2012

Expression of novel Alzheimer’s disease risk genes in control and Alzheimer’s disease brains

Celeste M. Karch
Washington University School of Medicine in St. Louis

Amanda T. Jeng
Washington University School of Medicine in St. Louis

Petra Nowotny
Washington University School of Medicine in St. Louis

Janet Cady
Washington University School of Medicine in St. Louis

Carlos Cruchaga
Washington University School of Medicine in St. Louis

See next page for additional authors

Follow this and additional works at: https://digitalcommons.wustl.edu/open_access_pubs

Recommended Citation
Karch, Celeste M.; Jeng, Amanda T.; Nowotny, Petra; Cady, Janet; Cruchaga, Carlos; and Goate, Alison M., "Expression of novel Alzheimer’s disease risk genes in control and Alzheimer’s disease brains." PLoS One. 7,11. e50976. (2012).
https://digitalcommons.wustl.edu/open_access_pubs/1258

This Open Access Publication is brought to you for free and open access by Digital Commons@Becker. It has been accepted for inclusion in Open Access Publications by an authorized administrator of Digital Commons@Becker. For more information, please contact vanam@wustl.edu.
Expression of Novel Alzheimer’s Disease Risk Genes in Control and Alzheimer’s Disease Brains

Celeste M. Karch, Amanda T. Jeng, Petra Nowotny, Janet Cady, Carlos Cruchaga, Alison M. Goate*

Department of Psychiatry and Hope Center for Neurological Disorders, Washington University School of Medicine, St Louis, Missouri, United States of America

Abstract

Late onset Alzheimer’s disease (LOAD) etiology is influenced by complex interactions between genetic and environmental risk factors. Large-scale genome-wide association studies (GWAS) for LOAD have identified 10 novel risk genes: ABCA7, BIN1, CD2AP, CD33, CLU, CR1, EPHA1, MS4A6A, MS4A6E, and PICALM. We sought to measure the influence of GWAS single nucleotide polymorphisms (SNPs) and gene expression levels on clinical and pathological measures of AD in brain tissue from the parietal lobe of AD cases and age-matched, cognitively normal controls. We found that ABCA7, CD33, and CR1 expression levels were associated with clinical dementia rating (CDR), with higher expression being associated with more advanced cognitive decline. BIN1 expression levels were associated with disease progression, where higher expression was associated with a delayed age at onset. CD33, CLU, and CR1 expression levels were associated with disease status, where elevated expression levels were associated with AD. Additionally, MS4A6A expression levels were associated with Braak tangle and Braak plaque scores, with elevated expression levels being associated with more advanced brain pathology. We failed to detect an association between GWAS SNPs and gene expression levels in our brain series. The minor allele of rs3764650 in ABCA7 is associated with age at onset and disease duration, and the minor allele of rs670139 in MS4A6E was associated with Braak tangle and Braak plaque score. These findings suggest that expression of some GWAS genes, namely ABCA7, BIN1, CD33, CLU, CR1 and the MS4A family, are altered in AD brains.

Citation: Karch CM, Jeng AT, Nowotny P, Cady J, Cruchaga C, et al. (2012) Expression of Novel Alzheimer’s Disease Risk Genes in Control and Alzheimer’s Disease Brains. PLoS ONE 7(11): e50976. doi:10.1371/journal.pone.0050976

Editor: Stephen D Ginsberg, Nathan Kline Institute and New York University School of Medicine, United States of America

Received June 1, 2012; Accepted October 29, 2012; Published November 30, 2012

Copyright: © 2012 Karch et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: Funding was provided by National Institutes of Health (NIH) P50AG05681 (John C. Morris-Knight Alzheimer’s Disease Research Center); NIH 5R01AG035083-02 (Alison Goate); and the American Health Assistance Foundation (Alison Goate). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: goatea@psychiatry.wustl.edu

Introduction

Late onset Alzheimer’s disease (LOAD) is the most common form of dementia. AD is pathologically defined by extensive neuronal loss and the accumulation of extracellular amyloid plaques and intracellular neurofibrillary tangles in the brain. While the familial form of AD is associated with heritable mutations in the APP, PSEN1, and PSEN2 family, LOAD onset and progression appears to be influenced by complex interactions between genetic and environmental risk factors. Apolipoprotein e4 (APOEe4) is the strongest genetic risk factor for LOAD [1–4] but only accounts for 10–20% of LOAD risk suggesting that susceptibility to LOAD involves additional genetic and environmental risk factors.

In recent efforts to identify additional genetic risk factors for LOAD, large-scale genome-wide association studies (GWAS) have identified single nucleotide polymorphisms (SNP) in 10 genes: ABCA7, BIN1, CD2AP, CD33, CLU, CR1, EPB41L1, MS4A6A, MS4A6E, and PICALM [5–10]. These genes fall into several functional pathways that are affected in AD: immune response (CLU, CR1, ABCA7, and MS4A6A family), cholesterol metabolism (CLU and ABCA7), and synaptic function (PICALM, BIN1, CD33, CD2AP, and EPB41L1).

Despite the identification of numerous SNPs that occur in genes that function in pathways relevant to AD, we still know little of the specific functional impact of the LOAD GWAS SNPs and the specific role of these genes in AD. Thus, we sought to measure the influence of GWAS SNPs on gene expression in a cohort of AD cases and age-matched, cognitively normal control brains. We found that ABCA7, BIN1, CD33, CLU, CR1, and MS4A6A expression are associated with clinical and neuropathological measures of AD. The GWAS SNPs, however, were not associated with gene expression. Thus, we found that the expression patterns of some GWAS genes are altered in AD brains.

