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A Systems View of the Differences between APOE ε4 Carriers and Non-carriers in Alzheimer’s Disease

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APOE ε4 is the strongest genetic risk factor for late-onset Alzheimer’s disease (AD) and accounts for 50–65% of late-onset AD. Late-onset AD patients carrying or not carrying APOE ε4 manifest many clinico-pathological distinctions. Thus, we applied a weighted gene co-expression network analysis to identify specific co-expression modules in AD based on APOE ε4 stratification. Two specific modules were identified in AD APOE ε4 carriers and one module was identified in non-carriers. The hub genes of one module of AD APOE ε4 carriers were ISOC1, ENO3, GDF10, GNB3, XPO4, ACLY and MATN2. The other module of AD APOE ε4 carriers consisted of 10 hub genes including ANO3, ARPP21, HPCA, RASD2, PCP4 and ADORA2A. The module of AD APOE ε4 non-carriers consisted of 16 hub genes including DUSP5, TNFRSF18, ZNF331, DNAJB5 and RIN1. The module of AD APOE ε4 carriers including ISOC1 and ENO3 and the module of non-carriers contained the most highly connected hub gene clusters. mRNA expression of the genes in the cluster of the ISOC1 and ENO3 module of carriers was shown to be correlated in a time-dependent manner under APOE ε4 treatment but not under APOE ε3 treatment. In contrast, mRNA expression of the genes in the cluster of non-carriers’ module was correlated under APOE ε3 treatment but not under APOE ε4 treatment. The modules of carriers demonstrated genetic bases and were mainly enriched in hereditary disorders and neurological diseases, energy metabolism-associated signaling and G protein-coupled receptor-associated pathways. The module including ISOC1 and ENO3 harbored two conserved promoter motifs in its hub gene cluster that could be regulated by common transcription factors and miRNAs. The module of non-carriers was mainly enriched in neurological, immunological and cardiovascular diseases and was correlated with Parkinson’s disease. These data demonstrate that AD in APOE ε4 carriers involves more genetic factors and particular biological processes, whereas AD in APOE ε4 non-carriers shares more common pathways with other types of diseases. The study reveals differential genetic bases and pathogenic and pathological processes between carriers and non-carriers, providing new insight into the mechanisms of the differences between APOE ε4 carriers and non-carriers in AD.

Keywords: Alzheimer’s disease, APOE ε4, APOE ε4 carriers and non-carriers, weighted gene co-expression network analysis (WGCNA), hub gene cluster
INTRODUCTION

Alzheimer’s disease (AD) is one of the leading causes of dementia and is characterized by cognitive decline with distinctive brain pathology such as amyloid plaques and neurofibrillary tangles (Selkoe, 2003). Rare familial AD (FAD) is an early onset disease and is caused by several definite and specific genes, such as amyloid precursor protein (APP) and presenilin 1 and 2 (PSEN1, PSEN2) (Scheuner et al., 1996; Campion et al., 1999). In contrast, the etiology of the most common form of non-familial late-onset AD appears to be more complicated. Thus far, many genetic loci have been identified to be risk factors of late-onset AD through genome-wide association studies (GWAS) (Harold et al., 2009; Naj et al., 2011; Kauwe et al., 2014). Moreover, alterations in many molecules and biological processes have been recognized in AD (Mattson, 2004). A biological process may involve dozens and even hundreds of molecules, and a molecule may participate in multiple biological processes. It seems impractical to uncover the relationships among all of these molecules and biological processes one by one. Thus, the exploration of the pathogenic and pathological mechanisms of AD in a systematic view may be more appropriate.

