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Replication Study of Genome-Wide Associated SNPs with Late-Onset Alzheimer's Disease

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Abstract

Late-onset Alzheimer's disease (LOAD) is a multifactorial disease with the potential involvement of multiple genes. Four recent genome-wide association studies (GWAS) have found variants showing significant association with LOAD on chromosomes 6, 10, 11, 12, 14, 18, 19 and on the X chromosome. We examined a total of 12 significant SNPs from these studies to determine if the results could be replicated in an independent large case-control sample. We genotyped these 12 SNPs as well the *E2/E3/E4 APOE* polymorphisms in up to 993 Caucasian Americans with LOAD and up to 976 age-matched healthy Caucasian Americans. We found no statistically significant associations between the 12 SNPs and the risk of AD. Stratification by *APOE**4 carrier status also failed to reveal statistically significant associations. Additional analyses were performed to examine potential associations between the 12 SNPs and age-at-onset (AAO) and disease duration among AD cases. Significant associations were observed between AAO and *ZNF224/rs3746319* ($p=0.002$) and *KCNMA1/rs16934131* ($p=0.0066$). *KCNMA1/rs16934131* also demonstrated statistically significant association with disease duration ($p=0.0002$). Although we have been unable to replicate the reported GWAS association with AD risk in our sample, we have identified two new associations with AAO and disease duration that need to be confirmed in additional studies.

Keywords

Alzheimer's disease; association; genome-wide association studies; replication; age-at-onset; disease duration

INTRODUCTION

Alzheimer's disease (AD) is the most common neurodegenerative disease and accounts for approximately two-thirds of individuals with late life dementia. AD was first described over a century ago by a German neurologist, Alois Alzheimer. However, only in recent years has research begun to reveal clues to the etiology of AD. Early-onset familial AD (FAD);

characterized by onset of clinical symptoms before the age of 60), usually follows an autosomal dominant pattern and accounts for less than 1% of all AD cases (Rocca et al. 1991). To date, mutations in three genes (*APP*, *PSEN1*, and *PSEN2*) have been implicated in early-onset of AD. Late-onset AD (LOAD) is a multifactorial disease that is caused by multiple genes and environmental factors. It is estimated that heritability of LOAD is between 58% and 79% (Gatz et al. 2006). Until recently, the only known significant genetic risk factor for LOAD was *APOE*. However, this does not explain the entire heritability of LOAD and for this reason researchers continue to search for additional contributing genetic factors in LOAD.

Recently several genome-wide association studies (GWAS) have been conducted to identify additional genes for LOAD (Beecham et al. 2009; Bertram et al. 2008; Carrasquillo et al. 2009; Harold et al. 2009; Lambert et al. 2009; Reiman et al. 2007). As replication of initial association studies in independent samples is necessary to confirm or refute these findings, we set out to replicate the top reported significant SNPs in our large case-control sample. We have already conducted a replication study of two GWAS (Harold et al. 2009; Lambert et al. 2009) and have confirmed the modest association of *CLU*, *PICALM* and *CR1* loci in our sample (Kamboh et al. 2010). The present study was aimed to follow-up, in our large case-control sample, the findings of the other four GWAS (Beecham et al. 2009; Bertram et al. 2008; Carrasquillo et al. 2009; Reiman et al. 2007) that reported significant associations of 12 SNPs with LOAD on chromosomes 6, 10, 11, 12, 14, 18, 19 and on the X chromosome. In addition to the assessing the association of these SNPs with AD risk, we also examined their associations with age-at-onset (AAO) and duration of disease which are quantitative measures of AD progression and have been found to be useful in association studies in conjunction with AD risk (Kamboh et al. 2006).

