A Cluster of Cholesterol-Related Genes Confers Susceptibility for Alzheimer's Disease

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Objective: Polygenic diseases are related to the complex interplay of genetic variations. We evaluated whether clusters of cholesterol- and lipid-related genetic variations are associated with Alzheimer's disease.

Method: We analyzed 12 cholesterol-related single nucleotide polymorphisms and 48 control polymorphisms in 545 study participants (Alzheimer's disease group N = 284; control group N = 261). Diagnoses of Alzheimer's disease were made according to the NINCDS-ADRDA criteria. Multi-locus genetic association analysis was done with the set-association method. Dates of data collection were from January 2000 to December 2003.

Results: We identified a cluster of polymorphisms in *APOE, SOAT1, APOE* 5'-untranslated region, *OLR1, CYP46A1, LPL, LIPA,* and *APOA4* conferring significant (p = .0002) susceptibility for Alzheimer's disease. This gene cluster reached a diagnostic accuracy of 74% and correlated significantly (p = .018) with the levels of the brain cholesterol catabolite 24S-hydroxycholesterol in the cerebrospinal fluid.

Conclusion: Our results establish a novel approach for the identification of disease-related genetic clusters and demonstrate the need for multilocus methods in the genetics of complex diseases.

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Alzheimer's disease (AD) is a common, age-related neurodegenerative disorder. The prevalence of AD in the general population increases from 1% in persons younger than 60 years to approximately 40% in nonagenarians.[1] Clinically, AD is characterized by progressive cognitive deficits such as impairment of memory and orientation. With disease progression, psychopathologic symptoms such as delusions, agitation, changes in personality, and mood disturbances occur frequently[2] and are a major cause of caregiver distress and nursing home placement.[3]

AD is a genetically complex trait and thereby results from the interplay between genetic variants and environmental factors.[4,5] In late-onset AD, high heritability estimates suggest that naturally occurring genetic variations have a major impact on the development and the psychopathologic manifestation of this devastating disorder.[2,6] Therefore, the identification of these genetic factors may have important clinical implications for future diagnosis and potential personalized treatments. However, of the many possible genetic polymorphisms, only the ε4 allele of the *APOE* gene has been so far reliably associated with disease risk. Results for additionally proposed susceptibility genes have not been consistently reproducible.

Besides some obvious reasons (e.g., studies in small populations, inaccurate phenotypes, genotyping errors), recent developments in complex genetics have identified 2 main causes for the existing ambiguities: First, genetic association studies on AD have either evaluated 1 genetic variation at a time or have analyzed multiple variations independently and have thereby ignored the polygenic nature of sporadic, late-onset AD. These approaches focus on marginal genotype effects, inflate the probability of false-positivity, and erroneously assume that the impact of the variation under study is comparable to a major gene effect. Second, the possibility of nonrandom genetic heterogeneity (i.e., hidden population structure) has not been thus far considered in AD genetics. However, a failure to detect and to control for hidden population structure may result in false-positive and/or false-negative results.[7] Importantly, recent advances in genotyping technology and computerized algorithms have resulted in practicable methods for the calculation of population structure.

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To address these issues and to identify AD-associated genetic patterns, we investigated the simultaneous impact of single nucleotide polymorphisms (SNPs) on the risk for developing AD and controlled for genetic heterogeneity of the study population. In this study, we focused our search on genes implicated in the cholesterol and lipid metabolism because brain cholesterol is tightly linked to the generation of the β-amyloid peptide and to AD risk[8,9] and because the best-established AD risk gene, *APOE*, is the major cholesterol transporter in the central nervous system (CNS). We also limited our search to only those cholesterol-related SNPs reportedly associated with AD in previous single-gene studies[10–19] to provide a reliable estimate of the power of the clustering method compared with the conventional SNP-by-SNP approaches.

The calculation of genetic risk was done by the set-association method, which evaluates polymorphic markers throughout the genome and results in a single test statistic.[20] By simultaneous analysis of several SNPs and extensive permutation testing, the set-association method controls for type I statistical error and considers the existence of gene-gene interactions. In addition, the power of the set-association method has been shown to be superior to the corresponding power of single-locus statistics[20]; however, specific power analyses are not available for this method.

Association studies in outbred populations such as the present one may be prone to false-positivity because nonrandom genetic heterogeneity within the study sample (i.e., hidden population structure) can lead to spurious associations between a candidate marker and a phenotype. Hidden population structure within the study sample may be a result of sampling bias when recruiting cases and their appropriate controls (an extreme example for such a condition would be to recruit white AD patients and control subjects of Asian origin). In that case, alleles that are in high frequency in one subpopulation would be associated with any disease that is most prevalent in that subpopulation. Thus, a subtle and unequal ethnic admixture in case-control samples may lead to spurious, false-positive associations with the phenotype of interest.