Materials and Methods

Subjects

Parietal lobes from European American, autopsy-confirmed AD (N = 73) and age-matched, cognitively normal control (N = 39) brains were obtained from the Charles F. and Joanne Knight Alzheimer’s Disease Research Center (Table 1). AD pathology was measured using Braak and Braak staging [11,12]. Clinical dementia rating (CDR) is a clinical measure of dementia, which incorporates six domains of cognitive and functional abilities: memory, orientation, problem solving, community involvement, home, and personal care [13].

The Washington University IRB reviewed the Knight ADRC Neuropathology Core (from whom the brains were obtained) operating protocol as well as this specific study and determined it was exempt from approval. In the state of Missouri, individuals can give prospective consent for autopsy. Our participants provide this consent by signing the hospital’s autopsy form. If the
participant does not provide future consent before death the DPOA or next of kin provide it after death. All data were analyzed anonymously.

RNA Extraction
RNA was extracted from brain tissue with an RNeasy kit (Qiagen) according to the manufacturer’s protocol. Extracted RNA (10 ug) was converted to cDNA by PCR using the High-Capacity cDNA Reverse Transcriptase kit (ABI). RNA integrity (RIN) was measured in an Agilent Bioanalyzer with an Agilent RNA Pico Kit (Table S1).

Real Time Reactions
Gene expression was analyzed by real-time PCR using an ABI-7900 real-time PCR system, Taqman real-time PCR assays were utilized to quantify expression for the following genes: ABCA7 (ABI: Hs01105094_m1), AIF1 (ABI: Hs00610419_g1), BIN1 (ABI: Hs00184913_m1), CD2AP (ABI: Hs00961451_m1), CD33 (ABI: Hs00233544_m1), CLI1 (ABI: Hs00156548_m1), CLI2 (ABI: Hs00971653_m1), CR1 (ABI: Hs00559342_m1), EPHA1 (ABI: Hs00178313_m1), GAPDH (ABI: Hs01105094_m1), APOE genotype (Table S2). After applying the appropriate covariates to the model, analysis of covariance (ANCOVA) was used to test for association between genotypes and gene expression. SNPs were tested using an additive model. All analyses were performed using statistical analysis software (SAS).

Replication Dataset
The replication dataset was obtained from Myers et al [15]. Brains were obtained from National Institute on Aging Alzheimer’s Centers and the Miami Brain Bank. The 193 brains came from 18 sites and were composed of 20% frontal lobe, 70% temporal lobe, and 1% parietal lobe. The sample was 46% female with a mean age of 81 (range 65–100) and an average post-mortem interval of 10 hours. Expression levels were measured on an Illumina Human Refseq-8 Expression Bead Chip System. To analyze expression levels, residual values were used that were log-transformed and incorporated site, brain region, post-mortem interval, age, APOE genotype, and hybridization date as covariates.

Results
Recent large-scale LOAD GWAS have identified SNPs in ABCA7, BIN1, CD2AP, CD33, CLI1, CR1, EPHA1, MS4A6A, MS4A6E, and PICALM [5–10]. To determine if gene expression is altered in AD, mRNA levels for each gene were measured by real-time PCR in the parietal lobe of AD case and age-matched, cognitively normal, control brains. All gene expression values were normalized to GAPDH, a housekeeping gene that accounts for total cell number. Because AD brains are characterized by neuronal loss, reactive gliosis, and microglial activation, we also corrected gene expression levels for specific subpopulations of cells (neurons [MAP2], microglia [AIF1], and astrocytes [GFAP]) to determine if there were cell specific effects on gene expression. ABCA7 expression was associated with CDR (p = 0.0304), where higher expression levels are correlated with elevated CDR (Table 2). CDR scores increase with cognitive and functional decline [13]. This association remained significant after correcting for subpopulations of cells (Table 2). After correcting expression for neuronal number, BIN1 expression was associated with age at onset (p = 0.0407) and disease duration (p = 0.0407), where higher expression levels are correlated with later age at onset and shorter disease duration (Table 2). The expression of the neuronal isoform of BIN1 (BIN1) was also associated with disease duration after correcting for total, neuronal, and microglial cell populations (Table 2). Correcting expression levels for neuronal and microglial cell populations produced significant associations between disease
Expression of Novel AD Risk Genes in Brain Tissue

status and CDR with CD33 and CR1 expression (Table 2). CorrectingCLU expression levels for neuronal number resulted in the association ofCLU expression with disease status after correcting for neuronal cell populations (p = 0.0159) (Table 2).CLU is alternatively spliced into two isoforms [16]. CLU isoforms containing exon 5 (CLU5) produced similar association patterns after correcting for neuronal and microglia cell populations (Table 2). Additionally, MS4A6A expression levels were weakly associated with Braak tangle and Braak plaque scores (p = 0.0564 and p = 0.0559, respectively), where higher expression levels are correlated with higher Braak scores (Table 2). Higher Braak scores are indicative of more extensive tau and amyloid pathology in the brain [11,12]. The association between MS4A6A expression and Braak tangle and Braak plaque scores was slightly stronger after brain [11,12]. The association between PICALM cholesterol metabolism (Braak tangle and Braak plaque scores was slightly stronger after brain [11,12]. The association between PICALM cholesterol metabolism (Braak tangle and Braak plaque scores (p = 0.0564 and p = 0.0559, respectively), where higher expression levels are correlated with higher Braak scores (Table 2). Higher Braak scores are indicative of more extensive tau and amyloid pathology in the brain [11,12]. The association between MS4A6A expression and Braak tangle and Braak plaque scores was slightly stronger after brain [11,12]. The association between PICALM cholesterol metabolism (Braak tangle and Braak plaque scores (p = 0.0564 and p = 0.0559, respectively), where higher expression levels are correlated with higher Braak scores (Table 2)).