MATERIALS AND METHODS

Subjects were 1969 self-reported Caucasian American individuals, including 993 LOAD cases (AAO ≥ 60 years) and 976 controls (age ≥ 60 years). Cases were derived from the University of Pittsburgh Alzheimer's Disease Research Center (ADRC) and included 67.1% female with a mean AAO of $72.9 \pm (\text{SD}) 6.3$ years; 8.3% of cases were autopsy-confirmed. Clinical diagnosis for definite or probable AD was made according to the NINCDS/ADRDA criteria. Our ADRC follows a standard evaluation protocol, including medical history, general medical and neurological examinations, psychiatric interview, neuropsychological testing and MRI scan. Cognitively normal controls were recruited from the same geographical area as the cases and underwent the same cognitive evaluation. The mean age of controls at baseline was 74.2 ± 6.2 years, and 60.0% were females. The subjects were recruited with informed consent, and the study was approved by the University of Pittsburgh Institutional Review Board.

DNA was isolated from blood using the QIAamp Blood DNA Maxi Kit protocol (Qiagen, Valencia, CA) or from brain tissue using the QIAamp DNA Mini Kit tissue protocol (Qiagen).

Information on 12 SNPs examined in this study is given in Table I. Taqman assays were available for 9 of the 12 SNPs, while Pyrosequencing was used for the remaining three (Table I). TaqMan SNP Genotyping Assays and Genotyping Master Mix were used according to manufacturer's protocol (Applied Biosystems, Foster City, CA). The analysis was performed on samples in 384-well plates. Each plate contained a mixture of cases and control samples, and approximately 10% of samples were repeated to assess error rate. Pyrosequencing was done on the PSQ HS 96 system (Qiagen, Valencia, CA). Pyrosequencing assays were performed using the following PCR (F,R) and sequencing (S)

primers. For *GAB2*/rs2373115: F-5'-AGACTTATGCGGACATGGATTT-3', R-5'-biotin-AGAAATGTTTCGACCATCAATACTC-3' and S-5'-GACATGGATTTATAGTCCG. For *FAM113B*/rs11610206: F-5'-GTTAATCTGCCTTGTGTCAATTTG-3', R-5'-biotin-ACTGGCACAATGGGCTAGGT-3' and S-5'-GGTGATCTTTAGCCTCC-3'. For *LOC100287804*/rs11159647: F-5'-GATGAGCTGGAGCCCATAAT-3', R-5'-biotin-AGGGCCCACTAAGTACTCATCT-3' and S-5'-CATATAGCAAAGCTGCA-3'. The Pyrosequencing analysis was performed on samples that were placed in 96 well plates. As with the Taqman sequencing, approximately 10% of samples were repeated to assess error rate.

Genotypes for *APOE* were determined either as previously described (Kamboh et al. 1995) or using TaqMan SNP genotyping assays.

Allele and genotype frequencies were calculated by the direct allele-counting method. Goodness of fit to Hardy-Weinberg equilibrium (HWE) was tested using the χ^2 test. Differences between genotype and allele frequencies in cases and controls were tested with the χ^2 or Fisher's exact tests appropriately. Differences between cases and controls stratified by *APOE**4 carrier and non-carrier status were also tested with the χ^2 or Fisher's exact tests appropriately.

Odds-ratios (OR), their 95% confidence intervals (CIs) and their significance were estimated using logistic regression with age (controls) or AAO (cases) and *APOE**4 carrier status as covariates. Association between the SNPs and AAO and duration of disease was examined by linear regression assuming an additive genetic model among AD cases. Disease duration was calculated as the difference between the age-at-death and the AAO and it was available on 86 AD cases. *APOE**4 carrier status was included as a covariate in the model for AAO, and sex and AAO were included as covariates in the model for disease duration.

Kaplan-Meier survival curve analysis was performed for SNPs found to be significant by linear regression for AAO. Survival analysis is more appropriately applied to time-to-event data (such as AAO) because such data is generally non-normally distributed due to the inherent censoring. As regression modeling commonly assumes that all variables are normally distributed (or at least nearly so), it is not necessarily appropriate for this type of data. Nonetheless, it is commonly used, and so we present results for both types of analyses. These analyses were performed in all cases as well as stratified by *APOE**4 carrier status. Kaplan-Meier survival analysis was also performed for a SNP to be significantly associated with disease duration in linear regression.