Because allele frequencies at random marker loci may differ among ethnic groups, a consistent pattern of allele-frequency differences between cases and controls will be detected if the cases and controls are not well matched. Conversely, if the cases and controls are well matched, significant allele-frequency differences will be located only near disease-susceptibility loci. In the present study, calculation of hidden population structure was done by the structured association method, which uses unlinked genetic markers to detect possible population stratification.[7] We therefore genotyped each subject for 48 random SNPs located in non-genic regions and distributed over the autosomes.

Table 1. Characteristics of Patients and Controls Recruited for a Study to Identify Alzheimer's Disease (AD)–Associated Genetic Patterns

Characteristic	Control Subjects (N = 261)	AD Patients (N = 284)
Age at examination, mean ± SD, y	71 ± 7	74 ± 7
Age at onset, mean ± SD, y		68 ± 7
Female gender, N (%)	130 (50)	163 (57)
MMSE score, mean ± SD	29 ± 1	20 ± 5
APOE3 allele frequency	0.748	0.625
APOE4 allele frequency	0.166	0.344
APOE2 allele frequency	0.086	0.031

Abbreviation: MMSE = Mini-Mental State Examination.

Here we report that a cluster of cholesterol-related SNPs confers significant susceptibility for AD.

MATERIALS AND METHOD

Populations

Genetic studies were conducted in 545 participants from 2 populations: a Swiss-German sample (366 participants) and a Mediterranean sample (179 participants from Northern Greece). Patients were recruited from the outpatient memory clinics of the participating institutions. The clinical diagnoses of AD (N = 284; 150 Swiss-German patients, 134 Greek patients) were made according to the NINCDS-ADRDA (National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer's Disease and Related Disorders Association) criteria[21] on the basis of medical interview, physical examination, neuropsychological testing, and brain magnetic resonance imaging or computed tomography, as well as blood and cerebrospinal fluid (CSF) tests. The mean ± SD age at onset of AD was 68 ± 7 years, and the mean Mini-Mental State Examination (MMSE) score was 20 ± 5. Of the AD patients, 163 (57%) were women (Table 1).

The control group (N = 261; 216 Swiss-German subjects, 45 Greek subjects) consisted of elderly individuals without severe medical conditions who were either the spouses of AD patients or subjects recruited from the outpatient clinics of the participating institutions. Dementia and memory deficits in the control group were excluded by neuropsychological testing, consisting of the CERAD (Consortium to Establish a Registry for Alzheimer's Disease) neuropsychological test battery[22] and the MMSE.[23] The mean age at examination was 71 ± 7 years, and the mean MMSE score was 29 ± 1. Among the control subjects, 130 (50%) were women.

CSF was collected from 21 nondemented elderly subjects (mean ± SD age: 66 ± 12 years; 7 women) who underwent spinal anesthesia before surgical intervention. Because our goal was to study the functional relevance of the genetic cluster independent of disease, we recruited nondemented individuals only. The levels of CSF 24S-

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hydroxycholesterol were determined as described previously.[24]

Informed consent was obtained from all participants, and the local human studies committees approved the study protocol.

Genotyping

Information on polymorphic sites was derived from the database of single nucleotide polymorphisms (dbSNP) established by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/index.html). Twelve SNPs in 10 genes were selected for gene clustering. SNP rs754203 in *CYP46A1*, rs1044925 in *SOAT1*, rs13500 in the *CH25H/LIPA* region, rs268 and rs328 in *LPL*, rs5104 in *APOA4*, rs1799986 in *LRP1*, rs405509 in the *APOE* promoter, rs1050286 in *OLR1*, rs1800206 in *PPARA*, and rs2228671 in *LDLR* were analyzed by Pyrosequencing AB, Uppsala, Sweden, on our Luc 96 machine. *APOE* genotyping was done on the LightCycler (Roche Diagnostics, Basel, Switzerland).[25]

In addition to SNP rs754203 in *CYP46A1* analyzed here, an SNP in intron 3 of the same gene has been also associated with AD risk.[26] We did not, however, use this SNP in this study because it is tightly linked to rs754203 and because it failed to exert independent effects on disease risk in our population.

Genotyping of the 48 unlinked control SNPs for the calculation of population structure was done by the Amplifluor method (Table 2).

