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Combined Genome-Wide CSF Aβ-42's Associations and Simple Network Properties Highlight New Risk Factors for Alzheimer's Disease

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Abstract The abnormal deposition of amyloid-β protein in the brain plays an important role in Alzheimer's disease (AD), being considered a potential clinical biomarker. To investigate genetic associations with amyloid-β we used biomarker data and genome-wide variants from individuals with AD and mild cognitive impairment in the Alzheimer's Disease Neuroimaging Initiative (ADNI) database. We used a standard linear model and retested the associations with a mixed linear model to correct the residual sample structure. Both methods' results showed two identical significant SNPs associated with the A β-42 levels in CSF (rs2075650 at intron region TOMM40 with p-value≥1×10–16 and rs439401 in the intergenic region of LOC100129500 and APOC1 with p-value≥1×10-9) and highlighted APOC1 and TOMM40, which are well-known genes previously associated with AD. Extending our analysis, we considered possible candidate genes mapped to SNPs with p-value≥1×10-6 to explore gene-set enrichment e gene-gene network analysis, which reveals genes related to synaptic transmission, transmission of nerve impulses, cell-cell signaling and neurological processes. These genes require fine mapping and replication studies to allow more detailed understanding of how they may contribute to the genetic architecture of AD.

Keywords Alzheimer's disease · Mild cognitive impairment · A β-42 · Alzheimer's Disease Neuroimaging Initiative (ADNI) · Biomarkers

Introduction

Alzheimer's disease (AD) is the most common progressive neurodegenerative disease and represents a major cause of disability for elderly patients with dementia worldwide (Alzheimer's Association 2013). AD can be divided into three phases, an initial presymptomatic phase, a prodromal stage known as mild cognitive impairment (MCI) and a third stage when patients show dementia with impairments in multiple domains and inability to perform daily activities (Trojanowski et al. 2010). Recently, the National Institute on Aging in collaboration with the Alzheimer's Association proposed changes to the guidelines to assess brain changes associated with dementias. It is noteworthy that more research is needed to confirm reliable biomarkers (Hyman et al. 2012; Alzheimer's Association 2013).

The neuropathological characteristics of AD include the deposition of β-amyloid peptide (Aβ) in the brain and blood vessels (forming amyloid or senile plaques) causing amyloid

Data used in the preparation of this article were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (adni.loni.ucla.edu). As such, the investigators within the ADNI contributed to its design and implementation and/or provided data but did not participate in the analysis or writing of this report. A complete listing of the ADNI investigators can be found at: http://adni.loni.ucla.edu/wpcontent/uploads/how_to_apply/ADNI_Acknowledgement_List.pdf.

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angiopathy and tau protein hyperphosphorylation forming neurofibrillary tangles (Braak and Braak 1991; Tarawneh and Holtzman 2010). With AD progression, there is a decrease in Aβ concentrations in the cerebrospinal fluid (CSF) and an increase in Aβ concentrations in the blood plasma, potentially clinical biomarkers (Graff-Radford et al. 2007; Rosemberg and Lyketsos 2008). Because it is detectable in the CSF and blood plasma, this biomarker has been used for clinical diagnosis (Koyama et al. 2012).

The individuals in the ADNI showed differences in Aβ-42 levels collected from the CSF and plasma, with Aβ-42 levels higher in the CSF. These differences prompted several studies based on the ADNI data (Kim et al. 2011; Trojanowski et al. 2010; Han et al. 2010; Shaw et al. 2009). For this study, our aims were to identify AD genes and validate candidate genes using an approach based on gene-gene interactions using GWAS hits as a starting point. This study differs from others in two points. First, we assessed the results from genome-wide scans of two linear methods; second, we investigated gene links and genetic interactions using a guilt-by-association method to assess the evidence of genes in the architectural genetic base of AD.

Materials and Methods

Subject and Biomarker Descriptions

Data used in the preparation of this article were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (adni.loni.ucla.edu). The ADNI was launched in 2003 by the National Institute on Aging (NIA), the National Institute of Biomedical Imaging and Bioengineering (NIBIB), the Food and Drug Administration (FDA), private pharmaceutical companies and non-profit organizations as a $60 million, 5-year public-private partnership. The primary goal of the ADNI has been to test whether serial magnetic resonance imaging (MRI), positron emission tomography (PET), other biological markers, and clinical and neuropsychological assessment can be combined to measure the progression of mild cognitive impairment (MCI) and early Alzheimer's disease (AD). Determination of sensitive and specific markers of very early AD progression is intended to aid researchers and clinicians to develop new treatments and monitor their effectiveness as well as lessen the time and cost of clinical trials.

