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Translating Alzheimer's disease-associated polymorphisms into functional candidates: a survey of IGAP genes and SNPs

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Abstract

The International Genomics of Alzheimer's Project (IGAP) is a consortium for characterizing the genetic landscape of Alzheimer's disease (AD). The identified and/or confirmed 19 single nucleotide polymorphisms (SNPs) are located on non-coding DNA regions, and their functional impacts on AD are as yet poorly understood. We evaluated the roles of the IGAP SNPs by integrating data from many resources, based on whether the IGAP SNP was (A) a proxy for a coding SNP or (B) associated with altered mRNA transcript levels. For (A), we confirmed that 12 AD-associated coding common SNPs and five nonsynonymous rare variants are in linkage disequilibrium with the IGAP SNPs. For (B), the IGAP SNPs in *CELF1* and *MA4A6A* were associated with expression of their neighboring genes, *MYBPC3* and *MA4A6A* respectively, in blood. The IGAP SNP in *DSG2* was an expression quantitative trait loci (eQTL) for *DLGAP1* and *NETO1* in human frontal cortex. The IGAP SNPs in *ABCA7*, *CD2AP*, and *CD33* each acted as eQTL for AD-associated genes in brain. Our approach for identifying proxies and examining eQTL highlighted potentially impactful, novel gene regulatory phenomena pertinent to the AD phenotype.

Keywords

WES; ADSP; ADGC; GWAS; neuroinflammation

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Disclosure statement

The authors declare no conflicts of interest.

1. Introduction

Dementia is a clinical state characterized by a loss of function in memory and behavior, and the clinical state is associated with underlying degeneration of central nervous system synapses and cells. Alzheimer's disease (AD) is the most common form of dementia, accounting for over 50% of dementia cases (Querfurth and LaFerla, 2010). Although it has been more than 100 years since Alois Alzheimer published "About a Peculiar Disease of the Cerebral Cortex" (Alzheimer, 1907), the exact cause of AD has not yet been defined. Amyloid β (Aβ) protein and hyperphosphorylated tau aggregates in brain are considered the key pathological hallmarks (Reitz, et al., 2011,Selkoe, et al., 2004). A widely held mechanistic hypothesis for AD pathogenesis is the "amyloid cascade hypothesis" wherein a key early pathogenetic role is played by parenchymal Aβ peptide accumulation, which causes or exacerbates downstream neuronal injury, enhanced neuroinflammation, tau hyperphosphorylation, and eventually the clinical symptoms of AD (Hardy and Selkoe, 2002).

Familial AD, which often occurs early in life, is linked to mutations in three genes: the amyloid precursor protein (*APP*) gene and the presenilin protein (*PSEN1* and *PSEN2*) genes (van Es and van den Berg, 2009). These genes are associated with altered processing of the APP protein, including a shift in Aβ peptide production from $A\beta_{40}$ to more neurotoxic $A\beta_{42}$ (e.g., Volga German mutation in *PSEN2* and Iberian mutation in *APP*) (Jayadev, et al., 2010,Levy-Lahad, et al., 1995,Lichtenthaler, et al., 1999,Walker, et al., 2005), increased total Aβ levels (Swedish mutation in *APP*) (Mullan, et al., 1992), and increased Aβ protofibril formation (Arctic mutation in *APP*) (Nilsberth, et al., 2001). In contrast, late-onset AD, which accounts for > 95% of all AD cases (Mancuso, et al., 2008), has a more complex genetic architecture. The ε4 allele of apolipoprotein E (*APOE*) gene is the most well-established susceptibility risk factor for late-onset AD.

A series of genome-wide association studies (GWAS) have identified AD-associated single nucleotide polymorphisms (SNPs) in addition to the *APOE* alleles (Harold, et al., 2009,Hollingworth, et al., 2011,Lambert, et al., 2009,Lambert, et al., 2013,Naj, et al., 2011,Seshadri, et al., 2010). The study with the largest number of AD and non-AD individuals was the International Genomics of Alzheimer's Project (IGAP), which capitalized on a large, multicenter study design to include 74,046 individuals (Lambert, et al., 2013). This study extended associations between the AD phenotype and genetics, finding 21 SNPs as significant by meta-analyzing genetic and phenotype data from four component consortia (Lambert, et al., 2013). These SNPs are in or close to *CR1*, *BIN1*, *INPP5D*, *MEF2C*, *CD2AP*, *NME8*, *EPHA1*, *PTK2B*, *PICALM*, *SORL1*, *FERMT2*, *SLC24A4-RIN3*, *DSG2*, *CASS4*, *HLA-DRB5-DBR1*, *CLU*, *MS4A6A*, *ABCA7*, *CD33*, *ZCWPW1*, and *CELF1* (Supplementary Table 1). Although GWAS have succeeded in revealing numerous susceptibility variants for AD, determining the functional impact of those gene variants and understanding how they contribute to AD pathogenesis represents a barrier to progress in the field.

Genetic variants located in coding regions constitute only ~1% of gene polymorphisms seen in humans (Rabbani, et al., 2014). However, there are many ways that genetic variants in non-coding regions can affect protein expression and structure, and thereby exert a protective or disease-inducing impact. Functional variants may be located in a coding region, an alternative splicing region, or a regulatory region such as promoter, operator, insulator, enhancer or silencer.

Nonsynonymous variants, by definition, alter the primary amino acid sequence of a protein and may have effects on the protein structure and function. Synonymous mutations occur in the coding region, but do not change the amino acid sequence. These variants were referred to as “silent mutations” until recently (Sauna and Kimchi-Sarfaty, 2011). Several synonymous mutations have been reported to affect mRNA splicing and stability, gene expression, and protein folding and function (Sauna and Kimchi-Sarfaty, 2011). Most of the non-*APOE* AD-associated genetic variants described to date are located in intronic or intergenic regions (i.e., non-coding regions), which may contain regulatory elements. Intronic and intergenic SNPs may act by regulating expression of disease-associated genes and/or modulating translation efficiency and stability (Mockenhaupt and Makeyev, 2015).

In the present study, we analyzed data from multiple sources to gain insights into the roles of non-coding SNPs identified in the IGAP study (including *CD33* and *DSG2*, although their associated SNPs, rs3865444 and rs8093731, respectively, did not reach statistical significance in the combined stages of that study (Lambert, et al., 2013)), hereafter referred to as “IGAP SNPs”. We hypothesized that each IGAP SNP is potentially: (1) a proxy for an exonic (coding) variant (Supplementary Figure 1A and 1B) that has not yet been identified; or (2) associated with altered transcript/mRNA levels (Supplementary Figure 1C). One approach to test the first hypothesis is to identify coding variants in strong linkage disequilibrium (LD) with the variant identified by GWAS, which indicates that the two gene loci are commonly co-inherited. For the second hypothesis, expression quantitative trait loci (eQTL) analyses can be used to assess the association between the gene variant and mRNA levels of various transcripts. Thus, eQTL are genetic loci that contribute to variation in gene expression. By mapping eQTL, we investigated how the variants regulate gene expression. Using these combined methods, and multiple data sources, we discovered new evidence of complex gene expression regulation mechanisms in association with previously identified IGAP SNPs.