Statistics

We used the SUMSTAT program (http://linkage.rockefeller.edu/ott/sumstat.html), which has been developed for statistical analysis with the set-association method.[20] The set-association method combines the information derived from measurements of allelic association and deviation from Hardy-Weinberg equilibrium into a single, genome-wide statistic. SNPs with high Hardy-Weinberg disequilibrium (HWD) values in the control population are set equal to zero ("trimming") and are not considered for further statistical analysis. For the remaining SNPs, effects of allelic association with disease and HWD values are combined into 1 statistic, because moderate HWD values in affected individuals are indicative of genetic association to a susceptibility locus. Genome-wide significance of this statistic is calculated by permutation tests. In the present study, 1 SNP (rs1800206 in *PPARA*) with high HWD value in control subjects ($p < .05$) was trimmed. The number of permutation tests was set at 50,000.

The calculation of the individual cholesterol-related genetic risk score (CREGS) was based on the combined set-association statistic for each cluster SNP. Thus, CREGS corresponds to the number of individual risk alleles weighted by the contribution (i.e., effect size) of each risk allele to the overall cluster. The genetic score of individuals carrying no risk allele was set equal to 0, which is the minimal possible CREGS value. Accordingly, CREGS of individuals possessing 1 risk allele was set equal to the corresponding set-association statistic for the particular SNP (e.g., 1.8 for *APOE4*, 0.3 for *OLR1*). For 2 or more risk alleles, set-association statistics of the corresponding SNPs were added. Depending on whether the *APOE* genotype was included in or excluded from CREGS, the maximal possible CREGS value is 2.5 (*APOE4* not included), 4.3 (*APOE4* included using the dominant model), and 6.1 (*APOE4* included using the additive model). An adjustment for a possible protective role of the *APOE2* allele was not performed because *APOE2* failed to be part of the gene cluster independent of *APOE4*.

The study sample was divided randomly into 2 equally large subsamples (i.e., a training set and an application set) prior to the calculation of the final individual genetic score. SNPs represented in the clusters of both sets were considered for the calculation of the final score.

Calculation of population structure was done using the STRUCTURE program[27] following the developers' instructions.[7]

RESULTS

Set-Association Results

Set-association analysis revealed that SNPs in the following genes and gene regions constituted a cluster with significant impact on AD risk: *APOE, SOAT1, APOE* promoter, *OLR1, CYP46A1, LPL, LIPA,* and *APOA4* (Figure 1). SNPs in *LDLR, LRP1,* and *PPARA* failed to contribute to the significance of the cluster. The significance level reached by the cluster SNPs was $p_{min} = .0001$. After 50,000 permutations, the final corrected significance was $p_{min-min} = .0002$. Set-association analysis in the Swiss cohort only yielded identical clustering results at a lower significance level ($p_{min-min} = .0008$), which is attributed to the lower statistical power. We did not perform a separate set-association analysis in the Greek cohort sample due to the low number of Greek control subjects.

Genetic Heterogeneity Control

To calculate and control for nonrandom genetic heterogeneity (i.e., hidden population structure) we genotyped each subject for 48 unlinked SNPs located in non-genic regions and distributed over all autosomes (Table 2). Structured association analysis[7] revealed a low allele-frequency divergence in our population and excluded nonrandom genetic heterogeneity as a potential source of false positivity (Figure 2). Of note, the genetic heterogeneity control was done in the Swiss sample only. Because inclusion of the Greek sample did not alter the set-association results obtained in the Swiss sample, we exclude the possibility that a potentially distinct genetic substructure of the Greek sample would have biased the results.

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Table 2. Characteristics of the 48 Unlinked Control Single Nucleotide Polymorphisms (SNPs) and Corresponding Primers for Which Each Subject Was Genotyped in a Study to Identify Alzheimer's Disease–Associated Genetic Patterns[a]