Together, we demonstrate that genes that fall into the same functional category involved in synaptic function (Figure 1J–K). Together, these results demonstrate that genes in a similar functional class are correlated. Expression of CD2AP, MS4A6A, and PICALM expression levels, however, were not associated with AD status or AD pathology (Table 2). Together, we demonstrate that in the absence of strong statistical associations between gene expression and clinical/neuropathological AD outcomes, accounting for subpopulations of cells reveals additional gene expression effects that are likely related to gene function and/or AD-specific cell loss.

The top LOAD risk genes fall into three functional categories: immune response (CLU, CR1, ABCA7, MS4A4, CD33, and EPHA1), cholesterol metabolism (CLU and ABCA7), and synaptic function (PICALM, BIN1, CD33, CD2AP, and EPHA1). We used the expression data for these genes to test whether expression levels of genes in a similar functional class are correlated. Expression of CD33 and MS4A6A, both of which function in immune response, were highly correlated (Figure 1A). Furthermore, expression of CD33 and MS4A6A were highly correlated with AIF1 expression, a marker for microglia, the immune cell of the brain (Figure 1B–C). Expression of genes related to synaptic function, BIN1, BIN1n, CD2AP, and PICALM, were highly correlated (Figure 1D–G). BIN1 and PICALM expression were also highly correlated with GFAP expression, an astrocytic marker (Figure 1H–I). ABCA7 expression, involved in immune response and cholesterol metabolism, was highly correlated with BIN1 and CD2AP expression, which are involved in synaptic function (Figure 1J–K). Together, these results demonstrate that genes that fall into the same functional category are related at the RNA level. Thus, their dysfunction may be linked in AD.

To determine if the LOAD GWAS SNPs influence gene expression, we analyzed the association of SNP genotype with gene expression using an ANCOVA and testing for association with an additive model, the model utilized when originally reporting association between these SNPs and risk for AD [5–10]. We failed to detect an association between GWAS SNPs and cis-acting expression quantitative trait loci (eQTL) after correcting for the total cell population (Table 3) or specific cell types (Table S3).

LOAD GWAS SNPs were identified based on their association with disease status. To determine if these SNPs contribute to AD pathology, independent of gene expression, we analyzed the association of each SNP with clinical (disease status, age at onset, disease duration, and CDR) and neuropathological (Braak tangle and Braak plaque score) measures of AD. The minor allele of rs3764650 in ABCA7 was associated with later age at onset and shorter disease course (p = 0.0040, p = 0.0040, respectively; Table 4; Figure 2). The minor allele of rs670139 in MS4A6E was associated with Braak tangle and Braak plaque score (p = 0.0411, p = 0.0501, respectively; Table 4). We failed to detect an association between the remaining GWAS SNPs and the clinical/neuropathological measures of AD (Table 4).

To replicate our findings, we analyzed a publically available AD dataset [15], in which RNA was measured by the Illumina Human Relseq-9 Expression Bead Chip System. Of the nine genes analyzed in our cohort, only five survived quality control measures in the replication dataset: ABCA7, BIN1, CLU, MS4A6E, and PICALM. We analyzed residual expression levels for association with disease status. MS4A6A and CLU expression levels were significantly associated with disease status (p = 0.0346 and p = 0.0334, respectively), where MS4A6A and CLU expression was up regulated in the AD brains compared with controls (Table 5). BIN1 expression levels were marginally associated with disease status (p = 0.0540), where expression was also up regulated in AD brains compared with controls (Table 5).

Discussion

AD is the most common form of dementia. AD etiology is influenced by complex interactions between genetic and environmental risk factors. APOE4 is the strongest risk factor for LOAD; however, variation in APOE accounts for only 10–20% of LOAD risk, suggesting that additional risk genes exist for LOAD. Recent LOAD GWAS genes have been identified that are involved in cholesterol metabolism, synaptic function, and immune response. Yet, the functional impact of these genes in LOAD remains to be determined. In this study, we measured the influence of LOAD GWAS SNPs and gene expression levels on clinical and neuropathological measures of AD in parietal brain tissue from AD cases and cognitively normal individuals. ABCA7, BIN1, CD33, CLU, and CR1 expression levels were associated with clinical measures of AD (disease status, age at onset, disease duration, and/or CDR), and MS4A6E expression levels were associated with neuropathological measures of AD (Braak tangle and Braak plaque score). We failed to detect an association between GWAS SNPs and gene expression levels. We found that the minor allele of rs3764650 in ABCA7 was associated with clinical measures of AD (age at onset and disease duration), and the minor allele of rs670139 in MS4A6E is associated with neuropathological (Braak tangle and Braak plaque score) measures of AD. Together, these findings demonstrate that ABCA7, BIN1, CD33, CLU, CR1, and the MS4A gene family are affected at the mRNA level in AD brains.

ABCA7, BIN1, CD33, CLU Gene Family Expression are Marginally Associated with AD Phenotypes, CR1, and MS4A

In this study, we found that ABCA7 expression levels are significantly associated with CDR, with higher expression levels of this gene being correlated with more extensive cognitive decline. We also demonstrated that the minor allele of rs3764650 in ABCA7 was associated with age at onset and disease duration, where the minor allele was associated with later age at onset and shorter disease duration. ABCA7 is an ATP-binding cassette transporter protein [17–19]. ABCA7 transports xenobiotics, metals, inorganic ions, carbohydrates, vitamins, amino acids, peptides, and lipids [20–23]. ABCA7 is highly expressed in the CA region of the hippocampus [24], where microglia express the protein at levels ten times greater than is observed in neurons [25]. ABCA7 has been predicted to stimulate the cellular cholesterol efflux to a lipid-free acceptor. ABCA7 may also play a role in phagocytosis [26].