2. Material and methods

2.1. Genetic datasets

Genetic data were obtained from multiple sources. Whole exome sequence (WES) data came from the Alzheimer’s Disease Sequencing Project (ADSP), composed of 18 cohorts from the Alzheimer’s Disease Genetic Consortium (ADGC) and six cohorts from the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium (Beecham, et al., 2017). We also used imputed SNP data (Supplementary Method) from the ADGC comprising 23 different cohorts (Supplementary Table 2). From these sources, there were 28,730 unrelated subjects with imputed GWAS SNP data in ADGC and 10,913 unrelated subjects with WES data in ADSP. We estimated identity-by-descent (IBD) to

identify any relatedness and duplicate individuals in the two datasets. Individuals were excluded with estimated IBD ≥ 0.1875 from ADGC datasets, and two independent datasets were created: an imputed ADGC dataset that excluded related individuals and those that potentially overlapped with those in ADSP (hereafter referred to as “ADGC”), and WES data in ADSP (hereafter referred to as “ADSP”) (Figure 1). We limited the included subjects to those who had AD diagnosis information and who were 65 years or older at the last visit or at death, yielding a total of 15,343 ADGC subjects with imputed SNP data in the discovery analysis and a total of 10,407 ADSP subjects with WES data in the replication analysis.

2.2. Gene expression datasets

Data were obtained from the Alzheimer’s Disease Neuroimaging Initiative (ADNI) database (adni.loni.usc.edu). The ADNI was launched in 2003 as a public-private partnership, led by Principal Investigator Michael W. Weiner, MD. The primary goal of 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). Data were included from 763 subjects aged 65 years or older who had both gene expression data from blood (Affymetrix Human Genome U219 Array platform) and whole genome sequencing (WGS) data available. Clinical status was determined based on the clinical evaluation at the last examination.

Human brain gene expression and genotype data were obtained from the North American Brain Expression Consortium (NABEC) (Hernandez, et al., 2012) and United Kingdom Brain Expression Consortium (UKBEC) (Trabzuni, et al., 2011). Details were as described in our previous report (Katsumata, et al., 2017). Briefly, the NABEC expression data for FCTX were available at Gene Expression Omnibus (GEO: https://www.ncbi.nlm.nih.gov/geo/) public repository and the genotype data were obtained from the database of Genotypes and Phenotypes (dbGaP: http://www.ncbi.nlm.nih.gov/gap). Standard quality control (QC) procedures were performed on the NABEC genotype data using PLINK v1.90a (Purcell, et al., 2007). Markers were excluded based on the following criteria: (1) minor allele frequency (MAF) < 1%; (2) call rate per variant (SNPs and indels) < 95%, (3) Hardy-Weinberg equilibrium test in controls < 10^{-5}. Samples were excluded based on the following criteria: (1) call rate per individual < 95%, (2) a high degree of relatedness per an estimated proportion of IBD > 0.1875, (3) excess of ± 3.0 standard deviations of heterozygosity rate. After performing QC, we imputed using the Michigan Imputation Server (https://imputationserver.sph.umich.edu/start.html) (Das, et al., 2016,Loh, et al., 2016) with the following parameters: 1000 Genome Phase 3 v5 reference panel, Eagle v2.3 phasing (Loh, et al., 2016), and EUR population. Of the 455 neurologically normal donors, 85 subjects who died at age 65 years or older and passed QC were included in the analysis (all were US Caucasians). The UKBEC gene expression for three brain regions (frontal cortex, FCTX; hippocampus, HIPP; and temporal cortex, TCTX) and genotype data were obtained from the BRAINEAC website (http://www.braineac.org/). Dosage genotype data were converted into PLINK file format using Genome-wide Complex Trait Analysis (GCTA) software version

1.24.4 (Yang, et al., 2011). Among the 134 neuropathologically normal individuals, 49 subjects who died at age 65 years or older were included in the present analyses.

Since the NABEC and UKBEC datasets do not have AD diagnosis information, we retrieved microarray datasets generated from Affymetrix Human Genome U133 Plus 2.0 Array platform (GPL570) regarding AD status from GEO to examine whether the levels of gene expression were different in association with AD status versus controls. We focused on gene expression in four brain regions affected by AD (entorhinal cortex (EC), FCTX, HIPP, and TCTX). We obtained two datasets for EC (GSE48350 (Berchtold, et al., 2008) and GSE5281 (Liang, et al., 2007)), four datasets for FCTX (GSE48350 (Berchtold, et al., 2008), GSE5281 (Liang, et al., 2007), GSE66333 (Simpson, et al., 2016), and GSE53890 (Lu, et al., 2014)), three datasets for HIPP (GSE48350 (Berchtold, et al., 2008), GSE5281 (Liang, et al., 2007), GSE28146 (Blalock, et al., 2011)), and two datasets for TCTX (GSE5281 (Liang, et al., 2007) and GSE29652 (Simpson, et al., 2011)). Included were 25 AD cases and 29 controls for EC, 52 cases and 56 controls for FCTX, 50 cases and 44 controls for HIPP, and 34 cases and 11 controls for TCTX, who were 65 years or older at death (Supplementary Table 3). The raw expression data downloaded from GEO (Affymetrix CEL files) were background-corrected and normalized by the RmaBackgroundCorrection and QuantileNormalization functions in “aroma.affymetrix” Bioconductor R package (Bengtsson, et al., 2008), and then log2-transformed. The normalized and log2-transformed expression data in each brain region were merged by the Combat function in “sva” Bioconductor R package (Leek, et al., 2012). Using principal component analysis (PCA), we confirmed that Combat successfully eliminated batch effects in each brain region. We removed one outlier identified in the PCA from GSE5281 in FCTX (Supplementary Figure 2).

Probes were excluded that targeted transcripts from different genes (i.e., probes with “_x” suffix) if a more reliable probe was available. We also excluded mono-allelically expressed genes including genes on chromosomes X and Y, and HLA- genes (i.e., *HLA-A*, *HLA-B*, *HLA-C*, *HLA*-DMA, HLA-DMB, HLA-DOA, HLA-DOB, HLA-DPA1, HLA-DPB1, HLA-DQA1, HLA-DQA2, *HLA-DQB1*, *HLA-DQB2*, *HLA-DRA*, *HLA-DRB1*, *HLA-DRB3*, *HLA-DRB4*, HLA-*DRB5*, *HLAE*, *HLA-F*, *HLA-G*, *HLA-J*, *HLA-P*, and *HLA-T*). The number of probes in each study is shown in Supplementary Table 4.