All statistical calculations were performed using PLINK 1.06 (Purcell et al. 2007) or R 2.7.2 (R Development Core Team 2007).

RESULTS

The estimated genotyping error rate for *GAB2*/rs2373115, *FAM113B*/rs11610206, *LOC100287804*/rs11159647 (genotyped by Pyrosequencing) were 2.3%, 1.7%, and 3.3%, respectively. The genotyping error rates for all remaining SNPs (genotyped by TaqMan) were estimated to be <0.6%. Although the error rate was slightly higher for three Pyrosequencing assays than the TaqMan assays, it seems acceptable as their genotyping call rate was >94%.

As expected, frequency of the *APOE**4 allele was significantly higher in cases than controls (33.3% vs. 10.7%; $p<0.0001$) and the frequency of the *APOE**2 allele was lower in cases than controls (3.4% vs. 8.7%; $p<0.0001$).

All SNPs included in this analysis were in HWE. Chi-squared analysis of genotype and allele frequencies revealed no significant differences between cases and controls for any of the SNPs examined. Stratification by *APOE**4 carrier status was also performed, which failed to reveal statistically significant associations (results not shown). The minor allele frequencies, adjusted odds ratios, and 95% CIs are presented in Table II. Logistic regression failed to reveal any associations between genotype and AD risk. ORs, 95% CIs and p-values can also be found in Table II. At $\alpha = 0.05$, we had 80% power to detect ORs greater than 1.2–1.59 and less than 0.68–0.83.

Linear regression analysis for AAO showed significant associations with rs16934131 in the *KCNMA1* gene on chromosome 10q22.3 ($p=0.045$) and rs3746319 in the *ZNF224* gene on chromosome 19q13.3 ($p=0.002$). Mean AAO values associated with genotypes of these two SNPs can be seen in Table III. The results of the Kaplan-Meier analyses can be found in Figure 1. *KCNMA1*/rs16934131 was found to be statistically significant in all subjects ($p=0.0018$, $n=993$) and specifically in non-*APOE**4 carriers ($p=0.001$, $n=423$). *ZNF224*/rs3746319 was found to be statistically significant only in *APOE**4 carriers ($p=0.036$, $n=566$). However, those findings have also not been replicated in three recent large GWAS (Harold et al. 2009; Lambert et al. 2008; Seshadri et al. 2010).

Disease duration was available on 86 LOAD cases. AAO and gender-adjusted disease duration showed significant association with *KCNMA1*/rs16934131 using both linear regression ($p=0.0009$ for three genotypes and $p=0.0002$ for two genotypes as explained in Table III) and Kaplan-Meier analysis ($p=0.0005$ for three genotypes and $p=0.0001$ for two genotypes; Fig. 2).

DISCUSSION

We did not observe a statistically significant association between any of the 12 SNPs examined and the risk of LOAD in our primary analysis. Our data indicate that the association of the 12 previously reported SNPs by Beecham et al. (2009), Bertram et al. (2008), Carrasquillo et al. (2009), and Reiman et al. (2007), if they exist, are not statistically significant in our study population.

There are several possible explanations for our inability to replicate the reported GWAS associations. Although we used a relatively large case-control sample, its power is still low to detect the reported modest effect sizes. Another reason could be that there are differences between the populations used in the initial studies and the population used in our study. The findings of the various GWAS may in fact be reflecting environmental covariables present in the initial studies' populations that were not present in our study population. Furthermore, heterogeneity in the sample populations may explain the discrepancies between our data and the results found by the various GWAS. Another possible explanation for the associations found by the original studies that were not replicated in our study could be the winner's curse. The winner's curse refers to a theory that it is likely that the first study to report a significant finding will have an effect size much larger than is seen in replication studies due to the necessary high level of significance in studies involving many tests, such as GWAS. These "false positives", will therefore not be replicated in subsequent studies, as these studies findings will tend to regress towards the mean (Nakaoka and Inoue 2009). Additionally, we examined only the index SNPs from the reported GWAS, which rarely are the causal variants; and often are in linkage disequilibrium (LD) with causal variants. A non-replication in our sample could be due to different LD structure in our sample than samples used in published GWAS. However, these findings have also not been replicated in three recent large GWAS (Harold et al. 2009; Lambert et al. 2009; Seshadri et al. 2010).