BIN1 and the neuron specific BIN1 isoform (BIN1n) expression levels were associated with clinical measures of AD, where elevated expression was associated with later age at onset and...
Table 2. Gene expression is associated with AD pathology.

| Gene  | Cell Type | Status | Age at Onset | Disease Duration | CDR | Braak Tangle Score | Braak Plaque Score |
|-------|-----------|--------|--------------|------------------|-----|-------------------|-------------------|
|       |           | P value | Beta         | P value          | Beta | P value            | Beta              |
|       |           |         |              |                  |      |                   |                   |
| ABCA7 | GAPDH     | 0.2151  | 0.19         | 0.5824           | 0.01 | 0.5824            | −0.01             |
| MAP2  |           | 0.1820  | 0.41         | 0.4067           | −0.03| 0.5236            | −0.01             |
| AIF1  |           | 0.1207  | 0.38         | 0.7996           | 0.01 | 0.7996            | −0.01             |
| GFAP  |           | 0.8641  | 0.03         | 0.7867           | 0.01 | 0.5577            | 0.02              |
| BIN1  | GAPDH     | 0.3490  | 0.15         | 0.1006           | 0.04 | 0.1006            | −0.04             |
| MAP2  |           | 0.1146  | 0.35         | 0.0407           | 0.06 | 0.3587            | −0.07             |
| AIF1  |           | 0.2104  | 0.30         | 0.1638           | 0.05 | 0.1638            | −0.05             |
| GFAP  |           | 0.7398  | −0.05        | 0.4874           | 0.01 | 0.7305            | −0.01             |
| BIN1n | GAPDH     | 0.3886  | −0.19        | 0.4042           | 0.01 | 0.0111            | −0.08             |
| MAP2  |           | 0.8622  | 0.04         | 0.2208           | 0.03 | 0.0061            | −0.09             |
| AIF1  |           | 0.9806  | −0.01        | 0.0368           | 0.08 | 0.0368            | −0.08             |
| GFAP  |           | 0.0915  | −0.38        | 0.1047           | 0.03 | 0.0961            | −0.05             |
| CD2AP | GAPDH     | 0.8737  | 0.02         | 0.9658           | −0.01| 0.9658            | 0.01              |
| MAP2  |           | 0.2703  | 0.2          | 0.9181           | 0.01 | 0.3334            | −0.02             |
| AIF1  |           | 0.4028  | 0.17         | 0.8332           | 0.02 | 0.8332            | −0.01             |
| GFAP  |           | 0.2235  | −0.18        | 0.5119           | −0.01| 0.1542            | 0.03              |
| CD33  | GAPDH     | 0.6291  | 0.06         | 0.5261           | 0.01 | 0.4950            | 0.01              |
| MAP2  |           | 0.0431  | 0.04         | 0.3260           | 0.01 | 0.9170            | 0.1               |
| AIF1  |           | 0.0174  | 0.27         | 0.9665           | 0.01 | 0.9665            | 0.01              |
| GFAP  |           | 0.3917  | −0.14        | 0.0889           | 0.04 | 0.0889            | 0.04              |
| CLU1  | GAPDH     | 0.3105  | 0.09         | 0.7466           | −0.01| 0.6191            | −0.01             |
| MAP2  |           | 0.0159  | 0.35         | 0.5680           | 0.01 | 0.2185            | −0.02             |
| AIF1  |           | 0.1051  | 0.27         | 0.8649           | 0.01 | 0.8649            | −0.01             |
| GFAP  |           | 0.4559  | −0.09        | 0.9527           | 0.01 | 0.1758            | 0.02              |
| CLU2  | GAPDH     | 0.0664  | 0.15         | 0.7600           | 0.01 | 0.6676            | −0.01             |
| MAP2  |           | 0.0036  | 0.42         | 0.4485           | 0.01 | 0.2147            | −0.02             |
| AIF1  |           | 0.0500  | 0.33         | 0.9039           | 0.01 | 0.9039            | −0.01             |
| GFAP  |           | 0.8104  | −0.03        | 0.8314           | 0.01 | 0.1798            | 0.03              |
| CR1   | GAPDH     | 0.2452  | 0.25         | 0.9715           | −0.01| 0.8680            | 0.01              |
| MAP2  |           | 0.0252  | 0.59         | 0.9118           | 0.01 | 0.9420            | −0.01             |
| AIF1  |           | 0.0303  | 0.51         | 0.4042           | −0.02| 0.4042            | 0.02              |
| GFAP  |           | 0.7829  | 0.05         | 0.8507           | 0.01 | 0.2024            | 0.04              |
| EPHA1 | GAPDH     | 0.0782  | −0.28        | 0.2299           | 0.01 | 0.2992            | −0.06             |
| MAP2  |           | 0.8657  | 0.03         | 0.4988           | 0.01 | 0.6580            | −0.01             |
| AIF1  |           | 0.9763  | 0.07         | 0.8337           | 0.01 | 0.8337            | 0.01              |
| GFAP  |           | 0.0596  | 0.45         | 0.8758           | −0.01| 0.2716            | 0.03              |
| MS4A6A| GAPDH     | 0.7251  | 0.06         | 0.5184           | 0.01 | 0.7308            | 0.01              |
| MAP2  |           | 0.1844  | 0.29         | 0.2252           | 0.02 | 0.8651            | −0.01             |
| AIF1  |           | 0.1332  | 0.25         | 0.7390           | 0.01 | 0.7390            | −0.01             |
| GFAP  |           | 0.4191  | −0.15        | 0.2453           | 0.03 | 0.2453            | 0.03              |
| PICALM| GAPDH     | 0.4682  | 0.10         | 0.1283           | 0.03 | 0.1283            | −0.03             |
| MAP2  |           | 0.1351  | 0.29         | 0.4987           | 0.01 | 0.0614            | −0.05             |
| AIF1  |           | 0.2380  | 0.26         | 0.2692           | 0.04 | 0.2692            | −0.04             |
| GFAP  |           | 0.4669  | −0.11        | 0.9884           | 0.01 | 0.9252            | −0.01             |
| MAP2  |           | 0.0224  | −0.27        | 0.2440           | 0.03 | 0.0203            | −0.03             |
| AIF1  |           | 0.2354  | −0.19        | 0.0203           | 0.03 | 0.0856            | −0.03             |
| GFAP  |           | 0.2658  | 0.19         | 0.7913           | −0.01| 0.1785            | −0.03             |