2.3. Statistical analysis

2.3.1. Hypothesis 1: identified IGAP SNPs are proxies for exonic/coding variants—

We applied two separate methods to identify potentially co-inherited SNPs with the IGAP SNPs: one was for common SNPs and one for rare variants. We first identified common SNPs in the nearby coding regions showing strong ($r^2 \geq 0.8$) or moderate ($0.4 \leq r^2 < 0.8$) LD with each of the IGAP SNPs by using 1000 Genomes Project Phase 3 in individuals of European ancestry (1000 Genomes EUR) (1000 Genomes Project Consortium, 2010). In the discovery analysis for common SNPs in ADGC, we performed association tests under an additive mode of inheritance (MOI) using logistic regression adjusted for age at last visit or death, sex, and the top five principal components (PCs) computed in PLINK v1.90a (Chang, et al., 2015,Purcell, et al., 2007). The coding SNPs

were evaluated for replication in independent individuals (within the ADSP dataset) to limit the possibility of imputation errors. We then identified potentially co-inherited rare variants with each of the IGAP SNPs using the Lewontin's D' estimates (Lewontin, 1964) in 1000 Genomes EUR (1000 Genomes Project Consortium, 2010). Due to the properties of LD metrics, a given common SNP can exhibit disparate patterns of LD (large D' but low r^2) between it and many rare variants. We thus focused on nonsynonymous rare variants which are more likely functional, and then applied the following criteria: MAF < 0.05, minor allele count ≥ 5, D' ≥ 0.9, the same direction of effect on AD, and within 1 Mb from the IGAP SNP. Fisher's exact test was used to examine the association between rare variants and AD in ADSP.

Variant Effect Predictor (VEP) (McLaren, et al., 2010) was used to annotate functional consequences of the common coding SNPs and rare variants identified in the association tests. The pathogenetic nature of nonsynonymous common SNPs/rare variants associated with AD was predicted by SIFT (http://sift.jcvi.org/) (Ng and Henikoff, 2003), PolyPhen-2 with HumDiv classifier (http://genetics.bwh.harvard.edu/pph2/) (Adzhubei, et al., 2010), and PROVEAN (http://provean.jcvi.org/index.php) (Choi, et al., 2012) to evaluate the effect of amino acid substitution on a protein function. We also used Genomic Evolutionary Rate Profiling (GERP)++ (http://mendel.stanford.edu/SidowLab/downloads/gerp/) (Davydov, et al., 2010) to examine evolutionary conservation for each of the associated nonsynonymous SNPs/rare variants. Higher score of rejected substitutions (RS) score indicates that a site is inferred to have a greater level of evolutionary constraint. We implemented these *in silico* algorithm tools except for PROVEAN for canonical transcripts that are defined as either the longest coding sequence or the longest cDNA in the UCSC Genome Browser (https://genome.ucsc.edu/) (Kent, et al., 2002).

2.3.2. Hypothesis 2: identified IGAP SNPs are eQTLs—The goal of these analyses was to evaluate whether the IGAP SNPs were eQTL. We first tested association between the IGAP SNPs and gene expression on the same chromosome as each of the SNPs, assuming an additive MOI as implemented in PLINK v1.90a (Chang, et al., 2015,Purcell, et al., 2007). We then examined whether the levels of gene expression associated with the IGAP SNP status were different from the association with AD phenotype. An analysis of covariance (ANCOVA) with age at the death and sex as covariates was applied to test for statistical significance.

For all analyses, we defined associations with false discovery rate (FDR) adjusted p-value < 0.05 as statistically significant using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995).

3. Results

For the current study, individuals with either prevalent or incident AD were considered as AD cases in ADSP. Descriptive characteristics of individuals in the two genetic datasets are shown in Supplementary Table 5. In ADGC and ADSP, 7,364 (48.0%) and 5,374 (51.4%) were AD cases, respectively.

3.1. Hypothesis 1: identified IGAP SNPs are proxies of coding variants

In the common SNP analyses, 10 exonic SNPs were in strong LD ($r^2 \geq 0.8$) and 16 exonic SNPs in moderate LD ($0.4 \leq r^2 < 0.8$) with IGAP SNPs based on 1000 Genomes EUR (Supplementary Table 6). We first analyzed the imputed genotype data from ADGC to replicate IGAP SNP association with AD and extended the analyses to exonic common SNPs. We confirmed that several proxy SNPs located in coding regions demonstrated statistically significant associations with AD phenotype. Of these coding SNPs, we replicated 12 loci (rs2296160 in *CR1*, rs1049086 in *HLA-DQB1*, rs2722372 and rs2598044 in *NME8*, rs2405442 and rs1859788 in *PILRA*, rs7982 in *CLU*, rs12453 and rs7232 in *MS4A6A*, and rs3752246, rs4147930, and rs4147934 in *ABCA7*) that surpassed the statistical significance level with FDR adjustment in the separate ADSP dataset (Table 1 and Supplementary Table 7). Of these 12 coding SNPs, six SNPs (rs2296160 in *CR1*, rs2722372 in *NME8*, rs1859788 in *PILRA*, rs7232 in *MS4A6A*, and rs3752246 and rs4147934 in *ABCA7*) are missense mutations on at least one of their transcripts (Supplementary Table 8).

In rare variant analyses using ADSP, five rare variants were identified that (i) had D' = 1 and the same direction of effect as an IGAP SNP (rs11575848 in *LY6G6C*, rs2070600 in *AGER*, rs62483572 in *EPO*, rs74547795 in *SYTL2*, and rs111986709 in *DSG3*) (Supplementary Table 9), and (ii) were missense mutations on at least one of their transcripts (Supplementary Table 10).

The nonsynonymous SNPs and rare variants in the canonical transcript were analyzed *in silico* with SIFT, PolyPhen-2, PROVEAN, and GERP++. Supplementary Table 11 shows the pathogenetic nature prediction only for the canonical transcripts. None of the common nonsynonymous SNPs except for rs7232 in *MS4A6A* were predicted to have functional impact; the minor allele of rs7232 was predicted to be possibly damaging to the MS4A6A protein according to PolyPhen-2. In contrast, all of the rare variants were predicted to have deleterious effects on protein function.

3.2. Hypothesis 2: identified IGAP SNPs are eQTLs

Table 2 shows transcript levels (gene expression) in blood that were significantly associated with the IGAP SNPs, each reaching FDR adjusted significance level. The risk allele of rs10838725 in *CELF1* was associated with increased *MYBPC3* expression, and the protective allele of rs983392 in *MA4A6A* was associated with decreased expression of *MS4A6A* itself. *MYBPC3* expression (probe ID: 11725151_at) and *MS4A6A* expression (probe ID: 11716846_a_at) were also significantly associated with AD status.

The significant associations between the IGAP SNPs and brain gene expression in NABEC and UKBEC are shown in Table 3. In FCTX data of NABEC, rs8093731 in *DSG2* acted as an eQTL for two genes, *DLGAP1* and *NETO1*, which were highly correlated ($r^2 = 0.69$). In UKBEC, the risk allele of rs4147929 in *ABCA7*, the risk allele of rs10948363 in *CD2AP*, and the protective allele of rs3865444 in *CD33* were associated with increased *EID2B* expression in FCTX, increased *AK9* expression in FCTX, and decreased *IER2* expression in TCTX, respectively.