SNP ID	Chromosome	Location	Primers
rs720413	1	46589740	GAAGGTGACCAAGTTCATGCTCCAGAGCTAGATGCATAGAACCTATAA GAAGGTCGGAGTCAACGGATTCAGAGCTAGATGCATAGAACCTATAG ACCCCAGGGAGGGGATTAAAAGTT
rs531924	1	73291219	GAAGGTGACCAAGTTCATGCTGGCCCTCTCTTAGAAGATCTAAATGAA GAAGGTCGGAGTCAACGGATTGCCCTCTCTTAGAAGATCTAAATGAG CCCAATGTTCTTTGCAGAGGCAAATCATTT
rs1693258	1	47853114	GAAGGTGACCAAGTTCATGCTCCATTTATAAAATGGCCATAATGAGACTTAT GAAGGTCGGAGTCAACGGATTCATTTATAAAATGGCCATAATGAGACTTAC TTCACCCTCACCACAACCTCATCAA
rs719154	2	235085990	GAAGGTGACCAAGTTCATGCTACATGTGCTGAAGTGAGAACTCCAA GAAGGTCGGAGTCAACGGATTCATGTGCTGAAGTGAGAACTCCAG GCTGGATAAGCAATACTGCCTGCTT
rs2173234	3	21045907	GAAGGTGACCAAGTTCATGCTAACTTTGCATCCTTTCAGCTGTTAGTA GAAGGTCGGAGTCAACGGATTCTTTGCATCCTTTCAGCTGTTAGTG CCAGGCTTCATATTTCATATCAGCATAT
rs1365113	3	188713641	GAAGGTGACCAAGTTCATGCTCCAGACAGAAGAAAGTGAGGAAAC GAAGGTCGGAGTCAACGGATTTCCAGACAGAAGAAAGTGAGGAAAA CCATGTTGTTCTCCCCACTGACAT
rs166315	3	22058552	GAAGGTGACCAAGTTCATGCTCCTAAATATGTTACTGAAGGTTTATCAGA GAAGGTCGGAGTCAACGGATTCCTAAATATGTTACTGAAGGTTTATCAGG CCAATTCTAGTCCCATGTTTGACCCAA
rs3846211	3	187627454	GAAGGTGACCAAGTTCATGCTGATTTTGCATCTAGCCATATTTCTTTATCTT GAAGGTCGGAGTCAACGGATTTTGCATCTAGCCATATTTCTTTATCTC ACTGGATTTCAACAAGATCTCTCAGCATA
rs1587120	4	21156512	GAAGGTGACCAAGTTCATGCTACGGTTTGAATGCCTGTGTC GAAGGTCGGAGTCAACGGATTGCTACGGTTTGAATGCCTGTGTG GATTAGGTTCCAACCTTAGAATTTTGAAGA
rs2867465	4	80080803	GAAGGTGACCAAGTTCATGCTCATGGAAGGGTAAAGAATCAACACATT GAAGGTCGGAGTCAACGGATTCATGGAAGGGTAAAGAATCAACACATG CTGGTTTGATATTTGTGTGACTTTTGC
rs1843486	4	22437069	GAAGGTGACCAAGTTCATGCTAAATGGAGAGGCAGAGGGAAGAATA GAAGGTCGGAGTCAACGGATTGGAGAGGCAGAGGGAAGAATC GGGCAACATCCCATAGTTTTCGGTA
rs2709847	4	81001221	GAAGGTGACCAAGTTCATGCTCAAGGGCTGAAATTCATGATTGAAAGA GAAGGTCGGAGTCAACGGATTAAGGGCTGAAATTCATGATTGAAAGC CTCAAGTTCAACACTTTGGAAAGCACAAA
rs1588625	5	100509786	GAAGGTGACCAAGTTCATGCTCCAAGAAATAGACACATACAAATAGAGTC GAAGGTCGGAGTCAACGGATTACCAAGAAATAGACACATACAAATAGAGTA CCTTTGTTCCTGTATCAAAGACCATTT
rs1382902	5	170972136	GAAGGTGACCAAGTTCATGCTCACATTAATCAAGGTGATTATTTAAGCCTT GAAGGTCGGAGTCAACGGATTCACATTAATCAAGGTGATTATTTAAGCCTC CTCACCATAACCCTTTCTCTATTTGTA
rs1020922	5	101515886	GAAGGTGACCAAGTTCATGCTATAGTTATGCCAGCCAAGGTGTATA GAAGGTCGGAGTCAACGGATTATAGTTATGCCAGCCAAGGTGTATG GTGTAGTAAAGACCAGAGTCTTGTTCTAA
rs793027	5	171017488	GAAGGTGACCAAGTTCATGCTCATCCCCACTTTCCAGGTAGCAA GAAGGTCGGAGTCAACGGATTATCCCCACTTTCCAGGTAGCAC CAATGCCACCTTGGGTGACCTTTAT
rs950286	6	374457	GAAGGTGACCAAGTTCATGCTATTGTACCCATTGCAACATTTCCCTTT GAAGGTCGGAGTCAACGGATTGTACCCATTGCAACATTTCCCTTC TCTGGAAGCTGTGAGGCTGGAA
rs1358883	6	167376721	GAAGGTGACCAAGTTCATGCTCAGCTTGCTCACTGCAATCCTAAC GAAGGTCGGAGTCAACGGATTCAGCTTGCTCACTGCAATCCTAAG ATTTCCACCGAGTGGCTGGCCTCA
rs196454	6	168830614	GAAGGTGACCAAGTTCATGCTCACCTCTCCCTGCTGGACCAT GAAGGTCGGAGTCAACGGATTCCTCTCCCTGCTGGACCAC TCAGCAAGGAAACTTTTCAAAGTCGCTAA
rs1589764	7	10140307	GAAGGTGACCAAGTTCATGCTGTGGAAACTTGGGTTCTGAGACA GAAGGTCGGAGTCAACGGATTGTGGAAACTTGGGTTCTGAGACG GCTGAGTCTCTCCTGAAACCAGTA
rs1208887	7	88531581	GAAGGTGACCAAGTTCATGCTAGCCAGCACATCTCAGAGTCTCAT GAAGGTCGGAGTCAACGGATTCCAGCACATCTCAGAGTCTCAC GCAATACCCAGTTAGTATCAAAAACCTTTG
rs764195	7	11361182	GAAGGTGACCAAGTTCATGCTCATAACTGTATTTTCACAGTACTTCTGGA GAAGGTCGGAGTCAACGGATTAACTGTATTTTCACAGTACTTCTGGG CAGCTAGATCTGTTTAGAAAGTCTTGGAT
rs723497	8	50436581	GAAGGTGACCAAGTTCATGCTGAATTTTAAAACTCATGATGTTCCCAGC GAAGGTCGGAGTCAACGGATTGAATTTTAAAACTCATGATGTTCCCAGG TGAGGACGGTATCCAGAAGCCAAAT
rs723597	8	51794768	GAAGGTGACCAAGTTCATGCTTTAAACAGCTTTATGTGAATTGCTATTTG GAAGGTCGGAGTCAACGGATTCTTTAAACAGCTTTATGTGAATTGCTATTTC TCCAGGCTGTGAGAAGAGATTTAAACATA
rs720767	9	129896217	GAAGGTGACCAAGTTCATGCTAGCCGTGAGACCAGACCCGA GAAGGTCGGAGTCAACGGATTCCGTGAGACCAGACCCGC GGTTTGGATTGAGCATCCTTA

