCML* and *HNT* genes are <80 kb apart.

Discussion

This study confirms earlier findings suggesting linkage of AD to a wide region that spans chromosome 1q21-31.[25,26,56] The 1q21 region yielded the most significant evidence of linkage over the genome in our study (HLOD = 5.2). This region was not replicated when testing for association with cognition in a series of 197 distantly related subjects. Although we cannot exclude the possibility of a false-positive finding, given the strength of the linkage signal and previous evidence, it is more likely that there is a rare mutation in a major gene in this region that could not be identified by association analysis in a small sample. This region contains the *NCSTN* gene, which binds presenilin and is required for γ-secretase activity and Aβ generation.[58] Mutations in this gene have been found to be related to early-onset AD, and we have reported association in a subgroup of patients with familial early-onset AD, particularly in those who lack the *APOE*4* allele.[59] We have sequenced all the exons and splice sites of this gene in six patients but have not found variants. Another obvious candidate gene in this region is the gene encoding C-reactive protein (*CRP* [MIM 123260]), which acts as a scavenger for chromatin released by dead cells during the acute inflammatory process.[60] We also sequenced the exons and splice sites of this gene in seven patients (5088, 5167, 5115, 5140, 5393, 5023, and 5394) (fig. 5*A*) and found that all patients except patients 5167 and 5393 carry the rare alleles of SNPs *rs1130864* (C→T) and *rs1417938* (T→A). The SNP *rs1130864* has been reported as a tagging SNP for a haplotype associated with higher levels of CRP.[61,62] We specifically tested the association of polymorphisms in *CRP* with cognitive function but failed to show any association (data not shown). Since CRP is a key protein involved in inflammation, a key process in life by itself, a major mutation in *CRP* seams unlikely for late-onset diseases, which suggests that another gene in the region may explain our high LOD score. In the 1q25 region, there was a second segregating haplotype. This region was confirmed in our association analysis by a SNP in intron 4 of *RGSL2,* which may be involved in the G-protein coupled receptor protein signaling pathway. Also, two SNPs upstream showed evidence of association. However, these SNPs were significant only for the TMTB and are intergenic, making it more likely that *RGSL2* is the relevant gene in the 1q25 region.

The second highest linkage signal was found at chromosome 3q22-24. This region was reported earlier to be linked to AD without tau pathology in a study of a small family with four affected relatives.[57] A significant LOD score of 4.1 between markers *D3S1569* and *D3S3554* was reported, whereas, in our study, *D3S1569* is also the marker that gives the highest HLOD over chromosome 3. In the study by Poduslo et al.,[57] no genomewide screen was conducted; only chromosomes 3 and 17 were screened, since the disease was expected to be related to frontotemporal dementia (FTD) and the phenotype was apparently considered to be compatible with that of FTD. Since we do not have pathology information for our patients, we cannot exclude the possibility that some of our patients also suffer from this atypical form of AD. However, all patients were carefully evaluated by a neurologist who specializes in FTD. A recent linkage-based genome scan of Caribbean Hispanic families revealed a new locus on chromosome 3q28, with a 2-point LOD score of 3.09 at marker *D3S2418*.[35] However, this region is ~50 cM downstream of the region we identified in our study. The linked region on chromosome 3q22-24 contains various possible candidate genes, including the *TF* gene, the gene encoding for butyrylcholinesterase (*BCHE* [MIM 177400]), the neprilysin gene (*MME* [MIM 120520]), and the somatostatin gene (*SST* [MIM 182450]). We screened these genes for mutations, but no variants were found. The SNP *rs952797* at 3q23 was consistently associated with cognitive function in the 197 unrelated subjects from the GRIP population. This SNP is 126 kb downstream of the *NMNAT3* gene encoding nicotinamide nucleotide adenylyltransferase 3 (NAD3). The coenzyme NAD and its derivatives are involved in hundreds of metabolic redox reactions and

are used in protein ADP-ribosylation, histone deacetylation, and in some Ca^{2+}-signaling pathways. NMNAT is a central enzyme in NAD biosynthesis, catalyzing the condensation of nicotinamide mononucleotide or nicotinic acid mononucleotide with the AMP moiety of ATP to form NAD or NaAD (Zhang et al.[63]); thus, the *NMNAT3* gene may relevant to AD. The SNP *rs952797* is 131 kb upstream of the *CLSTN2* gene. It has been reported recently that SNP *rs6439886,* a common T→C substitution within the first intron of *CLSTN2,* was significantly associated with memory performance.[64] Our SNP *rs952797* is 160 kb upstream of the reported SNP.