In comparison with non-AD samples using the merged datasets, AD cases had significantly lower expressions of *DLGAP1* and *NETO1* (potentially regulated by the *DSG2* SNP) in the four brain regions and of *EID2B* (potentially regulated by the *ABCA7* SNP) in HIPP. Significantly higher expression of *AK9* (potentially regulated by the *CD2AP* SNP) and *IER2* (potentially regulated by the *CD33* SNP) in AD cases was seen in EC/HIPP and EC/TCTX, respectively (Table 4).

4. Discussion

Although large GWAS have identified novel loci that are associated with altered AD risk, we have a relatively poor understanding of the functional impact of these loci. In this study, we examined possible functional effects of the IGAP SNPs on AD under two hypotheses: "the IGAP SNP is a proxy of a coding variant" and "the IGAP SNP is an eQTL". For the first hypothesis, rs6656401 in *CR1*, rs9271192 in *HLA-DRB5-DRB1*, rs2718058 in *NME8*, rs1476679 in *ZCWPW1*, rs9331896 in *CLU*, rs983392 in *MS4A6A*, and rs4147929 in *ABCA7* are proxies of common coding SNPs. Additionally, the IGAP SNPs rs9271192 in *HLA-DRB5-DRB1*, rs1476679 in *ZCWPW1*, rs10792832 in *PICALM*, and rs8093731 in *DSG2* may reflect the net effect of nonsynonymous rare variants. For the second hypothesis, rs8093731 in *DSG2*, rs4147929 in *ABCA7*, rs10948363 in *CD2AP*, and rs3865444 in *CD33* are associated with gene expression, although whether these SNPs are proxies for the functional regulatory SNP or functional themselves requires further studies.

4.1. *CR1* SNPs

There were two common coding SNPs, rs4844600 (synonymous) and rs2296160 (nonsynonymous), in strong LD with the IGAP SNP, although the association between rs4844600 and AD could not be assessed for replication because of the lack of WES data in ADSP. *CR1*, located on chromosome 1q32.2 within a cluster of complement-related genes, encodes complement receptor 1 which typically acts to bind complement-labeled proteins or complexes for their clearance by the immune system (Khera and Das, 2009). Regarding AD, the CR1 protein acts a receptor for complement fragments bound to Aβ, and thus the change in CR1 protein structure and expression levels may be related to Aβ clearance (Rogers, et al., 2006). The synonymous SNP rs4844600 (E60E) is located on exon 2, the IGAP SNP rs6656401 is located between exon 4 and 5, and the nonsynonymous SNP rs2296160, which causes an alanine-tothreonine amino acid substitution at codon position 2419 (A2419T), is located on exon 44. The SNP rs1408077 in *CR1*, which is in strong LD with the IGAP SNP rs6656401, was reported to be associated with loss of EC thickness (Biffi, et al., 2010), and carriers of the IGAP SNP rs6656401_A had smaller local gray matter volume in the EC in young health adults, which may lead to or reflect increased risk of late-onset AD (Bralten, et al., 2011). These results may indicate a causal relationship between *CR1* SNPs and AD development.

4.2. HLA-DRB5-DRB1 SNPs

The IGAP SNP rs9271192 is located in an intergenic region (chromosome 6p21.32), near HLA class II genes (HLA-DR, -DQ and -DP). There were four common coding SNPs which were in strong or moderate LD with the IGAP SNP; one nonsynonymous SNP rs9270303 in

HLA-DRB1, three synonymous SNPs rs2308759 in *HLA-DRB1*, rs1049092 and rs1049086 in *HLA-DQB1*. Because of missing data in ADGC, the association tests of rs9270303, rs2308759, and rs1049092 with AD could not be performed in the discovery analysis (Table 1 and Supplementary Table 7). We also identified two nonsynonymous rare variants, rs11575848 and rs2070600, that are potentially co-inherited with the IGAP SNP (D' = 1) and are located in the major histocompatibility complex (MHC) class III region. *LY6G6C* (rs11575848) encodes a leukocyte antigen-6 superfamily member, and *AGER* (rs2070600) encodes a receptor for advanced glycosylation end product (RAGE). The RAGE protein is a member of the immunoglobulin superfamily and may regulate Aβ transport across the blood brain barrier (Tarasoff-Conway, et al., 2015).

At least three classes of genes expressed from a single allele (mono-allelic) are recognized to exist (Chess, 2012,Gimelbrant, et al., 2007). One class is the autosomal imprinted genes regulated in a parent-of-origin specific manner. The second class is X-inactivated. The third class of these genes are located randomly in autosomes and include several immune system genes (Chess, 2012,Gimelbrant, et al., 2007). Because we excluded from the analysis *HLA*-genes that are randomly mono-allelically expressed, we did not examine the associations between the IGAP SNP rs9271192 and expression of *HLA*-genes including *HLA-DRB5* and *HLA-DRB1*. Given epigenetic association between DNA methylation in *HLA-DRB5* and AD pathology (Yu, et al., 2015), allele specific expression at these loci may have a strong impact on immunobiological function related to AD.

4.3. CD2AP SNPs

Increased expression of *AK9* in FCTX was associated with the risk allele of the *CD2AP* IGAP SNP, and *AK9* was significantly over-expressed in EC and HIPP brain regions of AD cases. *AK9*, located in chromosome 6q21 and more than 60Mb away from *CD2AP* locus, encodes a member of adenylate kinase family of enzymes. Adenylate kinase reversibly catalyzes the interconversion of adenine nucleotides (ATP + AMP ↔ 2 ADP) (Amiri, et al., 2013).

4.4. NME8 SNPs

Two common coding SNPs were in moderate LD with the IGAP *NME8* SNP; one nonsynonymous SNP rs2722372 and one synonymous SNP rs2598044. The nonsynonymous SNP rs2722372 causes arginine-to-lysine amino acid substitution at codon position 43 (R43K). These coding SNPs were significantly associated with AD and the associations were replicated. *NME8*, located on chromosome 7p14.1, encodes a protein with an N-terminal thioredoxin domain and C-terminal nucleoside diphosphate kinase domains. The NME8 protein is a member of NME/NM23 family. Although the function of this gene is poorly characterized, Liu et al. showed that the IGAP SNP rs2718058 had a neuroprotective effect against cognitive decline, elevated tau levels in cerebrospinal fluid (CSF), and hippocampal atrophy (Liu, et al., 2014).