While none of the SNPs examined in our study were found to be associated with AD risk, we observed statistically significant associations with AAO and disease duration. Our study found significant association of *ZNF224*/rs3746319 with AAO and *KCNMA1*/rs16924131 with disease duration and AAO. Both of these SNPs were found to be associated with AD risk by Beecham et al. (2009). Linear regression analysis of *APOE*-adjusted AAO showed significant associations with rs16934131 in the *KCNMA1* gene on 10q22.3 ($p=0.045$) and rs3746319 in *ZNF224* gene on 19q13.3 ($p=0.002$). There was a gene-dosage effect of the *ZNF224*/rs3746319 SNP on AAO with the GG genotype associated with the older onset (73.26 years), the AA genotype with the lowest AAO (71.15 years) and the GA genotype with intermediate AAO (72.08 years). Interestingly, Kaplan-Meier analysis showed that the *ZNF224*/rs3746319 effect on AAO was confined mainly to *APOE**4 carriers ($p=0.036$, $n=566$). Figure 1f shows obvious differences between the curve for the AA genotype and those for the GA and GG. These data suggest that the A allele of *ZNF224*/rs3746319 is associated with earlier AAO.

For the *KCNMA1*/rs16934131 SNP, the CC genotype was associated with earlier AAO than the TT and TC genotypes (70.9 ± 5.7 vs. 73.0 ± 6.1 years, $p=0.0066$). Kaplan-Meier analysis of AAO showed that the rs16934131 SNP was statistically significant ($p=0.002$, $n=993$), particularly among non-*APOE**4 carriers ($p=0.001$, $n=423$). Kaplan-Meier plots seen in Figure 1a and 1b show obvious differences between the curve of the CC genotype as compared to those of the TT and TC genotypes, particularly in non-*APOE**4 carriers. This SNP was not found to be statistically significant among *APOE**4 carriers alone ($p=0.138$, $n=570$). The fact that we are seeing a stronger effect of rs16934131 on AAO in non-*APOE**4 carriers seems biologically plausible considering that *APOE**4 is known to have such a strong effect on AAO that it may mask other effects. The *KCNMA1* gene on chromosome 10q22.3 is located close to a reported linkage peak for AAO of AD (Li et al. 2002) and thus may represent a genuine marker for AAO. Alternatively, it may be in linkage disequilibrium with a functional marker in this region. A detailed genetic characterization of this region in relation to AAO of AD may shed more light on this initial finding. Although Beecham et al. (2009) reported a significant association of this gene with AD risk, they did not report an analysis of AAO.

In addition to the association of the *KCNMA1*/rs16934131 SNP with AAO, this SNP analysis also revealed an association with disease duration ($p=0.0002$). The TC genotype was associated with longer disease duration than TT homozygotes (11.1 ± 4.0 vs. 7.7 ± 3.6 years; $p=0.0009$). Since there were only two individuals for whom disease duration was known with the CC genotype, it was difficult to interpret their mean value (8.23 ± 2.24), as it was similar to that of the TT genotype.