continued

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Table 2. Characteristics of the 48 Unlinked Control Single Nucleotide Polymorphisms (SNPs) and Corresponding Primers for Which Each Subject Was Genotyped in a Study to Identify Alzheimer's Disease–Associated Genetic Patterns[a] (cont.)

SNP ID	Chromosome	Location	Primers
rs2209299	10	10152239	GAAGGTGACCAAGTTCATGCTGACCTCAGTAAAGGGGATGCAGA GAAGGTCGGAGTCAACGGATTACCTCAGTAAAGGGGATGCAGG CTCAGGACAGCAACTTTTA
rs717799	10	11923870	GAAGGTGACCAAGTTCATGCTGGGCAGCAGGGACCAGGT GAAGGTCGGAGTCAACGGATTGGGCAGCAGGGACCAGGC GATGGAGCTGCAGCTGTAAATGTGTA
rs2173004	11	10801939	GAAGGTGACCAAGTTCATGCTCCTAGAATTCATCCCTCAACTTCA GAAGGTCGGAGTCAACGGATTCCTAGAATTCATCCCTCAACTTCG GGTAGAGAATGTATTGGACAGCAAGAGA
rs2168273	11	11786797	GAAGGTGACCAAGTTCATGCTAAGAAATCTCAGCTGGTGTTCTGAT GAAGGTCGGAGTCAACGGATTAAGAAATCTCAGCTGGTGTTCTGAC CAGAATGATCAGCCTATAGTTGGCATTTA
rs717996	12	82636288	GAAGGTGACCAAGTTCATGCTGCATACAGTGGCAAGAAACTGTCA GAAGGTCGGAGTCAACGGATTGCATACAGTGGCAAGAAACTGTCG GTAATCCTACATGAGAGATT
rs1452237	12	83682596	GAAGGTGACCAAGTTCATGCTTAAAACAAAGTCTTACTGGAGTAGAATGA GAAGGTCGGAGTCAACGGATTAAACAAAGTCTTACTGGAGTAGAATGG AAGGACATAAGTCATACTGAATTAAGGGC
rs727227	13	41123418	GAAGGTGACCAAGTTCATGCTGCAGCCAAGATTCAATGCTTGGAAAT GAAGGTCGGAGTCAACGGATTCAGCCAAGATTCAATGCTTGGAAAG CCCTTGTTTGTCCTCCAACTTAAACTT
rs1888302	13	42173330	GAAGGTGACCAAGTTCATGCTAGGAAGTTAGCTGTAATAGAACAATATGT GAAGGTCGGAGTCAACGGATTGGAAGTTAGCTGTAATAGAACAATATGC CCTTTTTCACCCACACAGTCTTTCTTAAA
rs728087	14	36202393	GAAGGTGACCAAGTTCATGCTGATAGCTAGCAGGTTAATTTACTCCA GAAGGTCGGAGTCAACGGATTGATAGCTAGCAGGTTAATTTACTCCG GGTTCATGGTTTTTCATTTGGAGGAA
rs1951218	14	37325407	GAAGGTGACCAAGTTCATGCTCCTAATCAGATAATCACCTGCTGCA GAAGGTCGGAGTCAACGGATTCTAATCAGATAATCACCTGCTGCG CCTTGTTTAGGAAGATCCTCTAGAGTTTA
rs1564492	15	71649535	GAAGGTGACCAAGTTCATGCTAGGCTTCCTGGCCATTTATGTTCT GAAGGTCGGAGTCAACGGATTAGGCTTCCTGGCCATTTATGTTCC AGGGAAGGGCCATTTGTCCTTT
rs2198843	15	72717047	GAAGGTGACCAAGTTCATGCTGGACTCCTAAATACCCTGCAG GAAGGTCGGAGTCAACGGATTCTGGACTCCTAAATACCCTGCAC CCTCCCCAAGCATCACAAGGGTT