4.5. ZCWPW1 SNPs

There were three common coding SNPs which were in strong or moderate LD with the IGAP *ZCWPW1* SNP; one nonsynonymous SNP rs1859788 (in *PILRA*) and two

synonymous SNP rs2405442 (in *PILRA*) and rs909152 (in *LRCH4*). The nonsynonymous SNP rs1859788 causes glycine-to-arginine amino acid substitution at codon position 78 (G78R). The coding SNPs which are in strong LD with the IGAP SNP were significantly associated with AD and we confirmed associations in the replication analysis. There was also one nonsynonymous rare variant rs62483572 (in *EPO*) with D' = 1, that causes an amino acid substitution of aspartic acid to asparagine at codon position 70 (D70N). This variant had a more protective effect than the nonsynonymous common SNP rs18597955 (OR = 0.53 for rs62483572 vs. OR = 0.89 for rs1859788 in ADSP). Erythropoietin (EPO) exhibits a neuroprotective effect under various conditions of neuronal damage such as hypoxia-ischemia, and thus the nonsynonymous rare variants may be involved in promoting maintenance of homeostasis (Siren, et al., 2001).

The IGAP SNP was associated with the expression of several genes including *GATS*, *TRIM4*, *PILRB*, *ZKSCAN1*, and *PVRIG* in blood. However, we did not find significant associations between expression of these genes and AD. *ZCWPW1*, located on chromosome 7q22.1, encodes zinc finger CW (zf-CW)-type and PWWP domain containing 1. Although the function(s) of this gene are unknown, zf-CW may be involved in epigenetics as it is regarded as a member of histone modification reader modules (He, et al., 2010).

The IGAP SNP was associated with the expression of several genes including *GATS*, *TRIM4*, *PILRB*, *ZKSCAN1*, and *PVRIG* in blood. However, we did not find significant associations between expression of these genes and AD. *ZCWPW1*, located on chromosome 7q22.1, encodes zinc finger CW (zf-CW)-type and PWWP domain containing 1. Although the function(s) of this gene are unknown, zf-CW may be involved in epigenetics as it is regarded as a member of histone modification reader modules (He, et al., 2010).

4.6. CLU SNPs

We confirmed that the synonymous *CLU* SNP rs7982 is in strong LD with the IGAP SNP rs9331896 and was protectively associated with AD. However, we found no evidence of gene expression regulation that was associated with either the *CLU* IGAP SNP or the proxy, synonymous SNP. *CLU* is located in chromosome 8p21.1, and encodes clusterin, also known as apolipoprotein J. Clusterin directly influences Aβ, regulating the conversion of Aβ into insoluble forms (Desikan, et al., 2014,Yu and Tan, 2012). *CLU* has two main isoforms, nuclear *CLU* (n*CLU*, isoform 1) and secretory *CLU* (s*CLU*, isoform 2) with different functions. The s*CLU* form is pro-survival, while n*CLU* is pro-apoptotic (Shannan, et al., 2006). Since the coding SNP rs7982 is synonymous, it may affect alternative splicing as Ling et al. showed that the protective SNP rs11136000 (which is in almost perfect LD with rs7982 in 1000 genomes EUR) was associated with increased n*CLU* expression level (Ling, et al., 2012).

4.7. CELF1 SNPs

We did not find evidence that the *CELF1* SNP rs10838725 and the proxy coding SNPs in LD with the IGAP SNP were associated with AD. However, rs10838725 acted as eQTL for *MYBPC3* expression in blood which was associated with AD. *MYBPC3* is located on chromosome 11p11.2, and encodes cardiac myosin binding protein C expressed in heart

muscle (Gautel, et al., 1995). Huang et al. reported that the protective allele of rs1057233 in *CELF1* (r^2 = 0.17 and D' = 0.97 with the IGAP SNP rs10838725 as shown in Supplementary Table 1) was associated with decreased *MYBPC3* and *SPI1* expressions and with the higher CSF $A\beta_{42}$ levels (Huang, et al., 2017). Our finding that the risk allele of IGAP SNP rs10838725 in *CELF1* was associated with the increased *MYBPC3* expression and also correlated with AD status is concordant with the previous study.

4.8. MS4A6A SNPs

The IGAP SNP rs983392 located downstream of *MS4A6A* was associated with several striking gene regulatory features. We found two coding SNPs, rs12453 and rs7232 in LD with the protective IGAP SNP rs983392. The coding SNP rs7232 is nonsynonymous, causing threonineto-serine amino acid substitution at codon position 213 (T213S), while the SNP rs12453 is synonymous (L137L). The IGAP SNP was associated with expression in blood of *MS4A6A* as well as other members of the *MS4A* gene family, Moreover, decreased *MS4A6A* expression was associated with AD risk (Figure 2). These results help illustrate that the two basic hypotheses we were testing (SNP is a proxy for a coding variant; and, SNP is an eQTL) are not mutually exclusive.

MS4A6A, located on chromosome 11q12.2, encodes a member of membrane-spanning 4A gene family (membrane-spanning 4A domains, subfamily A, member 6A). *MS4A* genes are highly expressed in hematopoietic cells, and involved in the regulation of calcium signaling (Ma, et al., 2015). Although functions of the MS4A6A protein are still incompletely understood, it is possible that the *MS4A6A* SNPs are linked to AD via deregulation of calcium signaling implicated in neurodegenerative diseases (LaFerla, 2002,Marambaud, et al., 2009).

4.9. PICALM SNPs

There was one nonsynonymous rare variant rs74547795 in *SYTL2* with D' = 1 for the IGAP SNP, that causes amino acid substitution of alanine to aspartic acid at codon position 825 (A825D). *PICALM* is located on chromosome 11q14.2, and encodes a phosphatidylinositol cinding clathrin assembly protein that may be involved in Aβ clearance (Zhao, et al., 2015) and synaptic neurotransmission release (Sleegers, et al., 2010). On the other hand, there is no evidence that *SYTL2* is associated with AD.

4.10. DSG2 SNPs

There was one nonsynonymous rare variant rs111986709 located in *DSG3* with D' = 1 for the IGAP SNP. The variant causes serine-to-phenylalanine amino acid substitution at codon position 771 (S771F), and the mutation was predicted to have an impact on the DSG3 protein. *DSG2* and *DSG3* encode members of the desmoglein family. The role of these genes in AD is unknown.

The expression of both *DLGAP1* and *NETO1* were strongly associated with the *DSG2* IGAP SNP and were highly correlated with each other in FCTX. Interestingly, these genes were significantly under-expressed in four brain regions of AD cases. Located in chromosome 18p11.31 more than 25Mb away from *DSG2*, *DLGAP1* encodes disks large-

associated protein 1 (also known as guanylate kinase- associated protein (GKAP)). *NETO1* is located on chromosome 18q22.3, more than 40Mb away from *DSG2,* and encodes neuropilin and tolloid like 1. Both *DLGAP1* and *NETO1* are mainly expressed in neurons of human brains (http://web.stanford.edu/group/barres_lab/brainseqMariko/brainseq2.html) (Bennett, et al., 2016), and may be involved in N-methyl-D-aspartate receptor-dependent synaptic plasticity (Ng, et al., 2009,Shin, et al., 2012).