The principal investigator of this initiative is Michael W. Weiner, MD, VA Medical Center and University of California-San Francisco. The ADNI is the result of efforts of many co-investigators from a broad range of academic institutions and private corporations, and the subjects were recruited from over 50 sites across the US and Canada. The initial goal of the ADNI was to recruit 800 adults, ages 55 to 90, to participate in the research with approximately 200 cognitively normal age-matched individuals to be followed for 3 years, 400 people with MCI to be followed for 3 years and 200 people with early AD to be followed for 2 years (www.adni-info.org).

For each individual, the ADNI provides measures of Aβ-42, and we observed significant differences between the CSF and plasma levels (see boxplot in Fig. 1). The levels were observed using data from 380 individuals for CSF and 654 individuals for plasma, collected at baseline diagnostic examinations. The measurements of Aβ-42 levels met all quality control (QC) requirements, and more details about the biomarkers have been previously reported in ADNI publications (Petersen et al. 2010). As previously mentioned, Aβ-42 levels in CSF are higher than in plasma. Considering the null hypothesis that the AB-42 levels are equal across each diagnostic (NC, MCI, AD), we used the Kolmogorov-Smirnov test to access differences between the Aβ-42 level distribution for NC, MCI and AD individuals. For Aβ-42 levels in the CSF, we performed a pairwise sample test for NC-MCI, NC-AD and MCI-AD that resulted in p-values of 5.231e–09, 1.676e–14 and 0.01, respectively. The distributions show significant differences in CSF between the groups. However, the null hypothesis was not rejected for the plasma level distribution. In the pairwise diagnostic analysis of the distributions, the Kolmogorov-Smirnov test did not show significance (p-values$\geq$0.05).

SNP Data Set

The ADNI used the Illumina Human 610-Quad BeadChip, which allows geneticists to investigate more than 610,000 SNPs. However, this study was restricted to autosomal SNPs with minor allele frequency (MAF) greater than 0.01 and SNPs with an 'rs' identifier and 95 % genotyping rate, and we considered SNPs in Hardy-Weinberg equilibrium. The MAF threshold was used to exclude rare SNPs for our analysis.

Statistical Association Analyses

We conducted genome-wide scans using two linear models to estimate the main effects of SNPs after basic filters. The first method is a standard linear regression model (SLRM) for quantitative trait association implemented in PLINK software (http://pngu.mgh.harvard.edu/_purcell/plink/), release v1.07 (Purcell et al. 2007). We retested the association analysis using EMMAX (Efficient Mixed-Model Association eXpedited), which is more robust than SLRM. EMMAX takes into account a kinship matrix to prevent false-positive associations by correcting for hidden sample relatedness of the ADNI Cohort. EMMAX builds a matrix with genetic relatedness measures for the modeling