APOE is the strongest susceptible gene of late-onset AD (Wijsman et al., 2011). APOE exists as three polymorphic alleles ε2, ε3, and ε4. Individuals with one or two copies of APOE ε4 have a higher risk of developing AD than the carriers of other isoforms (Corder et al., 1993). AD patients carrying or not carrying APOE ε4 manifest many clinico-pathological distinctions. Patients who are APOE ε4 carriers perform worse on memory tasks than non-carriers (Marra et al., 2004). Patients of APOE ε4 non-carriers exhibit impairments in naming, mental speed and executive function (van der Vlies et al., 2007; Wolk et al., 2010). A positron emission tomography study indicated different perfusion profiles in the brains of APOE ε4 carriers and non-carriers during a working memory task (Scarmeas et al., 2004). Moreover, AD APOE ε4 carrier’s display significantly reduced blood flow in the temporal and hippocampal areas (Suwa et al., 2015). In addition, APOE ε4 carriers have greater amyloid deposition and APOE ε4 can predict the atrophy rates across brain regions affected by AD (Jack et al., 2015; Hua et al., 2016). Moreover, previous studies have demonstrated that different drug responses are observed in APOE ε4 carriers and non-carriers. A neuroprotective agent facilitating brain noradrenergic and vasopressinergic activities have been shown to improve the Minimum Mental State Examination (MMSE) score in APOE ε4 carriers but not in non-carriers (Richard et al., 1997). A higher dose of bapineuzumab, an anti-Aβ monoclonal antibody, is needed to decrease cerebrospinal fluid phospho-tau concentration in APOE ε4 non-carriers than in carriers (Salloway et al., 2014). All the evidence indicates that different pathogenic and pathologic processes are involved in the disease progression of AD patients with different APOE ε4 statuses. APOE ε4 has been shown to affect Aβ aggregation, promote neurofibrillary tangle formation and impair synaptic plasticity (Bu, 2009), which are all pathological hallmarks of AD. However, these harmful effects of APOE ε4 do not fully explain the clinico-pathological phenotypic distinctions between APOE ε4 carriers and non-carriers of AD patients. Furthermore, APOE ε4 only accounts for 50–65% of late-onset AD. Therefore, patient stratification based on APOE ε4 status can allow for the exploration of the underlying mechanisms of clinico-pathological distinctions between APOE ε4 carriers and non-carriers and may further help to elucidate the molecular mechanisms of AD which could be masked when combining APOE ε4 carriers and non-carriers together.

The transcriptome bridges the gap between genetic variation and the function of the molecules. Knowing its structure permits the exploration of the comprehensive function of molecules that could be influenced by genetic and non-genetic factors. With the availability of a transcriptome dataset (Webster et al., 2009), we analyzed the transcription profiles of AD patients based on APOE ε4 status by applying a systems biology approach, weighted gene co-expression network analysis (WGCNA) (Langfelder and Horvath, 2008). WGCNA allows for the identification of groups of genes (called modules) whose expression is highly correlated within a network. Then, specific co-expression modules can be obtained by comparing the modules identified in different sub-datasets. Using this method, we identified different co-expression modules and revealed different biological processes involved in AD APOE ε4 carriers and non-carriers.

MATERIALS AND METHODS

Microarray Dataset

The microarray dataset was downloaded from Gene Expression Ominibus (GEO)1. The dataset, GSE15222, measured on GPL2700 using Illumina Sentrix Human Ref-8 Expression Bead Chip which covers 24534 probesets consists of human cerebral cortex samples of 188 controls and 176 patients with diagnosis of late-onset AD. The original expression data were rank-invariant by BeadStudio software available from Illumina and the Illumina custom error model was used. Rank-invariant-normalized expression data were log2 transformed, and missing data were encoded as missing, rather than as a zero level of expression. Chips with average detection scores less than 0.99 (5% of control chips, 8% of late-onset AD chips) were excluded from the analysis. Transcripts that were detected in less than 90% of the case or 90% of the control series were excluded from our study. Finally 8650 high quality transcripts were obtained (Webster et al., 2009). Of the 188 controls, 40 were APOE ε4 carriers and 148 were non-carriers. Of the 176 patients, 121 were APOE ε4 carriers and 55 were non-carriers.

Sub-dataset-specific Co-expression Module Detection by WGCNA

The WGCNA package which provides a robust set of R functions was used to detect sub-dataset-specific modules (Langfelder and Horvath, 2008). GSE15222 was divided into four sub-datasets: patients carrying APOE ε4, patients not carrying APOE ε4, controls carrying APOE ε4 and controls not carrying APOE ε4.

Outliers were filtered out using Euclidean distance as the similarity measure and average linkage as an agglomeration method. For each sub-group, subjects were clustered based on their dissimilarity, and any arrays with average inter-subject correlation less than 2 standard deviations below the mean were removed. This process was repeated until no arrays needed to be removed (Oldham et al., 2008). One outlier was detected in AD APOE ε4 non-carriers and was excluded before the construction. A demographic depiction of the subjects was performed to examine the similarity of sub-datasets. To construct the network, Pearson correlation coefficients were calculated for all possible gene pairs, and then the coefficients were powered by an exponent β in each sub-group stratified by APOE ε4 and phenotypes. A high β maintains high adjacencies but pushes lower adjacencies toward zero. An optimal exponent β can reduce the false positive rate within the network to the utmost extent (Zhang and Horvath, 2005). The dynamic tree-cutting algorithm was then used to identify modules after the summarization of similar patterns of connectivity among genes as topological overlap matrix (TOM) (Langfelder et al., 2008). Modules with similar module eigengenes (ME) were merged together. Unsupervised average linkage hierarchical clustering identified 18 modules in AD APOE ε4 carriers, 21 modules in AD APOE ε4 non-carriers, 10 modules in control APOE ε4 carriers and 8 modules in control APOE ε4 non-carriers. Next, consensus modules (AD APOE ε4 carriers and non-carriers, AD and control APOE ε4 carriers, and AD and control APOE ε4 non-carriers) were constructed with a similar procedure to that illustrated above. A detailed description of consensus module construction can be found at the WGCNA tutorial website.