4.11. ABCA7 SNPs

Of six coding SNPs in strong or moderate LD with the IGAP SNP, we confirmed that two nonsynonymous SNPs (rs3752246 causing alanine-to-glycine amino acid substitution at codon position 1527 (A1527G) and rs4147934 causing serine-to-alanine amino acid substitution at codon position 2045 (S2045A)) and one synonymous SNP (rs4147930 (L1995L)) were associated with AD. The IGAP SNP acted as an eQTL for *EID2B* expression (the risk allele was associated with increased *EID2B* expression in FCTX). However, decreased expression of *EID2B* in HIPP was associated with AD risk (i.e., the association directions are in conflict). *ABCA7*, located in chromosome 19p13.3, encodes a member of the super family of ATP-binding cassette transporters. *ABCA7* is expressed in hippocampal CA1 neurons and in microglia (Kim, et al., 2006). ABCA7 is involved in lipid efflux from cells to lipoproteins and has been associated with Aβ accumulation (Kim, et al., 2013).

4.12. CD33 SNPs

There were two nonsynonymous SNPs in strong or moderate LD with the IGAP *CD33* SNP, rs12459419 causing alanine-to-valine amino acid substitution at codon position 14 (A14V) and rs35112940 causing glycine-to-arginine amino acid substitution at codon position 304 (G304R). Although we did not find sufficient evidence that the proxy coding SNPs in LD with the IGAP SNP rs3865444 were associated with AD, the protective allele of rs3865444 was associated with decreased *IER2* expression in TCTX and, further, decreased *IER2* expression in EC and TCTX had a protective effect on AD. *IER2* is located on chromosome 19p13.2 more than 35Mb away from *CD33*, and encodes immediate early response 2. IER2 may function as a transcription factor (Takaya, et al., 2009).

Malik et al. reported that the IGAP SNP rs3865444 modulated exon 2 splicing by showing that the proportion of *CD33* expressed as a *CD33* isoform lacking exon 2 was increased in the protective allele of rs3865444; the proxy nonsynonymous SNP rs12459419 was shown to modulate exon 2 splicing efficiency (Malik, et al., 2013). Additional studies are warranted to examine the association between *CD33* isoform and *IER2* expression.

There are limitations to this study. We aggregated data from many rich resources that aid in establishing a confluence of related information; however, these datasets are heterogeneous and can exhibit biases from their respective parent study designs, analytic protocols, and participant pools. A major limitation of our study is that we have limited our assessment to subjects with European-type genomic characteristics, which is connected to the fact that many research centers and clinics that contribute to the study share this underlying bias.

Also, according to the commonly used, but inexact convention, we focused on genes closest to the identified IGAP SNP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ADGC	Alzheimer's Disease Genetic Consortium
ADNI	Alzheimer's Disease Neuroimaging Initiative
ADSP	Alzheimer's Disease Sequencing Project
GWAS	Genome-wide association studies
IGAP	International Genomics of Alzheimer's Project
LD	Linkage disequilibrium
NABEC	North American Brain Expression Consortium
UKBEC	United Kingdom Brain Expression Consortium

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- The IGAP SNPs are located on non-coding regions.
- The functional impacts of the IGAP SNPs are poorly understood.
- Some of the IGAP SNPs are proxies of coding SNPs.
- Some of the IGAP SNPs acted as eQTL for AD-related genes' expression.

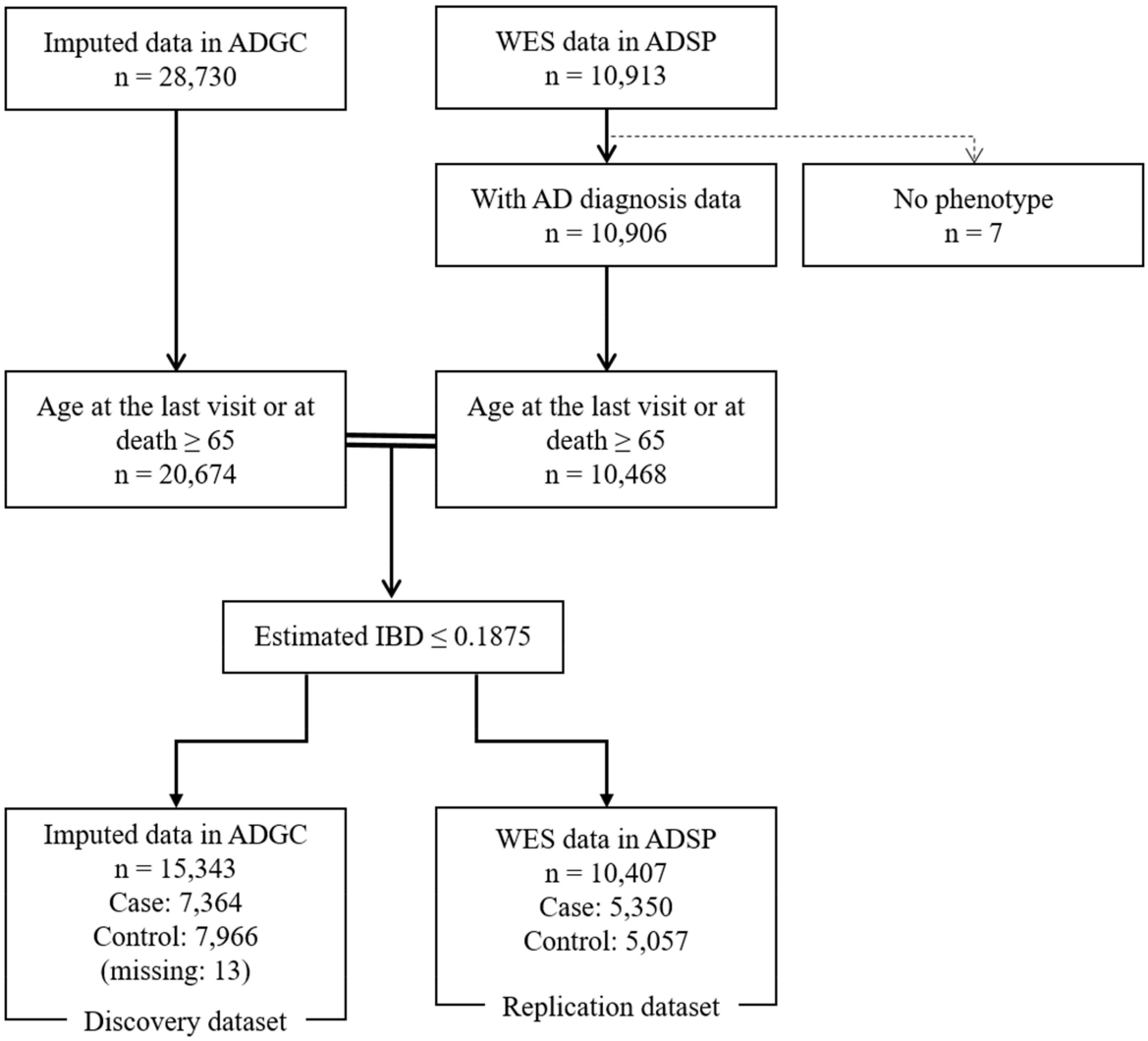

Figure 1.