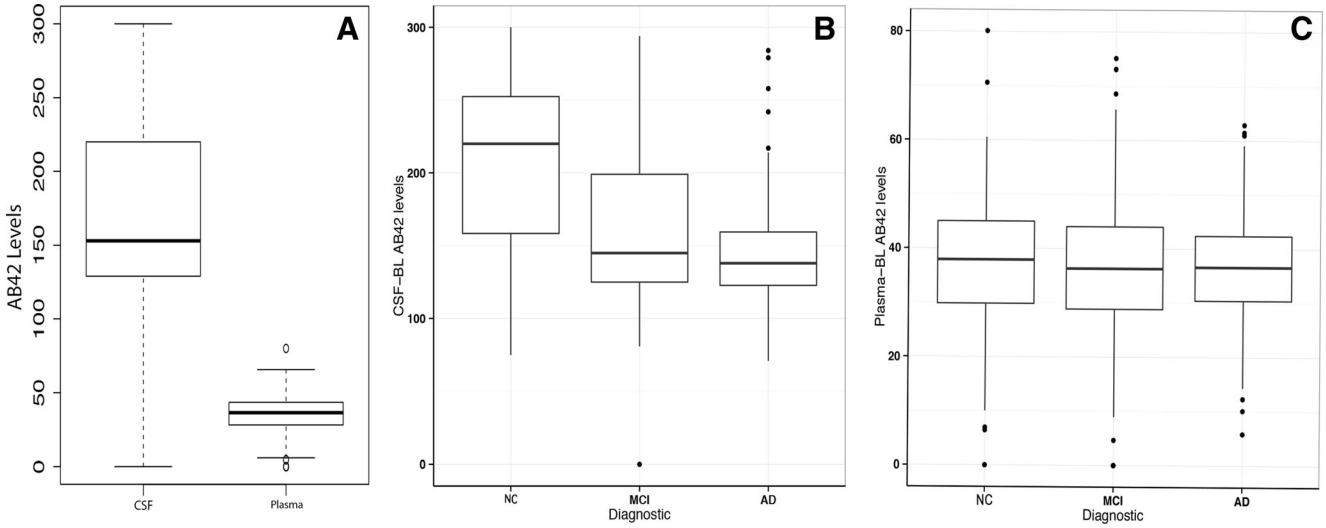

Fig. 1 Distribution of Aβ-42 levels in the CSF and plasma. To illustrate the distributions we considered 346 individuals from the ADNI cohort common to both sources (more details in Material and Methods). Wilcoxon signed-ranked test was used in statistical comparison to show significant differences (p-value=2×2E-16)

sample structure based on all autosomal SNPs and estimates the contribution of the individual's relatedness matrix to phenotypic variance (Kang et al. 2010). By conducting the statistical association tests with SLM and EMMAX, we restrict the most significant results to the expected threshold p-value≤1×10-8.

Enrichment Gene Set Analysis and Functional Analysis

First, we use DAVID (Database for Annotation, Visualization and Integrated Discovery) for enrichment analyses of all mapped genes, which contain significant SNPs (p-value≤1×10^{-5}), in a common way to investigate the functional terms (Huang et al. 2009a, b). We used the Functional Annotation Clustering module from DAVID to map gene sets. Despite the broad set of parameters, we performed DAVID by setting only the classification stringency parameter to its highest level. Second, network-based approaches were used to discover relations between candidate genes from genome-wide scans and well-known disease genes. We use GeneMANIA (Montojo et al. 2010) by Cytoscape (Saito et al. 2012) to explore gene-gene networks constructed from heterogeneous data. GeneMANIA was proposed for predicting gene functions in real time, and it is composed of a heuristic algorithm derived from a ridge regression to integrate multiple networks through a process of label propagation. Using a guilt-by-association approach, GeneMANIA bases its prediction on data from a wide variety of public and curated databases, including Gene Expression Omnibus, BIOGRID and Pathway Commons (Edgar et al. 2002; Cerami et al. 2011; Chatr-Aryamontri et al. 2013). The networks generated by GeneMANIA can be treated as multi-graphs, and often six types of edges compose each network, including co-expression, co-localization, genetic interaction, physical interaction, predicted function and shared protein domain edges. Using GeneMANIA we queried links between the set of genes (with SNPs that reached a p-value≤1×10^{-5}) highlighted by the association analysis and the list of Alzheimer's disease genes highlighted in the meta-analyses of the AlzGene database. We restricted the neighborhood to only the two gene lists and treated each type of data with equal weight to construct our networks.

Results

Genome-Wide Association Analysis

First we analyzed the genotype and baseline CSF Aβ-42 levels for 380 individuals. We then considered the genotype data and plasma Aβ-42 levels for 654 individuals (see a summary of the individuals in Table 1).

We conduct our GWAS analysis with SLM and EMMAX, both of which identify the same significant top SNPs (considering the expected threshold p-value≤1×10-8). The two SNPs significantly associated with CSF Aβ-42 levels at baseline were rs2075650 in the intron region of TOMM40 and rs439401 in the intergenic region of LOC100129500 and APOC1. Results for both linear analyses are presented in Table 2. SNPs were significant for both analyses even considering a rigid Bonferroni correction. The SNP rs2075650 reached a p-value of 9.24E-16 for EMMAX analysis, a p-value of 9.34E-16 using SLM and a Bonferroni p-value of 5.039E-10, whereas rs439401 achieved a p-value of 4.84 E-09 for EMMAX, a p-value of 8.23E-09 for SLM and Bonferroni p-value of 0.004. Annotations for these SNPs are presented in Table 2.