Covariates included in analyses are reported in Table S2. CLU1, probe spans exons 3–4. CLU2, probe spans exons 4–5.
doi:10.1371/journal.pone.0050976.t002
shorter disease duration. Bin1 is implicated in receptor-mediated endocytosis and recycling of endosomes in the cell. Bin1 knockout mice do not exhibit deficiency in synaptic vesicle recycling [27,28] but have less age-associated inflammation [29].

CD33 and CR1 expression levels were associated with clinical measures of AD, where elevated expression levels were associated with AD after correcting for neuron and microglia number in the brain. CD33 and CR1 function in immune response pathways. CD33 is a transmembrane receptor expressed on cells from the myeloid lineage. CD33 functions in the innate and adaptive immune response [30], and it may play a role in receptor-mediated endocytosis independent of clathrin [31]. CR1 plays an essential role in the adaptive immune response. CR1 is highly expressed in red blood cells [32], where it mediates cell binding to particles and immune complexes. CR1 is a negative regulator of the complement cascade; mediates immune adherence and phagocytosis; and inhibits the classical and alternative complement pathways [33].

CLU expression levels are associated with clinical measures of AD, where elevatedCLU levels occur in individuals with AD. Clusterin (ApoJ) exists as two isoforms and is highly expressed in astrocytes [16]. Clusterin is secreted from cells where it is reported to have several roles in the cell: chaperone function [34,35], lipid trafficking [36,37], and inhibition of the complement cascade [38]. Clusterin inhibits complement activation and the membrane attack complex [30], which is relevant to AD in that neuroim-

Figure 1. Expression of genes involved in immune response and synaptic function are highly correlated in brain tissue. Relative expression (RE) was plotted for the indicated genes. Genes involved in immune response (A-C). Genes involved in synaptic function (D-I). Genes involved in cholesterol metabolism and synaptic function (J-K). doi:10.1371/journal.pone.0050976.g001
flammation is a key feature of the disease. Clusterin has been implicated in AD in its ability to assist in refolding of misfolded proteins [35], bind to fibrillar proteins [39,40], clearance of Aβ [41], and interact with ApoE [41]. Neuritic dystrophy and fibrillar amyloid deposits are markedly reduced when \( \text{CLU} \) is knocked out in PDAPP mice [42], suggesting that \( \text{CLU} \) may have deleterious effects when upregulated in AD brains. However, in the absence of APOE and \( \text{CLU} \), PDAPP mice have accelerated disease onset, elevated CSF and ISF beta-amyloid levels, and more extensive amyloid deposition in the brain [41]. Thus, the role of clusterin in the brain is complex and influenced by other genes.

In our cohort, genes in the \( \text{MS4A} \) gene cluster showed association with clinical and neuropathological measures of AD. \( \text{MS4A6A} \) expression levels were found to be associated with elevated Braak tangle and Braak plaque scores. Additionally, the minor allele of rs670139 in \( \text{MS4A6E} \) was associated with CDR, Braak tangle score, and Braak plaque score. The \( \text{MS4A} \) family of genes is reported to play a role in the immune response via expression on high affinity IgE receptors [43]; however, little is known about the function of each family member. While several genes in the \( \text{MS4A} \) gene cluster have been identified in recent LOAD GWAS [9,10], we only measured expression levels of the \( \text{MS4A6A} \) gene. Due to extensive sequence conservation between the \( \text{MS4A} \) genes, we were unable to identify Taqman probes in other \( \text{MS4A} \) genes that would specifically detect a single gene; thus, we are limited in our interpretation of the role of each of the \( \text{MS4A} \) genes in AD brains. While our replication data set only contained the \( \text{MS4A6A} \) gene, we were able to replicate the association with disease status.

Factors Contributing to the Absence of Robust Findings

The associations we describe in this study are only marginal and would not survive multiple test correction. We interpret these findings to point to subtle effects in gene expression. However, type 1 errors are also a possible explanation. Our observations that the association of gene expression with clinical and neuropathological measures of AD can change after correction for neuronal, astrocytic, and microglial subpopulations indicates that cell specific gene expression plays an important role in disease.

We chose to examine measures of AD (disease status, CDR, Braak plaque score and Braak tangle score) because each trait represents a different, not completely overlapping, aspect of Alzheimer’s disease. AD status, a dichotomous trait, is assigned at autopsy based on several criteria, including clinical dementia, neuronal death and Braak plaque and Braak tangle scores. CDR,

| SNP      | Gene   | MAF   | P value | Beta  |
|----------|--------|-------|---------|-------|
| rs3764650| ABCA7  | 0.13  | 0.6471  | 0.07  |
| rs744373 | BIN1   | 0.34  | 0.7720  | 0.03  |
| rs59335482| BIN1  | 0.31  | 0.2879  | 0.12  |
| rs744373| BIN1n  | 0.34  | 0.2666  | 0.17  |
| rs59335482| BIN1n| 0.31  | 0.1217  | 0.24  |
| rs9349407| CD2AP  | 0.26  | 0.0610  | −0.18 |
| rs3865444| CD33   | 0.29  | 0.3071  | 0.10  |
| rs7982  | CLU1   | 0.38  | 0.1324  | −0.09 |
| rs7982  | CLU2   | 0.38  | 0.1452  | −0.08 |
| rs670173| CR1    | 0.01  | 0.9630  | −0.02 |
| rs3818361| CR1   | 0.22  | 0.1753  | −0.20 |
| rs11767557| EPHA1| 0.16  | 0.1989  | 0.17  |
| rs610932| MS4A6A | 0.43  | 0.5130  | −0.13 |
| rs3851179| PICALM | 0.38  | 0.2791  | −0.09 |

Covariates included in analyses are reported in Table S2.