Flow diagram of the subjects included in the analyses.

Key: ADGC, Alzheimer's Disease Genetics Consortium; ADSP, Alzheimer's Disease Sequencing Project; IBD, identity-by-descent

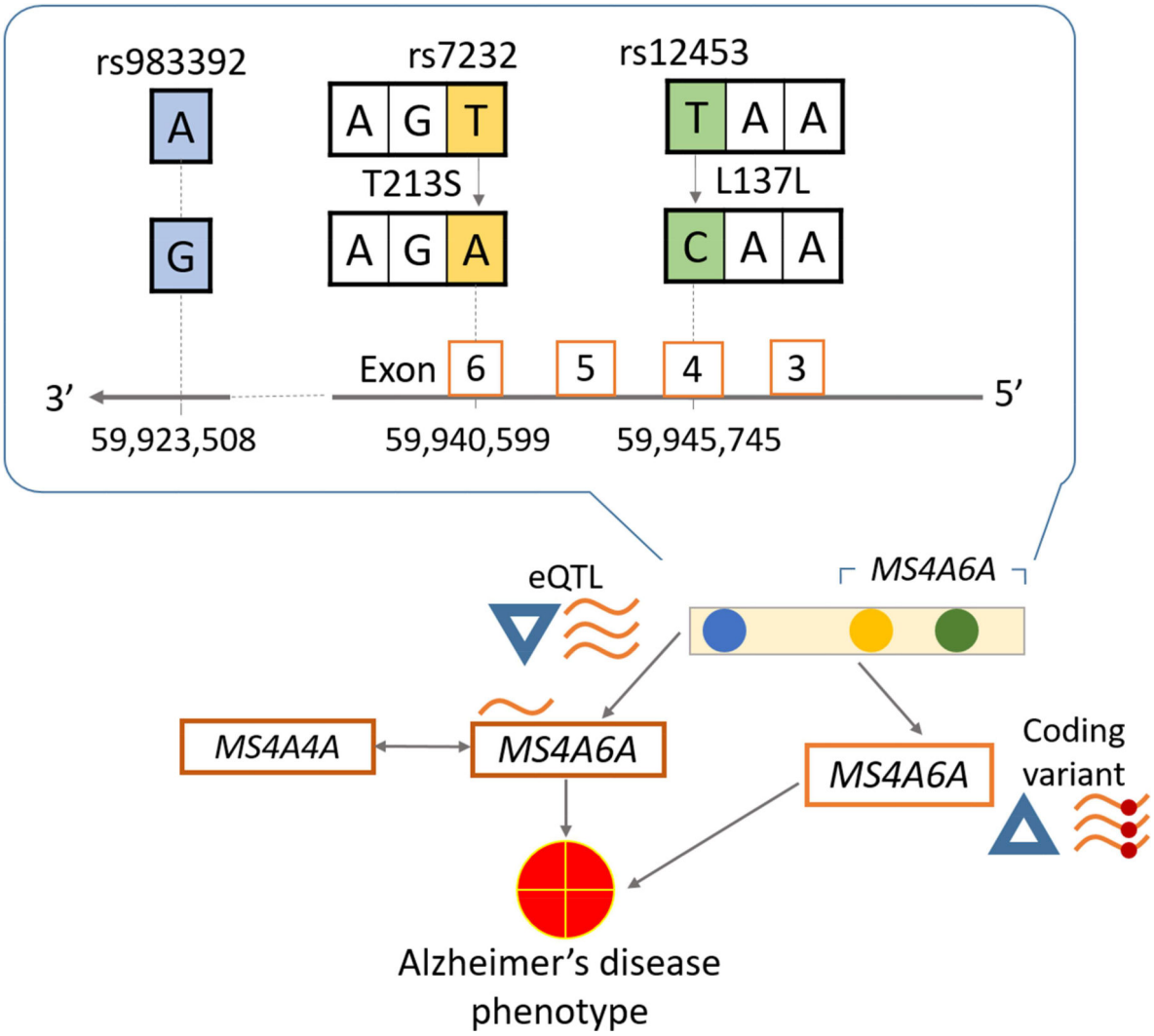

Figure 2.

Potential pathway for the relationship between *MS4A6A* SNPs, blood gene expression and Alzheimer's disease phenotype. The relationship between *MS4A6A* IGAP SNP rs983392, blood gene expression, and Alzheimer's disease phenotype are complex and possibly multifactorial. The rs983392 SNP constitutes an eQTL for *MS4A6A*, and also a proxy for a nonsynonymous exonic *MS4A6A* SNP rs7232. It is possible that the nonsynonymous SNP rs7232 is not, or is only partially responsible, for the eQTL phenomena, which may indicate parallel gene regulatory mechanism(s).

Key: SNP, single nucleotide polymorphism, IGAP, International Genomics of Alzheimer's Project, eQTL, expression quantitative trait locus

Table 1.

Association of IGAP SNPs and the coding SNPs strongly correlated with the IGAP SNPs with Alzheimer's disease in two datasets: ADGC [a] and ADSP [b]

IGAP SNP				Exonic SNP				
SNP	Closest gene	ADGC [a]		SNP ID	ADGC [a]		ADSP [b]	
		OR	P-value		OR	P-value	OR	P-value
Strong LD ($r^2 \geq 0.8$)								
rs6656401	*CR1*	1.17	8.49×10^{-7}	rs4844600	1.17	**4.22×10^{-7}**	-	-
				rs2296160	1.18	**4.24×10^{-8}**	1.11	**8.56×10^{-3}**
rs9271192	*HLA-DRB5*	1.11	2.66×10^{-4}	rs9270303	-	-	1.15	**2.50×10^{-4}**
rs1476679	*ZCWPW1*	0.92	1.41×10^{-3}	rs2405442	0.92	**2.12×10^{-3}**	0.89	**1.09×10^{-3}**
				rs1859788	0.93	**5.62×10^{-3}**	0.89	**1.12×10^{-3}**
rs9331896	*CLU*	0.92	1.02×10^{-3}	rs7982	0.91	**3.64×10^{-4}**	0.90	**1.62×10^{-3}**
rs10838725	*CELF1*	1.05	0.054	rs2293576	1.03	0.24	1.07	0.040
rs983392	*MS4A6A*	0.86	7.22×10^{-9}	rs12453	0.86	**1.34×10^{-9}**	0.89	**3.60×10^{-4}**
rs4147929	*ABCA7*	1.12	1.39×10^{-3}	rs3752246	1.11	**2.70×10^{-3}**	1.18	**9.89×10^{-5}**
rs3865444	*CD33*	0.91	4.49×10^{-4}	rs12459419	0.91	**4.02×10^{-4}**	0.94	0.090

[a] Imputed genotype data from ADGC

[b] Whole exome sequencing data from ADSP

Bold p-value represents the statistical significance after false discovery rate adjustment.