Table 1 Details of experimental designs performed

CSF					
Diagnostic	AB-42 levels		Individuals	Age mean	
	Mean	SD		Mean	SD
NC	205.67	55.72	N_{NC}=107; male=56; female=51	75.44	5.16
MCI	163.00	55.41	N_{MCI}=179; male=120; female=59	74.81	7.62
AD	142.82	39.56	N_{AD}=94; male=54; female=40	75.08	8.02
Plasma					
Diagnostic	AB-42 levels		Individuals	Age mean	
	Mean	SD		Mean	SD
NC	37.19	11.66	N_{NC}=192; male=106; female=86	75.75	4.95
MCI	36.26	12.54	N_{MCI}=314; male=209; female=105	75.29	7.19
AD	36.43	10.50	N_{AD}=148; male=80; female=68	75.54	7.49

Abbreviations: *CSF* cerebrospinal fluid, *AD* Alzheimer's disease, *MCI* mild cognitive impairment, *NC* normal control, *N* number, *SNP* single nucleotide polymorphism, *SD* standard deviation

For the CSF Aβ-42 levels, the genomic inflation factor (GIF) based on the median chi-squared statistic was 1.10 and the mean chi-squared statistic was 1.11, suggesting population stratification or residual sample relatedness. The GIF for plasma Aβ-42 levels based on the median chi-squared statistic was 1, and the mean chi-squared statistic was 0.989, representing a low risk of confounding factors. We focused on two SNPs with the highest *p*-values for graphic analysis. SLM and EMMAX presented two identical SNPs. We then drew the main basic GWAS plots for the significant associations provided by EMMAX analysis (see Fig. 2a). Both SNPs were not in linkage disequilibrium (see Fig. 2D1 and D2) considering the SNP genotype data set for 380 individuals and 645 individuals of plasma samples.

Table 2 Top SNPs with *p*-value$\leq 10^{-5}$

CSF-BL-Aβ42									
EMMAX					Standard linear model				
CHR	GENE	SNP	P	CLASS	CHR	GENE	SNP	P	CLASS
19	TOMM40	rs2075650	9.24E-16	INTRON	19	TOMM40	rs2075650	9.34E-16	INTRON
4	LOC100129500 | APOC1	rs439401	4.84E-09	INTERGENIC	19	LOC100129500 | APOC1	rs439401	8.23E-09	INTERGENIC
19	TOMM40	rs157580	1.48E-07	INTRON	19	TOMM40	rs157580	1.74E-07	INTRON
8	PXDNL	rs2915495	7.32E-07	INTRON	8	PXDNL	rs2915495	1.89E-06	INTRON
14	PRIMA1 | C14orf86	rs4900200	1.66E-06	INTERGENIC	10	LHPP	rs7090933	2.30E-06	INTRON
10	LHPP	rs7090933	7.20E-06	INTRON	14	PRIMA1 | C14orf86	rs4900200	2.59E-06	INTERGENIC
7	WDR60 | LOC154822	rs2527214	8.74E-06	INTRON	7	WDR60 | LOC154822	rs2527214	6.20E-06	INTERGENIC
14	ADCK1 | NRXN3	rs11628271	9.88E-06	INTERGENIC	13	LOC100287432 | LOC144776	rs1948851	6.50E-06	INTERGENIC
1	FMO4 | BAT2D1	rs7523796	9.92E-06	INTERGENIC	9	STOM | GGTA1	rs7044653	8.36E-06	INTERGENIC
					16	GRIN2A | LOC100287628	rs13332694	8.49E-06	INTERGENIC
PL-BL-Aβ42									
EMMAX					Standard linear model				
CHR	GENE	SNP	P	CLASS	CHR	GENE	SNP	P	CLASS
21	C21orf131 | NCAM2	rs2186867	7.51E-07	INTERGENIC	21	C21orf131 | NCAM2	rs2186867	6.45E-07	INTERGENIC
4	UGT2B7	rs7375178	5.97E-06	INTRON	4	UGT2B7	rs7375178	5.54E-06	INTRON
1	LOC728510 | SLC30A10	rs12121613	9.68E-06	INTERGENIC	1	LOC728510 | SLC30A10	rs12121613	9.68E-06	INTERGENIC