rs1560107	16	12675017	GAAGGTGACCAAGTTCATGCTCCAGCACGTATTGATGGATGTGTT GAAGGTCGGAGTCAACGGATTCCAGCACGTATTGATGGATGTGTC GGTGGTCAGAACACAACTGGCTTT
rs2031084	16	13685956	GAAGGTGACCAAGTTCATGCTTATGGTACTATGTCTATTGCACCACA GAAGGTCGGAGTCAACGGATTATGGTACTATGTCTATTGCACCACG CCATTTACCTAGACAAAAGTATAGGTGATA
rs1465650	17	8784384	GAAGGTGACCAAGTTCATGCTCACTGCTTCCATCATCCACCAGA GAAGGTCGGAGTCAACGGATTACTGCTTCCATCATCCACCAGG AGTTCCTCCCTCTCCCACTCACATT
rs1426692	18	34393649	GAAGGTGACCAAGTTCATGCTGTCCTCTTCCCAATTAGCTGAACTA GAAGGTCGGAGTCAACGGATTCCTCTTCCCAATTAGCTGAACTG CTCCAGATGGAAATTGACTTCAGGATT
rs1788930	18	35373810	GAAGGTGACCAAGTTCATGCTGGGTGTCTTTAAATGGAAATACACACT GAAGGTCGGAGTCAACGGATTGGGTGTCTTTAAATGGAAATACACACA GCCCATGTTAGTTTTATGACTGATCAGTA
rs1384936	19	5374643	GAAGGTGACCAAGTTCATGCTGAGAAGAGATGGCTTCACTGATAAAT GAAGGTCGGAGTCAACGGATTGAGAAGAGATGGCTTCACTGATAAAC ATAGGTGGATGGACAGGTGAGTCT
rs348384	19	6503386	GAAGGTGACCAAGTTCATGCTCAGAGATTTATCAAATTGGCAGGTTACA GAAGGTCGGAGTCAACGGATTAGAGATTTATCAAATTGGCAGGTTACG CCTGGCTCTCCCAGGAGGTTTT
rs2410417	21	41578882	GAAGGTGACCAAGTTCATGCTACACATTAAAGATCACATTAATAAACAATAGT GAAGGTCGGAGTCAACGGATTACACATTAAAGATCACATTAATAAACAATAGG CTGATCCCATCTATGTGTCTGTTT
rs461466	21	40117964	GAAGGTGACCAAGTTCATGCTGTAGAACTCCACCTTCTTTGTTCACA GAAGGTCGGAGTCAACGGATTAGAACTCCACCTTCTTTGTTCACG AGCACTATTTGGTCAAACTTACAAACTCAT
rs137257	22	33480563	GAAGGTGACCAAGTTCATGCTGATGAAGGTGGAGATGCTTTAAAGG GAAGGTCGGAGTCAACGGATTATGATGAAGGTGGAGATGCTTTAAAGT GCTTATTTAATTAACAGGCATAAATTCCAT
rs132692	22	34850979	GAAGGTGACCAAGTTCATGCTGCGAAGTGTGTTAGCTCATGACA GAAGGTCGGAGTCAACGGATTGCGAAGTGTGTTAGCTCATGACG GGAGCATCATGTGGATTCCTTTCCAT

[a]SNP ID is given by the reference cluster number (rs#) in the NCBI database for single nucleotide polymorphisms (dbSNP, build 121; http://www.ncbi.nlm.nih.gov/SNP/index.html). "Location" indicates the distance in bases from pter of the corresponding chromosome according to dbSNP build 121. In the "primers" column, the first 2 rows represent the primers used for allele-specific amplification with the last nucleotide indicating the polymorphic region. The third row represents the common amplification primer. All primers are listed in 5' – 3' direction.