Chromosome 10q22-24 is the third highest peak over the genome. This finding is consistent with previous findings about AD[15,20–22,24] and plasma amyloid β42 levels.[65] Our finding of linkage to late-onset AD at 10q22-24 is the first replication with use of a data set fully independent of the National Institute of Mental Health (NIMH) sample. So far, it has been difficult to identify the causal mutation(s) in this region. A series of genes has been densely genotyped, and several genes have been noted to be susceptibility genes for AD, including *IDE, CH25H* (MIM 604551), *PLAU* (MIM 191840), and *LIPA* (MIM 278000). In our linkage analysis, there was not a single haplotype segregating in the region, suggesting that multiple mutations in one or multiple genes may contribute to the linkage. In our association analysis, the most significant evidence of association with cognitive function was seen for this region. Three SNPs:—*rs17129662, rs11185978,* and *rs7071717,* together at 91.7 Mb—showed association with multiple cognitive domains in the 197 unrelated subjects from the GRIP population. These SNPs are intergenic SNPs of known genes. All three SNPs are <1 Mb upstream of the *CH25H* gene and the *LIPA* gene and <3 Mb downstream of the *IDE* gene. The genes most closely flanking these SNPs are the *MPHOSPH1* gene and the *HTR7* gene, which encodes 5-hydroxytryptamine receptor 7. These two genes, however, have not been extensively investigated. Another associated SNP, *rs4110517* at 116.8 cM, is surrounded by four similar genes—*CYP2C18* (MIM 601131), *CYP2C19, CYP2C9,* and *CYP2C8* (MIM 601129)—in a range of <350 kb. Since no significant association was found between the *CYP2C19* gene and patients with familial AD in a previous study,[66] it is more likely that the SNP *rs4110517* is in LD with the causal gene(s) in the region.

We also found suggestive evidence of linkage to chromosome 11q25. Blacker et al.[22] previously described this region in their study of the NIMH sample, including 437 families with AD. Recent evidence suggests the *SORL1* gene may be responsible.[30] Our linkage peak is, however, ~23 cM downstream of the *SORL1* gene. We specifically tested the association between polymorphisms flanking *SORL1* and cognitive function but failed to detect consistent associations (data not shown), suggesting that our linkage peak may be explained by other gene(s). The association for SNP *rs11223225,* a C→T substitution at 11q25, is one of the most promising results from our association analysis. The T allele of this SNP is consistently associated with reduced cognitive performance on multiple domains, and this SNP is an intronic SNP of the *OPCML* gene, which encodes the opioid-binding protein. There is evidence that the opioidergic system is affected in AD.[67] Furthermore, performance on immediate memory and mental flexibility tasks has been suggestively linked to 11q25 in a recent genomewide linkage study of 260 families.[68] Four other close SNPs at 11q25 also showed association with cognitive function. These SNPs are in intron 1 of the *HNT* gene, which encodes neurotrimin. Notably, the *OPCML* and *HNT* genes are separated by <80 kb.

In summary, we confirmed two previously well-described linkage regions for late-onset AD on chromosomes 1q21-25 and 10q22-24. With cognitive function as an endophenotype of AD, our study specifies the *RGSL2, RALGPS2,* and *C1orf49* genes at 1q25. Our analysis of chromosome 10q22-24 points to the *HTR7, MPHOSPH1,* and *CYP2C* cluster. To our knowledge, this is the first genomewide screen that showed significant linkage to chromosome 3q23 markers. For this region, our analysis identified the *NMNAT3* and *CLSTN2* genes. Our findings confirm linkage to chromosome 11q25. We were unable to confirm *SORL1*; instead, our analysis points to the *OPCML* and *HNT* genes.

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Web Resources

The URLs for data presented herein are as follows:

AlzGene Database and Alzheimer Research Forum, http://www.alzgene.org/

GENEHUNTER, http://linkage.rockefeller.edu/soft/gh/ (for multipoint linkage analysis with the Lander-Green algorithm)

Merlin, http://www.sph.umich.edu/csg/abecasis/Merlin/download/ (for detecting unlikely double-recombination events and haplotype construction)

MGA, http://mga.bionet.nsc.ru/soft/index.html (for PedCut)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for *PSEN1, PSEN2, APP, APOE, ACE, CHRNB2, CST3, ESR1, GAPDHS, IDE, MTHFR, NCSTN, PRNP, TF, TFAM, TNF, SORL1, HTR7, CYP2C19, CYP2C9, NMNAT3, OPCML, HNT, CRP, BCHE, MME, SST, CH25H, PLAU, LIPA, CYP2C18,* and *CYP2C8*)

Pedfiddler, http://www.medicine.mcgill.ca/statgene/software.html (v. 0.5, for drawing large pedigrees)

Statgen, http://watson.hgen.pitt.edu/register/ (for PedCheck [for detecting Mendelian errors] and SIMWALK [for multipoint linkage analyses with the Markov chain–Monte Carlo method])

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