SNPs associated with CSF and Plasma BL-Aβ42

Abbreviations: *CSF* cerebrospinal fluid, *BL* baseline, *Chr* chromosome, *SNP* single nucleotide polymorphism

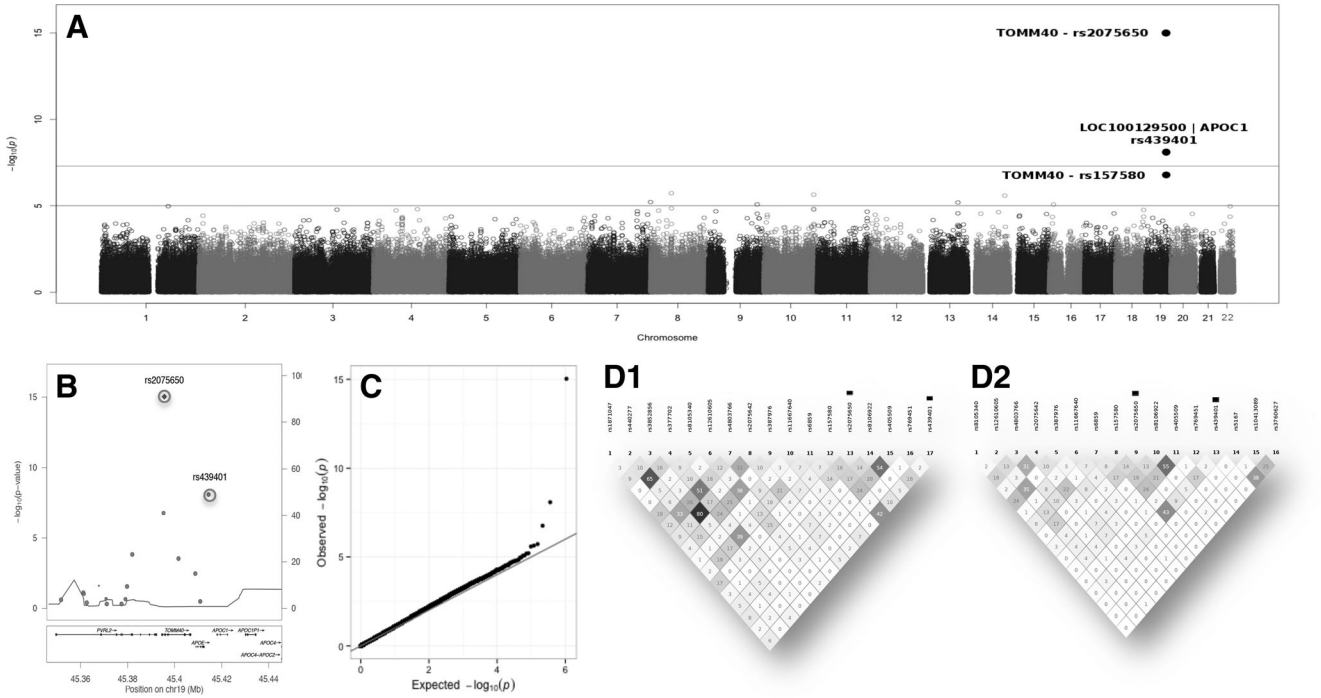

Fig. 2 **a** Manhattan plot from EMMAX association analyses displays the three most significant associated SNPs with CSF Aβ-42 levels for baseline diagnostics. The *blue and red lines* represent the $-\log10(10^{-6})$ and $-\log10(10^{-8})$ statistical significance threshold. **b** The regional association plot was drawn using LocusZoom software and shows the two most significant SNPs (*red circles*) and their genes (*dark blue arrows*) from EMMAX association analysis. **c** Q-Q plot of EMMAX *p*-values. **D1** and **D2** are plots of the linkage disequilibrium structure for the two significant SNPs. For draw **D1** we considered 380 individuals with CSF Aβ-42 levels, and **for draw D2** we considered 654 individuals with plasma Aβ-42 levels. The linkage disequilibrium structures were created by Haploview on chromosome 19 and were considered a 50-kb window between each SNP to calculate the D' values

Gene-Set Enrichment Analysis of Top Results

We carried out an analysis with DAVID to explore functional terms associated without a set of associated genes. DAVID results (see Table 3) show the functional enrichment terms with high significance when compared with the theoretical threshold (1.3) (Huang et al. 2009a, b). The two clusters of annotations are directly related to the neurological processes, including synaptic transmission and transmission of nerve impulses, and trait class, which includes psychiatric traits.