Table 3. AD GWAS SNPs do not modify gene expression in the parietal lobe of human brains.

Figure 2. Rs3764650 in \( \text{ABCA7} \) is associated with age at onset. Kaplan-Meier curve. AAO, age at onset in years. SNPs were analyzed using an additive model. G, minor allele. Blue line, TT (11). Red line, TG (12). Green line, GG (22).

doi:10.1371/journal.pone.0050976.t003

doi:10.1371/journal.pone.0050976.g002
absence of association across phenotypes is an issue of statistical
the other phenotypes are ordinal, it remains possible that the
tangle scores are also ordinal traits, each representing an aspect of
problem solving, community involvement, home and personal
domains of cognitive and functional abilities including memory, orientation,
however, represents a clinical diagnosis that measures six domains
mental factors may also contribute to or obscure gene expression
disease course could produce additional associations. Environ-
are susceptible to AD pathology at earlier time points in the
study was limited to the parietal lobe, where AD pathology occurs
that changes are occurring in these genes during disease that we
are unable to capture in our cohort. With a sample size of 112, this
that changes are occurring in these genes during disease that we
neuropathological measurements of AD in some of the genes
Covariates included in the analysis are marked: *Age, APOE, $PMI$, $CDR.
Table 4. Gene SNPs do not significantly influence AD brain pathology.

| SNP      | Gene | Status* | P value | Beta | CDR*  | P value | Beta | Age at Onset* | P value | Beta | Disease Duration* | P value | Beta | Braak Tangle Score* | P value | Beta | Braak Plaque Score* | P value | Beta |
|----------|------|---------|---------|------|-------|---------|------|---------------|---------|------|-------------------|---------|------|-------------------|---------|------|-------------------|---------|------|
| rs3746450| ABCA7| 0.1524  | 0.13    | 0.2966| 1.87  | 0.0040  | 2.58| 0.0040        | 2.58   | 0.5875| −0.18            | 0.7356  | −0.11|
| rs744373 | BIN1 | 0.8570  | 0.01    | 0.2245| 1.85  | 0.5234  | 0.49| 0.5234        | 0.49   | 0.4395| −0.21            | 0.4194  | 0.21|
| rs59335482| BIN1 | 0.7857  | 0.02    | 0.3509| 1.38  | 0.9192  | 0.08| 0.9192        | 0.08   | 0.1477| −0.38            | 0.5852  | 0.14|
| rs9349407 | CD2AP| 0.8372  | 0.02    | 0.1217| 2.52  | 0.0507  | 1.56| 0.0507        | 1.56   | 0.5909| 0.16             | 0.4127  | 0.20|
| rs3865444 | CD33 | 0.4485  | 0.06    | 0.8427| 0.34  | 0.4515  | 0.64| 0.4515        | 0.64   | 0.6188| −0.15            | 0.9381  | 0.02|
| rs7982   | CLU  | 0.8533  | 0.01    | 0.9251| −0.14| 0.4431  | 0.57| 0.4431        | 0.57   | 0.8560| 0.05             | 0.1357  | −0.34|
| rs670173 | CR1  | 0.3697  | 0.18    | 0.3205| 3.62  | 0.7683  | −0.52| 0.7683        | 0.52   | N/A   | N/A              | N/A     | N/A |
| rs3818361| CR1  | 0.3315  | 0.08    | 0.9699| −0.07| 0.2829  | 0.95| 0.2829        | 0.95   | 0.2082| −0.35            | 0.6167  | 0.13|
| rs11767557| EPHA1| 0.3221  | 0.19    | 0.4902| 1.19  | 0.7123  | 0.31| 0.7123        | 0.31   | 0.1470| −0.45            | 0.8202  | 0.07|
| rs610932 | MS4A6A| 0.2860  | 0.07    | 0.9290| −0.12| 0.1532  | 0.97| 0.1532        | 0.97   | 0.1520| 0.34             | 0.0998  | 0.43|
| rs670139 | MS4A6E| 0.1624  | 0.09    | 0.5966| 0.82  | 0.9753  | −0.02| 0.9753        | 0.02   | 0.0411| −0.53            | 0.0581  | −0.47|
| rs3851179| PICALM| 0.6162  | 0.03    | 0.6355| −0.73| 0.3357  | −0.74| 0.3357        | 0.74   | 0.8133| 0.06             | 0.1133  | 0.41|

The Complexities of Defining the Functional Impact of LOAD GWAS SNPs

In this study, we analyzed genotype association with gene expression level to determine if the LOAD GWAS SNPs were functionally relevant. We failed to identify any SNPs that influence gene expression levels independent of disease status. Thus, it is possible that the functional polymorphisms that exist within these genes are rare, alter gene splicing, or impact inducible expression rather than constitutive expression. These findings fit with our previous study: we were unable to identify statistically significant associations of GWAS SNPs and SNPs in linkage disequilibrium with GWAS SNPs with CSF tau and $A\beta$ levels [44]. Thus, it is essential to exploit deep sequencing techniques to identify functional variants in these genes.

LOAD GWAS Genes are Functionally Linked

The genes identified in recent LOAD GWAS fall into three functional categories: immune response (CLU, CR1, ABCA7, MS4A family, CD33, and EPHA1), cholesterol metabolism (CLU and ABCA7), and synaptic function (PICALM, BIN1, CD33, CD2AP, and EPHA1). The genes with the most significant association with clinical and neuropathological measures of AD function in immune response and cholesterol metabolism. Despite an absence of association with the remaining GWAS genes, it is possible that these genes are affected at the protein level in AD brains.