The modules constructed using the sub-dataset of AD APOE ε4 carriers were compared with the consensus modules of AD APOE ε4 carriers and non-carriers, and with the consensus modules of AD and control APOE ε4 carriers to detect specific modules of AD APOE ε4 carriers. The modules constructed using the sub-dataset of AD APOE ε4 non-carriers were compared with the consensus modules of AD APOE ε4 carriers and non-carriers, and with the consensus modules of AD and control APOE ε4 non-carriers to detect specific modules of AD APOE ε4 non-carriers. Then, the module preservation statistic Z_summary was utilized to further examine the sub-dataset-specificity of the modules (Langfelder et al., 2011). Unlike the cross-tabulation test, Z_summary takes into account both overlaps in module membership and the density and connectivity patterns of the modules. The following recommended significant thresholds for Z_summary were adopted: Z_summary < 2 implies no evidence of module preservation, 2 < Z_summary < 10 implies weak to moderate evidence of module preservation, and Z_summary > 10 implies strong evidence for module preservation. Greater module preservation corresponds to lesser specificity of the module to the sub-dataset. Thus, a Z_summary less than 2 indicates strong evidence of specificity of the module, a Z_summary between 2 and 10 indicates modest evidence of specificity of the module and a Z_summary more than 10 indicates no evidence of specificity of the module. Module networks were graphically depicted using the program Cytoscape.

Then random samplings with two different levels (50 and 75%) were performed with each level carried out five times. The criterion of replication is that more than 80% of genes within one of the modules (violet, dark magenta and light cyan) detected among the total samples should be included in one module detected in random samplings. Moreover, modules that were obtained from random samplings and whose genes are overlapped more than 80% with violet, dark magenta or light cyan modules should be AD APOE ε4 carrier or non-carrier-specific.

Hub genes were identified by WGCNA via measures of intramodular connectivity and module membership (Zhang and Horvath, 2005; Dewey et al., 2011; Langfelder et al., 2011; de Jong et al., 2012). Intramodular connectivity measures how a given gene is connected, or co-expressed, with the genes of a particular module. Module membership measures the membership of the i-th gene with respect to a given module. Hub genes tend to own high values of intramodular connectivity and module membership.

Module eigengene values adjusted for phenotype, age and sex were used to test differential expression of conserved modules between AD and control APOE ε4 carriers and between AD and control APOE ε4 non-carriers, and the values adjusted for APOE ε4 status, age and sex were used to test differential expressions of conserved modules between AD APOE ε4 carriers and non-carriers.

**Hub Gene Complete Graph Detection**

R package graph was utilized to detect whether there was/were complete graph(s) comprised of hub genes in sub-dataset-specific modules.

**Primary Neuron Culture and Quantitative RT-PCR**

Primary neurons were derived from the hippocampus of Sprague–Dawley (SD) rats at postnatal day 1 as previously described (Kam et al., 2010). Neurons were plated at a density of 2 × 10^6 cells/well on poly-L-lysine-treated 6-well plate. The neurons were treated with recombinant human APOE ε4 or ε3 at a concentration of 5 µg/ml or without APOE treatment on DIV19 and collected for RNA extraction after 24, 48, and 72 h of treatment. Total RNA was extracted using a Total RNA kit 1 from Sigma (St. Louis, MO, USA). RNA was reverse transcribed to synthesize cDNA by using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed using SYBR Green (Roche Diagnostics, Basel, Switzerland). GAPDH was used as the normalization control. The relative mRNA levels were calculated by a comparative Cp value. The primers used were: ENO3: sense: AGCTGCTACCTAGGCCAC TCT; antisense: GGTTCGTCGTCAGGCTCAATCA; GN3B: sense: TTTTACTGCGCCAGAGTCA; antisense: CCTCTCGTGGAG TAGGGCTGT; XPO4: sense: GGAATATCCGACAGGAGGAGA; antisense: TAGTGTTTTTGGAGGAGAAATTC; GDF10: sense: AATCATCAAGGCTGCGGCAA; antisense: CTCGAC