Gene-Gene Interactions Network Analysis

GeneMANIA was used to construct the gene-gene networks (Figs. 4a and b). First, we carried out GeneMANIA to explore relations between all mapped genes for SNPs, which represents the main association results. Not all genes are recognized by GeneMANIA. Fifteen genes remained in Fig. 4a. Genetic interaction edges and co-expression edges support the major part of the relations between genes. One exception can be highlighted for the gene pair NCAM2 and GRIN2A, which shows a physical interaction edge.

Table 3 Functional annotation clustering by DAVID

Annotation cluster 1	Enrichment score: 2.4	*p*-value
GOTERM_BP_FAT	Synaptic transmission	8.1E-4
GOTERM_BP_FAT	Transmission of nerve impulse	1.3E-3
GOTERM_BP_FAT	Cell-cell signaling	6.0E-3
GOTERM_BP_FAT	Neurological system process	4.0E-2
Annotation cluster 2	Enrichment score: 1.89	*p*-value
GOTERM_BP_FAT	Regulation of catabolic process	1.7E-3
GENETIC_ASSOCIATION_DB_DISEASE_CLASS	Psych	3.3E-2
GENETIC_ASSOCIATION_DB_DISEASE_CLASS	Neurological	3.6E-2

Terms related to the list of genes presented in the Table 2

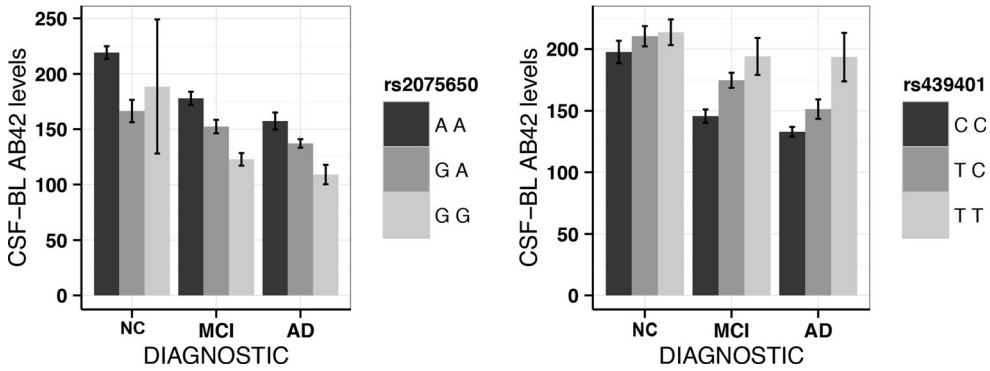

Fig. 3 Mean of Aβ-42 levels in the function of genotypes over all diagnostic groups (NC, MCI and AD) for the most significant associated SNPs

Using GeneMANIA we extended our analysis to discover relations between well-known Alzheimer's disease genes, reported in AlzGene, and genes from association results. The resulting network (Fig. 4-b) supports the genetic interaction and co-expression edges. In addition, GeneMANIA presents the shared protein domain edge links between MS4A4E and MS4A6A, APOE and APOC1, and BIN1 and CD2AP. Physical interaction edges are presented for the following gene pairs: NCAM2 and GRIN2A, STOM and MS4A6A, and APOE and FMO4. Finally, one pathway edge appears between BIN1 and PICALM.

We compute two topology network properties, the node degree and number of multi-edged node pairs (Table 4). These simple measures help to quantify the evidence of interactions between the associated genes and the most associated AD genes highlighted by AlzGene. The degree expresses the number of edges with other genes in the network, whereas the number of multi-edge node pairs expresses how often a node is linked with other nodes by more than one type of edge. The gene GRIN2A is the most central gene, which is the gene with more edges than the others, interacting with the neighborhood of genes with 17 edges, being 2 genes in the neighborhood partners of multi-edges. APOC1 and TOMM40 interact by eight and seven edges, respectively. APOC1 has five multi-edge partners, including APOE. This may suggest co-participation of the genes in the Alzheimer's disease pathway.