Changes in genes that influence immune response may be difficult to identify in autopsied brain tissue, as the immune response can be transient. Additionally, alterations of the immune response in AD may primarily occur in organs other than the brain. CD2AP is localized in the cytoplasm where it has several

Table 5. CLU and MS4A6A expression are associated with AD status in a replication dataset.

| Gene     | Status | P value | Beta |
|----------|--------|---------|------|
| ABCA7    | 0.3471 | 0.07   |
| BIN1     | 0.0540 | 0.09   |
| CLU      | 0.0334 | 0.11   |
| MS4A6A   | 0.0346 | 0.19   |
| PICALM   | 0.1405 | 0.09   |

doi:10.1371/journal.pone.0050976.t005

doit:10.1371/journal.pone.0050976.t004

Expression of Novel AD Risk Genes in Brain Tissue
axogenesis, and dendritic outgrowth in neurons [51]. EphA1 is highly expressed in the adult brain, where it participates in forward signaling in receptor-bearing cells and reverse signaling in ligand-bearing cells by binding to GPI-linked A ephrins, which together facilitates axon guidance and communication between neighboring cell populations [52–57]. CD2AP knockout also mice exhibit deficiencies in receptor trafficking to the lysosome.

Conclusions

This study provides evidence for the involvement of ABCA7, BIN1, CD33, CLU, CR1, and MTH1 gene family in AD brain pathology. As AD is a complex disorder, it is likely that many genes are affected at the RNA and protein levels and that an understanding of the complex interactions that may occur between these genes is essential to understanding and treating AD.

Supporting Information

Figure S1 CT values for expression assays. Non-normalized CT values for each gene expression assay were averaged for AD (white) and non-demented control (black) brains.

Figure S2 GAPDH and PPIA expression are highly correlated. Average CT was plotted for each sample. A. GAPDH-FAM versus PPIA. B. GAPDH-VIC versus PPIA. C. GAPDH-FAM versus GAPDH-VIC.

References

1. Bertram L, Lange C, Mullin K, Parkinson M, Hsiao M, et al. (2008) Genome-wide association analysis reveals putative Alzheimer’s disease susceptibility loci in addition to APOE. American journal of human genetics 83: 623–632.
2. Goon KD, Myers AJ, Craig DW, Webster JA, Pearson JV, et al. (2007) A high-density whole-genome association study reveals that APOE is the major susceptibility gene for sporadic late-onset Alzheimer’s disease. The Journal of clinical psychiatry 68: 613–618.
3. Pericak-Vance MA, Behrouz JL, Gaskell PC Jr, Yamaoka LH, Hung WY, et al. (1991) Linkage studies in familial Alzheimer disease: evidence for chromosome 19 linkage. American journal of human genetics 46: 1034–1050.
4. Schmechel DE, Saunders AM, Strittmatter WJ, Hulette CM, et al. (1993) Increased amyloid beta-peptide deposition in cerebral cortex as a consequence of apolipoprotein E genotype in late-onset Alzheimer disease. Proceedings of the National Academy of Sciences of the United States of America 90: 9649–9653.
5. Jun G, Naj AC, Beecham GW, Wang LS, Burco J, et al. (2010) Meta-analysis confirms CR1, CD33, and PICALM as Alzheimer disease-risk loci and reveals interactions with APOE genotypes. Archives of neurology 67: 1473–1481.
6. Seshadri S, Fitzpatrick AL, Irahama MA, De Stefano AL, Gutman AS, et al. (2010) Genome-wide analysis of genetic loci associated with Alzheimer disease. JAMA: the journal of the American Medical Association 303: 1832–1840.
7. Harold D, Abraham R, Hollingsworth P, Sims R, Gerrish A, et al. (2009) Genome-wide association study identifies variants at CLU and PICALM associated with Alzheimer’s disease. Nat Genet 41: 1038–1040.
8. Lambert JC, Heath S, Even G, Campion D, Sleegers K, et al. (2009) Genome-wide association study identifies variants at MS4A4E/MS4A6E, CD2AP, CD33 and EPHA1 are associated with Alzheimer’s disease. Nature genetics 43: 429–435.
9. Naj AC, Jun G, Beecham GW, Wang LS, Vardarajan BN, et al. (2011) Common variants at MS4A4/MS4A6E, CD2AP, CD33 and EPHA1 are associated with late-onset Alzheimer’s disease. Nature genetics 43: 436–441.
10. Braak H, Baczek E (1991) Neuropathological staging of Alzheimer-related changes. Acta neuropathologica 82: 239–259.
11. Braak H, Alafuzoff I, Arzberger T, Kretschmar H, Del Tredici K (2006) Staging of Alzheimer disease-associated neurofibrillary pathologizing by paraflin sections and immunocytochemistry. Acta neuropathologica 112: 309–404.
12. Murre JC (1993) The Clinical Dementia Rating (CDR): current version and scoring rules. Neurology 43: 2142–2144.
13. Muller PY, Janowjak H, Miserez AR, Dobbie Z (2002) Processing of gene expression data generated by quantitative real-time RT-PCR. BioTechniques 32: 1372–1374, 1376, 1378–1379.

Figure S3 Normalization of gene expression by log transformation. Log transformed values of relative expression values for each LOAD GWAS genes are illustrated in a histogram. Red line, normal density curve. Gray line, fitted density curve.

Table S1 Average RNA integrity number for case and control brains.

Table S2 Covariates that were included in analysis.

Table S3 AD GWAS SNPs do not modify gene expression in the parietal lobe of human brains after correcting for cell-specific gene expression.