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2 http://www.cytoscape.org
with a missing genotype rate

(Lambert et al., 2013) was downloaded from http://www.pasteur-

7 Neuroimaging Initiative (ADNI) (Mueller et al., 2005) was
dbGaP

Disease (GenADA) (Li et al., 2008) was downloaded from
Study for Genotype-Phenotype Associations in Alzheimer's
replication, the GWAS dataset from the Multi-Site Collaborative
was directly downloaded from the Myers laboratory
5

34129.8 frequency (MAF) < 0.01 and a Hardy–Weinberg equilibrium
(HWE) < 0.001. Finally, after quality control, 364048, 431284,
and 567199 SNPs from the Myers, GenADA and ADNI datasets,
respectively, were included for APOE ε4 carriers, and 374149,
427678, and 566459 SNPs from the Myers, GenADA and ADNI
datasets, respectively, were included for APOE ε4 non-carriers.

GWAS dataset preparation and quality control procedures
were performed using the software package PLINK4, release
v 1.07 (Purcell et al., 2007). Processed data of IGAP were
downloaded from the following link: http://www.pasteur-

A previously reported procedure of GWAS signal enrichment
test, called i-GSEA4GWAS, was employed (Zhang et al., 2010).
First, the maximum -log(P-value) of the SNPs located between
20 kb upstream and downstream of a gene boundary was
assigned to represent the gene, and the min P-value method,
which does not exclusively penalize large genes if causative
associations are proportionally more common in smaller genes,
has been widely used in systems biology (Baranzini et al., 2009;
Jia et al., 2010). Instead of the commonly used phenotype
label permutation, SNP label permutations were implemented
to generate the distribution of the enrichment score (ES), and
then the gene set’s significance proportion based enrichment
score (SPES) was calculated based on genes’ rank using the
following equation: SPES = k/K × ES, where k is the proportion
of significant genes of the gene set and K is the proportion
of significant genes of the total number of genes in the GWAS
(Zhang et al., 2010). Gene-length bias was eliminated by applying
adaptive permutation in PLINK before i-GSEA4GWAS. False
discovery rate was applied for multiple comparison correction.
Then, a comparative quantile-quantile (QQ) plot was used to
demonstrate the differences among all genes and genes
within the interested modules of APOE ε4 carriers, and the
genomic dispersion factor, λ, was used to assess the strengths
of genetic association signals and to quantify the differences when
compared with the signals of all genes. The comparative QQ plot
was produced by plotting the ranked -log(P-value) against the
expected order statistic, −log10(i(i+1)/L), where i is the rank for
each SNP P-value (1 = smallest and L = largest), and L is the
number of SNPs. Function estlambda() within the R package
GenABEL was utilized to estimate the genomic dispersion
decay factor λ. If a co-expression module was genetically associated,
significant SNPs within 20 kb upstream and downstream of the
gene boundaries within the module in the sub-group in which
it belongs (APOE ε4 carrier sub-group or APOE ε4 non-carrier
sub-group) were identified, and the significance of these SNPs in
its opposite sub-group were also calculated.

Phylogenetically Conserved Promoter Motif Identification in Hub Genes of the Complete Graph

The conserved promoter motif(s) was/were identified by
PhyloCon. PhyloCon, which stands for Phylogenetic Consensus,
is one of the first motif-finding algorithms to combine the power

http://www.ingenuity.com

http://labs.med.miami.edu/myers/LFuN/data%20ajhg.html

http://www.ncbi.nlm.nih.gov/projects/gap/cgi-

http://adni.loni.usc.edu/

http://pngu.mgh.harvard.edu/~purcell/plink/
of phylogenetic conservation and gene co-regulation (Wang and Stormo, 2003; Wang, 2007). PhyloCon first aligns conserved regions of orthologous sequences into multiple sequence alignments, or profiles, and then compares profiles representing non-orthologous sequences. Then, motifs represented by matrices or IUPAC strings emerge as common regions in these profiles (Wang, 2007). An online web server named WebLogo was used to generate the sequence logos of the motifs (Crooks et al., 2004). In the present study, orthologous sequences of the species Homo sapiens, Rattus norvegicus, Mus musculus, and Canis lupus familiaris were utilized. For promoter regions, the 4 kb segments centered on the annotated transcription start site (TSS) of each human RefSeq gene were extracted according to a previous study’s method (Xie et al., 2005). If the annotated translation start codon was within 2 kb of the TSS, the shorter region that did not overlap the protein-coding sequence was selected. For genes with alternatively spliced first exons, all promoters were included. Details for promoter region detection are given in a previous study (Xie et al., 2005).