Discussion

In this study, we investigated CSF Aβ-42 as a quantitative biomarker to discover genetic variant associations using genotype data obtained from the ADNI cohort. The use of phenotypes as quantitative traits in GWAS has been successfully carried out to test associations between biomarkers and SNPs. Additionally, the biomarkers have increased power over case-control designs (Potkin et al. 2009; Han et al. 2010; Kim et al. 2011).

TOMM40, translocase of outer mitochondrial membrane 40 homolog is essential for mitochondrial protein import. Aging decreases the number of mitochondria and also increases the risk of developing AD (Humphries et al. 2005). *TOMM40* alleles have been associated with an increased risk of developing LOAD (Devi et al. 2006; Roses et al. 2010). *TOMM40* is adjacent, approximately 15 kb upstream of *APOE* and established as one of the susceptibility genes for LOAD (Corder et al. 1993). *TOMM40* variable-length poly-T

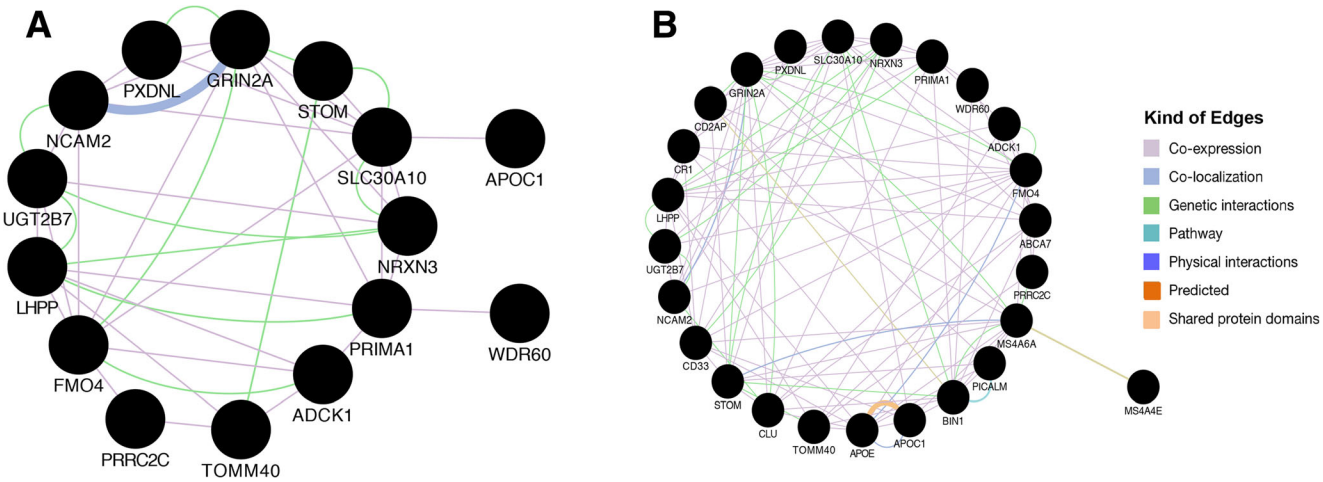

Fig. 4 GeneMANIA networks showing the interaction results of associated genes and highly associated AD genes

Table 4 Topological properties of the GeneMANIA networks (see Fig. 4)

No.	Gene symbol	Degree	Partners of multi-edges
1	GRIN2A	17	2
2	FMO4	15	1
3	MS4A6A	14	1
4	SLC30A10	14	3
5	BIN1	13	2
6	LHPP	13	2
7	APOE	11	3
8	STOM	11	0
9	CD33	10	2
10	ABCA7	9	0
11	CLU	9	0
12	PRIMA1	9	2
13	NRXN3	9	1
14	APOC1	8	5
15	NCAM2	8	2
16	CR1	7	1
17	TOMM40	7	1
18	UGT2B7	7	1
19	CD2AP	7	1
20	ADCK1	6	3
21	PICALM	6	0
22	PXDNL	4	0
23	PRRC2C	4	0
24	WDR60	3	3
25	MS4A4E	1	0