The two SNPs we found in our study to be associated with AAO and/or disease duration, *KCNMA1*/rs16934131 and *ZNF224*/rs3746319, were found originally to be associated with AD risk by Beecham et al. (2009). While our study did not find associations between these SNPs and risk of AD, the associations seen in our study may be consistent with the findings of Beecham et al. If an individual has a genetic factor that predisposes them to have a longer disease duration, they will live longer with AD, and therefore there may be a greater chance that such an individual would be chosen as a case rather than a control. Similarly, if an individual has a genetic factor that causes earlier disease onset, they would be affected with AD at an earlier age and increase the likelihood that they would be classified as an AD case. Therefore, depending on how old individuals are when they are enrolled in a study, what was seen in our study as a difference in disease duration or AAO, may be reflected in another study as a difference in case/control status. Subjects recruited as controls who subsequently develop AD are reclassified as cases in our data set.

It is interesting to note that *KCNMA1*/rs16934131 was found to be associated with both AAO and disease duration in this study. It is possible that this SNP does impact the two measures independently, or perhaps that it has one common underlying effect that is reflected in both measures. Alternatively, this could be a genetic factor that causes earlier AAO but does not cause AD to progress any more rapidly. Therefore, the earlier AAO could lead to a longer time between diagnosis and death, thus longer disease duration.

The *KCNMA1* gene has been suggested to be associated with innate immunity, cochlear hearing impairment, vascular dysfunction, epilepsy, and mental retardation. Knockout mice engineered by targeted disruption of the *KCNMA1* gene manifested ataxia, moderate vascular dysfunction, and other neurologic deficits, including cerebellar dysfunction in the form of abnormal eye-blink reflexes, abnormal motion, and deficiency in motor coordination (Sausbier et al. 2004). Considering the evidence that this gene functions in the brain and could potentially impact mental capacity, it seems biologically plausible that alterations in this gene could have an effect on an individual's degree of cognitive impairment.

In summary, we were unable to replicate the reported GWAS signals in our large case-control samples. Additional analyses revealed statistically significant associations of ZNF224/rs3746319 with AAO and significant association of *KCNMA1*/rs16934131 with disease duration and AAO. We have presented nominal p-values for these two traits for hypothesis generation purpose and not corrected for multiple testing. Thus, these findings should be considered provisional until confirmed in independent samples. If confirmed, the mechanisms by which they modify disease characteristics should be examined for potential adaptation for possible therapeutic intervention.

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Figure 1.
Kaplan-Meier Analysis of rs16934131 and rs3746319, all cases and all cases stratified by *APOE**4. The curves displayed in (a), (b) and (f) are statistically significant.

Figure 2.
Kaplan-Meier Analysis of the Effect of rs16934131 on Disease Duration Adjusted for Sex and Age-at-Onset

Table I

Gene Location and Genotyping Method Used for the Study SNPs

dbSNP/Major→Minor Allele	Chromosome Location	Mapped ID	Genotyping Method	Reference
rs179943/C→T	6p23	<i>ATXN1</i>	TaqMan	Bertram et al. 2008
rs7893928/C→T	10q11.21	200kb 5' of <i>CXCL12</i>	TaqMan	Beecham et al. 2009
rs16934131/T→C	10q22.3	<i>KCNMA1</i> Intron	TaqMan	Beecham et al. 2009
rs7894737/A→C	10q25.1	<i>SORCS3</i> Intron	TaqMan	Beecham et al. 2009
rs11244841/T→C	10q26.3	<i>ADAM12</i> Intron	TaqMan	Beecham et al. 2009
rs2373115/G→T	11q14.1	<i>GAB2</i>	PSQ ^a	Reiman et al. 2007
rs11610206/T→C	12q13	9 kb 3' of <i>FAM113B</i>	PSQ ^a	Beecham et al. 2009
rs11159647/G→A	14q31	100kb from <i>LOC100287804</i>	PSQ ^a	Bertram et al. 2008
rs2049161/A→C	18p11.3	150kb 5' of <i>GAPDHL1</i>	TaqMan	Bertram et al. 2008
rs3746319/G→A	19q13.2	<i>ZNF224</i>	TaqMan	Beecham et al. 2009
rs3826656/A→G	19q13.3	1.5 kb 5' of <i>CD33</i>	TaqMan	Bertram et al. 2008
rs5984894/T→C	Xq21.3	<i>PCDH11X</i> Intron	TaqMan	Carrasquillo et al. 2009