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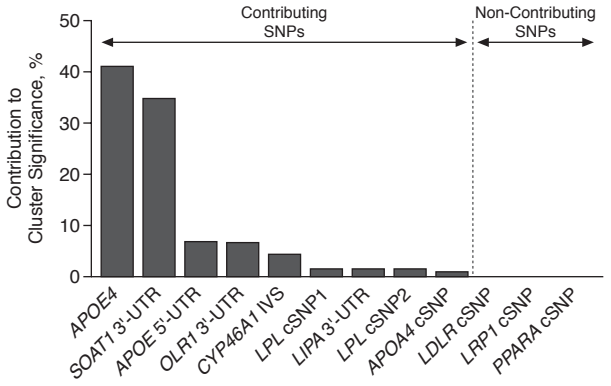
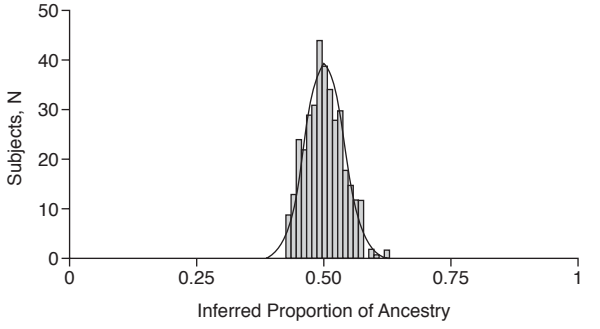
Figure 1. Cluster of Cholesterol- and Lipid-Related Single Nucleotide Polymorphisms (SNPs) Associated With Alzheimer's Disease[a]



[a]SNPs are added to the model according to their relative contribution to overall cluster significance ($p_{min-min} = .0002$) with the most significant SNP added first. The last 3 SNPs (*LDLR*, *LRP1,* and *PPARA*) failed to contribute to overall cluster significance.
Abbreviations: cSNP = SNP in coding region, IVS = intronic SNP, UTR = untranslated region of the gene.

Figure 2. Genetic Structure of a Population Enrolled in a Study of Alzheimer's Disease–Associated Genetic Patterns[a]

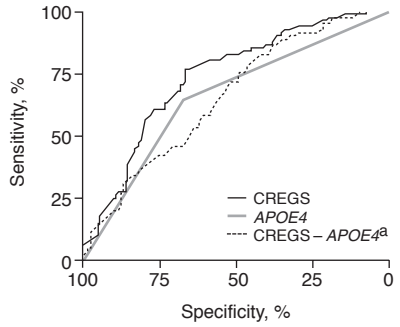


[a]Estimates of the ancestry of study subjects under the a priori assumption of $K = 2$ discrete subpopulations. The histogram shows the number of individuals with distinct proportions of ancestry in subpopulation 1. Using 48 unlinked single nucleotide polymorphisms, structured association analysis revealed that the allele-frequency divergence between the 2 subpopulations was low (Kullback-Leibler distance = 0.15). The superimposed curve indicates normal distribution of the data (p = .4, Kolmogorov-Smirnov-test). Identical results were obtained under the a priori assumption of $3 \leq K \leq 6$ discrete subpopulations.

Genetic Risk Score

SNPs composing the significant cholesterol cluster were considered for calculation of the individual CREGS, which corresponds to the number of risk alleles present in a subject weighted by the effect size of the risk alleles. Receiver operating characteristics (ROC) analysis revealed that CREGS differentiated highly significantly between AD patients and healthy control subjects ($p < .001$, Figure 3). The area under the ROC curve (AUROC) reached a value of 0.74, indicating that the proportion of correct diagnostic classifications based on CREGS information only was 74%. In addition to CREGS, the *APOE4* allele alone and CREGS excluding the *APOE4* effect also differentiated between AD patients and healthy control subjects ($p < .001$, Figure 3) with an AUROC of 0.66 for both curves. However, the areas under these curves were significantly lower than the AUROC of CREGS ($p < .05$).

Figure 3. Area Under the Receiver Operating Characteristic Curve Indicating the Ability of the Individual CREGS to Discriminate Between AD Patients and Healthy Control Subjects[a]



[a]CREGS without considering the *APOE4* effect.
Abbreviations: AD = Alzheimer's disease, CREGS = cholesterol-related genetic risk score.