Acknowledgments

We would like to thank Jen Wang for thoughtful advice in the preparation of this manuscript. We also thank the Clinical and Neuropathological Cores of the Charles F. and Joanne Knight Alzheimer’s Disease Research Center for generously sharing clinical data, pathological data, and brain tissue and the Genetics Core for APOE genotypes.

Author Contributions

Conceived and designed the experiments: CMK CC AMG. Performed the experiments: CMK ATJ PN JC. Analyzed the data: CMK. Wrote the paper: CMK AMG.
30. Crocker PR, Hartnell A, Munday J, Nath D (1997) The potential role of siaoadhesin as a macrophage recognition molecule in health and disease. Glycocompounds journal 14: 601–609.

31. Tateno H, Li H, Schur M, Bovin N, Crocker PR, et al. (2007) Distinct endocytic mechanisms of CD22 (Siglec-2) and Siglec-F reflect roles in cell signaling and innate immunity. Molecular and cellular biology 27: 5699–5710.

32. Zanjani H, Finch CE, Kemper C, Atkinson J, McKeel D, et al. (2005) Complement activation in very early Alzheimer disease. Alzheimer Dis Assoc Disord 19: 55–66.

33. Morgan BP, Rushmere NK, Harris CL (1998) Therapeutic uses of recombinant complement receptors. Biochemical Society transactions 26: 49–54.

34. Poon S, Easterbrook-Smith SB, Rybchyn MS, Carver JA, Wilson MR (2000) Clusterin is an ATP-independent chaperone with very broad substrate specificity that stabilizes stressed proteins in a folding-dependent state. Biochemistry 39: 15953–15960.

35. Zinkel M, Kruse FE, Junemann AG, Naumann GO, Schlotzer-Schrehardt U (2006) Clusterin deficiency in eyes with pseudoexfoliation syndrome may be implicated in the aggregation and deposition of pseudoexfoliative material. Investigative ophthalmology & visual science 47: 1982–1990.

36. Jenne DE, Lowin B, Peitsch MC, Bottcher A, Schmitz G, et al. (1991) Clusterin (complement lysis inhibitor) forms a high density lipoprotein complex with apolipoprotein A-I in human plasma. The Journal of biological chemistry 266: 11030–11036.

37. Calevo G, Restagno A, Matsubara E, Zikovic B, Frangione B, et al. (2000) Apolipoprotein J (clusterin) and Alzheimer’s disease. Microscopy research and technique 50: 305–315.

38. Kirszbaum L, Bozas SE, Walker ID (1992) SP-40,40, a protein involved in the control of the complement pathway, possesses a unique array of disulphide technique 50: 305–315.

39. Kumita JR, Poon S, Caddy GL, Hagan CL, Dumoulin M, et al. (2007) The extracellular chaperone clusterin potently inhibits human lysozyme amyloid formation by interacting with prefibrillar species. Journal of molecular biology 369: 157–167.

40. Yerbury JJ, Poon S, Meehan S, Thompson B, Kumita JR, et al. (2007) The potential mapping of genetic variants in BIN1, CLU, CR1 and PICALM for association with cerebrospinal fluid biomarkers for Alzheimer’s disease. PLoS ONE 6: e15918.

41. Dustin ML, Olszowy MW, Holdorf AD, Li J, Bromley S, et al. (1998) A novel adaptor protein orchestrates receptor patterning and cytoskeletal polarity in T-cell contacts. Cell 94: 667–677.

42. Huber TB, Hartleben B, Kim J, Schmidts M, Schermer B, et al. (2005) Neprhin and CD2AP associate with phosphoinositide 3-OH kinase and stimulate AKT-dependent signaling. Molecular and cellular biology 25: 4917–4928.

43. Schaffer M, Mandel P, Shaw AS, Bottinger EP (2004) A novel role for the adaptor molecule CD2-associated protein in transforming growth factor-beta-induced apoptosis. The Journal of biological chemistry 279: 37004–37012.

44. Cormont M, Meton I, Mari M, Monzo P, Keslair F, et al. (2003) CD2AP/CM3 regulates endosome morphology and traffic to the degradative pathway through its interaction with Rab4 and c-Cbl. Traffic 4: 97–112.

45. Kobayashi S, Sawano A, Nojima Y, Shibuya M, Maru Y (2004) The c-Cbl-CD2AP complex regulates VEGF-induced endocytosis and degradation of Flt-1 (VEGFR-1). The FASEB journal: official publication of the Federation of American Societies for Experimental Biology 18: 928–931.

46. Bushlin I, Petralia RS, Wu F, Harel A, Mughal MR, et al. (2000) Clathrin assembly protein AP180 and CALM differentially control axonogenesis and dendrite outgrowth in embryonic hippocampal neurons. The Journal of neuroscience: the official journal of the Society for Neuroscience 20: 10257–10271.

47. Himanen JP, Nikolov DB (2003) Eph receptors and ephrins. The international journal of biochemistry & cell biology 35: 130–134.

48. Kollander K, Klein R (2002) Mechanisms and functions of Eph and ephrin signalling. Nature reviews Molecular cell biology 3: 473–486.

49. Pasquale EB (2005) Eph receptor signalling casts a wide net on cell behaviour. Nature reviews Molecular cell biology 6: 462–475.

50. Wilkinson DG (2000) Eph receptors and ephrins: regulators of guidance and assembly. International review of cytology 196: 177–244.

51. Polakow A, Corrion A, Wilkinson DG (2004) Diverse roles of eph receptors and ephrins in the regulation of cell migration and tissue assembly. Developmental cell 7: 465–480.

52. Noren NK, Pasquale EB (2004) Eph receptor-ephrin bidirectional signals that target Ras and Rho proteins. Cellular signalling 16: 655–666.