Key: IGAP, International Genomics of Alzheimer's Project; SNP, single nucleotide polymorphism; ADSP, Alzheimer's Disease Sequencing Project; ADGC, Alzheimer's Disease Genetic Consortium; OR, odds ratio; LD, linkage disequilibrium

Table 2.

Significant association of the IGAP SNPs with gene expression in blood in ADNI

IGAP SNP	Closest gene	Probe set ID [a]	Gene	eQTL association		AD association
				$\hat{\beta}$	P-value [b]	P-value [c]
rs6733839	*BIN1*	11719631_s_at	*BIN1*	0.071	1.51×10^{-7}	0.28
		11746895_a_at	*BIN1*	0.084	2.05×10^{-6}	0.56
rs111418223	*HLA-DRB5*	11730933_a_at	*AGPAT1*	0.120	1.83×10^{-11}	0.91
		11750187_a_at	*AGPAT1*	0.114	6.96×10^{-9}	0.68
		11751668_a_at	*AGPAT1*	0.119	1.71×10^{-8}	0.94
rs1476679	*ZCWPW1*	11722909_a_at	*GATS*	0.177	1.53×10^{-17}	0.53
		11736388_a_at	*TRIM4*	−0.129	3.53×10^{-9}	0.95
		11743311_a_at	*PILRB*	−0.115	4.15×10^{-9}	0.85
		11730023 s at	*PILRB*	−0.107	1.58×10^{-8}	0.78
		11730022_a_at	*PILRB*	−0.128	3.77×10^{-8}	0.89
		11760665_at	*ZKSCAN1*	0.147	2.30×10^{-7}	0.15
		11730247_a_at	*PVRIG*	0.086	6.07×10^{-5}	0.82
rs11771145	*EPHA1*	11755327 s at	*LOC154761*	0.109	3.68×10^{-6}	0.40
rs28834970	*PTK2B*	11720981_a_at	*PTK2B*	0.114	6.86×10^{-18}	0.40
		11720982 s at	*PTK2B*	0.086	1.18×10^{-17}	0.91
		11720980_a_at	*PTK2B*	0.094	7.33×10^{-12}	0.47
		11723344_at	*TRIM35*	−0.070	2.31×10^{-6}	0.45
rs10838725	*CELF1*	11725151_at	*MYBPC3*	0.140	1.07×10^{-7}	8.13×10^{-5}
rs983392	*MS4A6A*	11716846_a_at	*MS4A6A*	−0.082	2.34×10^{-12}	4.97×10^{-3}
		11751570_a_at	*MS4A4A*	−0.150	1.15×10^{-6}	0.85
		11732865_a_at	*MS4A4A*	−0.179	1.59×10^{-6}	0.68

[a] Probe set IDs on Affymetrix Human Genome U219 Array

[b] P-values less than significance level after false discovery rate adjustment were displayed

[c] P-values calculated by analysis of covariance with the outcome of gene expression and the predictor of Alzheimer's disease status (normal/mild cognitive impairment/AD)

Key: IGAP, International Genomics of Alzheimer's Project; SNP, single nucleotide polymorphism; ADNI, Alzheimer's Disease Neuroimaging Initiative; eQTL, expression quantitative trait locus

Table 3.

Significant association of the IGAP SNPs with brain gene expression in NABEC and UKBEC

IGAP SNP	Closest gene	Probe set ID [a]	Gene expression	Brain region	$\hat{\beta}$	P-value [b]
NABEC						
rs8093731	*DSG2*	ILMN_23 80779	*DLGAP1*	FCTX	0.770	1.36×10^{-8}
		ILMN_1783168	*NETO1*	FCTX	0.706	1.11×10^{-5}
UKBEC						
rs4147929	*ABCA7*	t3862068	*EID2B*	FCTX	0.259	3.94×10^{-6}
rs10948363	*CD2AP*	t2969159	*AK9*	FCTX	0.343	1.64×10^{-5}
rs3865444	*CD33*	t3822216	*IER2*	TCTX	-0.255	1.96×10^{-5}

[a] Probe set IDs on HumanHT-12_v3 Expression BeadChips in NABEC (platform = GPL6947) and on Affymetrix Exon 1.0 ST Arrays in UKBEC (platform = GPL5175)

[b] P-values less than significance level after false discovery rate adjustment are displayed.

Key: IGAP, International Genomics of Alzheimer's Project; SNP, single nucleotide polymorphism; NABEC, North American Brain Expression Consortium; UKBEC, United Kingdom Brain Expression Consortium; FCTX, frontal cortex; TCTX, temporal cortex

Table 4.

Associations between gene expressions identified in NABEC and UKBEC and Alzheimer's disease status in the merged dataset

Probe set ID [a]	EC		FCTX		HIPP		TCTX	
	$\hat{\beta}$	P-value	$\hat{\beta}$	P-value	$\hat{\beta}$	P-value	$\hat{\beta}$	P-value
Identified in NABEC								
DLGAP1								
206489_s_at	−0.495	**5.68×10^{-3}**	−0.294	**0.013**	−0.635	**2.77×10^{-6}**	−0.318	**1.58×10^{-4}**
206490_at	−0.225	0.071	−0.202	0.039	−0.280	**3.18×10^{-4}**	−0.212	0.052
210750 s at	−0.343	**0.013**	−0.135	0.13	−0.084	0.27	0.214	0.071
NETO1								
1552736_a_at	−0.433	**5.36×10^{-3}**	−0.210	0.14	−0.597	**1.53×10^{-5}**	−0.088	0.58
1552904 at	−0.411	**2.70×10^{-4}**	−0.115	0.085	−0.359	**5.23×10^{-6}**	−0.119	0.085
1562713_a_at	−0.548	**2.95×10^{-3}**	−0.255	**8.99×10^{-3}**	−0.615	**1.97×10^{-5}**	−0.276	**4.73×10^{-4}**
Identified in UKBEC								
EID2B								
242470_at	−0.383	0.051	−0.319	0.015	−0.424	**6.70×10^{-4}**	−0.166	0.33
AK9								
1552299_at	0.039	0.54	0.014	0.82	0.001	0.99	−0.179	0.048
1564002_a_at	0.197	**5.23×10^{-3}**	0.092	0.084	0.287	**6.38×10^{-3}**	0.083	0.41
IER2								
202081 at	0.292	**7.11×10^{-4}**	0.141	0.060	0.061	0.52	0.949	**4.48×10^{-5}**

[a] Probe set IDs on Affymetrix U133 Plus 2.0 array (platform = GPL570)

Bold p-value represents the statistical significance after FDR adjustment.

Key: NABEC, North American Brain Expression Consortium; UKBEC, United Kingdom Brain Expression Consortium; EC, entorhinal cortex; HIPP, hippocampus; FCTX, frontal cortex; TCTX, temporal cortex