^aPyrosequencing

Table II

Association Results of the Study SNPs, evaluated by logistic regression

Gene/SNP/Minor Allele	<i>n</i>		MAF ^a		Adjusted Odds Ratios (ORs) ^b		Unadjusted ORs ^b	
	Cases	Controls	Cases	Controls	OR (95% CI)	<i>p</i>	OR (95% CI)	<i>p</i>
ATXN1/rs179943/T	907	914	0.072	0.084	0.85 (0.65–1.10)	0.2218	0.85 (0.66–1.08)	0.1765
CXCL12/rs7893928/T	989	972	0.150	0.152	1.04 (0.86–1.26)	0.6620	0.98 (0.83–1.17)	0.8540
KCNMA1/rs16934131/C	993	976	0.250	0.259	0.90 (0.77–1.05)	0.1815	0.95 (0.83–1.10)	0.5183
SORCS3/rs7894737/C	979	976	0.400	0.389	1.00 (0.87–1.15)	0.9615	1.05 (0.92–1.19)	0.4996
ADAM12/rs11244841/C	980	965	0.261	0.249	1.09 (0.93–1.27)	0.3016	0.99 (0.92–1.23)	0.3907
GAB2/rs2373115/T	923	915	0.187	0.189	1.00 (0.83–1.20)	0.9900	0.99 (0.84–1.17)	0.8991
FAM113B/rs11610206/C	957	909	0.092	0.090	1.06 (0.83–1.35)	0.6264	1.02 (0.82–1.28)	0.8531
LOC100287804/rs11159647/A	942	913	0.458	0.463	1.00 (0.87–1.15)	0.9814	0.98 (0.86–1.12)	0.7744
GAPDH11/rs2049161/C	777	840	0.186	0.172	1.11 (0.92–1.34)	0.2786	1.10 (0.92–1.32)	0.3010
ZNF224/rs3746319/A	980	966	0.183	0.187	0.94 (0.79–1.12)	0.4785	0.97 (0.82–1.14)	0.7047
CD33/rs3826656/G	811	875	0.231	0.241	0.90 (0.76–1.07)	0.2513	0.95 (0.81–1.11)	0.5218
PCDH11X/rs5984894 ^c /C	977	966	0.486	0.486	0.98 (0.84–1.14)	0.7945	1.00 (0.87–1.15)	0.9963

^aMAF = Minor allele frequency

^badjusted for age (age-at-onset in cases) and APOE

^ccontrols included 378 males and 588 females, cases included 320 males and 657 females; this SNP is located on X-chromosome

Table IIIAdjusted mean quantitative traits ($\pm$ SD), evaluated by linear regression

<i>SNP</i>	<i>Genotype^a</i>			<i>p-value</i>
<i>ZNF224/rs3746319</i>	GG	GA	AA	
AAO ^b	73.3 $\pm$ 5.9 (645)	72.1 $\pm$ 6.5 (288)	71.2 $\pm$ 6.1 (31)	0.0020
<i>KCNMA1/rs16934131</i>	TT	TC	CC	
AAO ^b	73.1 $\pm$ 6.4 (554)	72.9 $\pm$ 5.7 (358)	70.9 $\pm$ 5.7 (65)	0.0447 (0.0066) ^d
Disease Duration ^c	7.7 $\pm$ 3.6 (47)	11.1 $\pm$ 4.0 (33)	8.23 $\pm$ 2.24 (2)	0.0009 (0.0002) ^e

^a Number in parentheses indicate number of subjects in each genotype.^b Adjusted for *APOE**4^c Adjusted for AAO and sex^d *p*-values in parentheses are for comparison between the TT + TC and CC genotypes.^e *p*-values in parentheses are for comparison between the TT and TC + CC genotypes.