This table presents a ranking of genes by the node degree. A higher node degree expresses more interactions between the gene and the neighborhood. The column partners of multi-edge express how often a node is liked with other nodes by more than one type of edge

sequence polymorphism (rs10524523) in combination with *APOE* alleles (E2, E3, E4) has been reported to influence LOAD (Roses et al. 2010). Recently, the impact of *TOMM40* poly-T in combination with APOE alleles (E2, E3, E4) on LOAD incidence was assessed in a group of 414 LOAD patients, 173 centenarians and 305 neurologically healthy individuals. The study demonstrated poly-T affecting the LOAD risk in some analyses (Maruszak et al. 2012).

Using quantitative traits in a GWAS, the *TOMM40* gene, specifically SNP rs2075650, has been identified as a susceptible putative locus associated with AD (Han et al. 2010). However, CSF biomarkers (Aβ-42, t-tau, p-tau181p, p-tau181p/Aβ-42 and t-tau/Aβ-42) from the ADNI data show rs2075650 (*TOMM40*) associated with Aβ-42, p-tau181p/Aβ-42 and t-tau/Aβ-42 (Kim et al. 2011). An intronic SNP (rs2075650; *TOMM40*) is associated with Aβ-42 in AD and MCI subjects. rs2075650 has previously been described as having a positive association with *APOE* as a quantitative trait (Potkin et al. 2009).

Apolipoprotein C1 (ApoC1) encoded by APOC1 is a member of the apolipoprotein family. According to the Allen Institute Human Brain Atlas, APOC1 presents a selective pattern of expression in the hippocampus, a region known to be involved in AD. APOC1 appears to be regulated in the temporal and visual cortex (http://human.brain-map.org/). Moreover, APOC1 has been studied in microarray expression analysis of post mortem samples from patients with LOAD and matched controls. However, few studies have correlated the APOC1 gene with LOAD, suggesting their role in the AD pathology of this neurodegenerative disease (Ki et al. 2002; Zhou et al. 2014).

The product of proline-rich membrane anchor 1, PRIMA1, functions to organize acetylcholinesterase (AChE) into tetramers and to anchor AChE at the neural cell membranes, having therapeutic relevance for Alzheimer's disease, as shown in knockout mice (García-Ayllón et al. 2014). Although the relevance of the PRIMA1 gene to Alzheimer's disease has been reported, no study has addressed the rs4900200 polymorphism, first reported in this study.

As shown in Table 2, the association test allowed us to identify eight polymorphisms (rs2915495, rs7090933, rs2527214, rs11628271, rs7523796, rs1948851, rs7044653 and rs13332694) that have not been previously reported in association with AD or neurodegenerative diseases. Furthermore, we also present the interaction of genes already described in the literature to be associated with Alzheimer's disease and suggest new candidate genes via network approaches.

Network approaches were essential for assisting our analysis of genetic association data from different points of view. Our study used GeneMANIA as an integrative tool that found curated relationships between the above-discussed genes. We are convinced that if a gene is related to a disease, this might rule significant biological mechanisms involved in the pathophysiology and lead to susceptibility to developing complex diseases such as AD. We assume that if a gene has the same co-expression and the gene is in the same biological pathway, there is strong evidence of complementarity in the progressive development of the phenotype.

Conclusion

Our genome-wide scan analysis of the ADNI cohort identified some putative loci that are in genetic association with Aβ-42 levels in CSF and moderately associated with Aβ-42 levels in plasma. Extending our analysis using network approaches could emphasize new potential targets, which need more extensive molecular understanding concerning what is truly causal for LOAD. Thus, our analysis demonstrates that quantitative traits observing the Aβ-42 biomarker level and using genome-wide screening can reveal additional insights into the

mechanism that connects these biomarkers with potentially new candidate genes for AD and MCI.

Further studies with independent, larger sample sizes will be important to confirm these findings. In addition, further studies might confirm whether a panel of genetic markers can be combined with CSF and plasma analyses to better predict longitudinal outcomes or responses to emergent therapeutics. In future studies, it will also be important to consider the interaction of SNPs, diagnosis and gene analyses to further investigate the associations with biomarker levels.

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Compliance with Ethical Standards

Conflict of interest There is no conflict of interest in this study.

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