CSF 24S-Hydroxycholesterol

CREGS represents a joint score of genes involved in different pathways of the cholesterol metabolism. Therefore, we hypothesized that CREGS might be related to the final step of this pathway and analyzed the CSF levels of the final catabolite of brain cholesterol metabolism, 24S-hydroxycholesterol, in 21 independently recruited non-demented subjects. CREGS correlated significantly and positively with the CSF levels of 24S-hydroxycholesterol ($r = 0.510$, $p = .018$, Figure 4).

DISCUSSION

The risk for the development of AD is related to a complex interplay of genetic variations. Whereas the AD phenotype is per definition characterized by the presence of cortical β-amyloid depositions,[28] AD-related genetic variations are not restricted to the amyloid precursor protein gene[29] but are also localized in genes related to such biological pathways as proteolysis, neuroinflammation, and the lipid metabolism. This diversity of genetic susceptibility factors along with the recent development of high-throughput genotyping enables multi-locus analyses for the localization of complex human trait genes. Multi-locus analyses acknowledge the polygenic nature of the trait under study, provide reliable sets of trait-associated genetic markers, and control for multiple testing.

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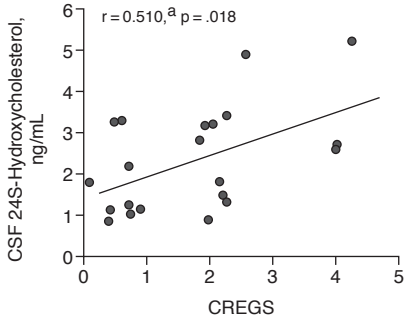
Figure 4. CREGS Correlates Significantly With CSF Levels of 24S-hydroxycholesterol in 21 Nondemented Elderly Subjects



[a]Pearson correlation coefficient.
Abbreviations: CREGS = cholesterol-related genetic risk score, CSF = cerebrospinal fluid.

Importantly, multi-locus analyses and the resulting individual genetic risk clusters may have important clinical implications for future diagnosis, prognosis, and potential personalized treatments.

In the present study, we analyzed 12 SNPs across 10 cholesterol-related genes in 545 participants and applied multi-locus statistics to extract a pattern of AD-associated SNPs. We also controlled for hidden population structure by analyzing 48 additional, nonfunctional SNPs distributed over the autosomes. Seven of the 10 examined genes generated an SNP cluster with significant contribution to the risk for AD. Interestingly, the SNP cluster contained genes (*OLR1, LPL, APOA4*) that failed to exert significant effects by conventional, single-locus χ^2 analysis in our study population (data not shown). These SNPs would have been erroneously reported as replication failures although they confer modest but significant risk within the identified SNP cluster. These results demonstrate the importance of the use of powerful multi-locus methods in the genetics of complex traits.

The value of identifying clusters of trait-associated genes is further demonstrated by the considerable discriminatory ability (74%) of the individual genetic risk score, which mirrors the number of risk alleles present in each subject. Importantly, the genetic risk score calculated after inclusion of all cluster SNPs performed significantly better than the *APOE4* allele alone or the genetic score excluding the *APOE4* effect. This result demonstrates that adding relevant candidate alleles improves the discriminatory ability of the gene cluster. The significant correlation of the genetic risk score with the CSF levels of the cholesterol catabolite 24S-hydroxycholesterol further suggests that the extracted genetic cluster is functionally related to cholesterol metabolism.

As in any genetic association study, significant associations may be a consequence of type I statistical error. We believe that this possibility is unlikely in the present study. First, we used the set-association method, which controls for type I error by permutation. Second, we excluded hidden population structure by analyzing 48 control SNPs and by applying the structured association method. Third, whereas 9 of the 12 candidate SNPs were significantly associated with AD, none of the 48 control SNPs came close to the significance level (data not shown). Fourth, CREGS correlated significantly with CSF 24S-hydroxycholesterol in an independently recruited sample of nondemented subjects, who were not considered for the calculation of the genetic cluster.

In conclusion, we identified a cluster of cholesterol-related SNPs associated with AD and provided a proof-of-principle for the usefulness of multi-locus statistics in the associative genetics of AD. Analysis of additional SNPs in the genes examined in this study as well as in other cholesterol-related genes will provide complete information on the impact of the cholesterol-related genetic cluster in AD. The next step will be to characterize the functional pathways related to the extracted SNP cluster. Finally, a systematic SNP analysis in genes related to such distinct pathogenetic pathways as amyloid precursor protein processing, β-amyloid degradation, tau phosphorylation, proteolysis, protein misfolding, neuroinflammation oxidative stress, and lipid metabolism may improve the sensitivity and specificity of this method and thereby contribute to the characterization of functionally distinct genetic risk profiles related to AD.

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