JASPAR CORE database and miRWAlk were utilized to identify common human transcription factors (TFs), TF binding sites (TFBS) and common miRNAs of the conserved motif(s) and these highly connected genes. The JASPAR CORE database, which contains a curated, non-redundant set of profiles, is derived from published collections of experimentally defined transcription factor binding sites for eukaryotes. The matrices and IUPAC strings were then input into the JASPAR CORE database to identify common human TFs and the TF binding sites (TFBS) of these co-expressed genes (Mathelier et al., 2014). miRWAlk, a comprehensive database that incorporates miRNA-targets interactions information produced by eight prediction programs, it was regarded to bind to the gene with high probability.

AD and Parkinson’s Disease (PD), Bipolar Disease (BD) Consensus Pathogenic Module Identification

GEO dataset GSE202959 for PD and GSE1265410 for BD were extracted. The procedures for PD and BD co-expression network construction and module detection were the same as those of AD. Then, each PD- or BD-specific module was compared with AD-specific modules via hypergeometric tests which examined whether the number of genes overlapped between PD or BD and AD was significantly larger than that expected by chance.

RESULTS

Specific Co-expression Module Detection in AD APOE ε4 Carriers and Non-carriers

The demographic characteristics of the enrolled subjects after excluding outlier samples demonstrated no differences (Supplementary Table S1). By applying WGCNA, we identified two specific co-expression modules (violet and dark magenta) in AD APOE ε4 carriers and one module (light cyan) in AD APOE ε4 non-carriers (Figure 1). The permutation-based preservation statistic, Zsummary, demonstrated that the Zsummary statistics of the identified three modules were all less than 2 when assessing the violet and dark magenta modules of AD APOE ε4 carriers in AD APOE ε4 non-carriers and control APOE ε4 carriers and when assessing the light cyan module of AD APOE ε4 non-carriers in AD APOE ε4 carriers and control APOE ε4 non-carriers, showing no evidence of module preservation in these sub-datasets (Figure 1; Supplementary Table S2). These results strongly indicated that the three co-expression modules were sub-dataset-specific. Network visualizations of the violet and dark magenta modules in AD APOE ε4 carriers and the light cyan module in AD APOE ε4 non-carriers are shown in Figures 2A–C, respectively (Supplementary Tables S3–S5 for full list of genes of violet, dark magenta and light cyan modules respectively). The identified hub genes (most centered genes) are shown in Supplementary Tables S3–S5. Moreover, six of the seven hub genes (ISOC1, ENO3, GDF10, GNB3, XPO4 and ACY1, Supplementary Table S3) of the violet module and all the hub genes of the light cyan module (Supplementary Table S5) were found to be highly connected as demonstrated to form a complete graph respectively, which represents the most highly connected gene cluster in biological network and tends to be very important (Jeong et al., 2001; Horvath and Dong, 2008) (Figure 2D; Supplementary Table S6 for the pairwise Pearson correlation coefficients for the violet module and Figure 2E; Supplementary Table S7 for the pairwise Pearson correlation coefficients for the light cyan module).

Furthermore, we examined whether the non-specific co-expression modules were differentially expressed between AD and control APOE ε4 carriers, between AD and control APOE ε4 non-carriers, and between AD APOE ε4 carriers and non-carriers. In the results, no differences were found (data not shown), demonstrating that these non-specific co-expression modules were completely conserved.

Due to the unavailability of other AD-associated datasets with APOEε4 status, we carried out random samplings for replication. All three modules could be replicated (Supplementary Table S8).

Highly Connected Hub Genes Detected in the Violet and Light Cyan Modules Co-expressed in a Time-Dependent Manner Dependent on APOE Types

The highly connected hub genes in the complete graph implicate that the expression of these genes is correlated. Thus, we

examine the co-expression patterns of these genes in primary cultured neurons treated with APOE ε3 or APOE ε4. As shown in Figure 3A, the mRNA expressions of all six genes (ISOC1, ENO3, GDF10, GNB3, XPO4, and ACLY) in the complete graph of the violet module of AD APOE ε4 carriers demonstrated a similar time-dependent patterns in neurons treated with physiological concentration of APOE ε4, whereas, no such patterns were observed in neurons treated with APOE ε3 or in untreated neurons (Figures 3B,C). The pairwise Pearson correlation coefficient matrix of the expressions showed the high correlations between either two genes expressed in the APOE ε4-treated neurons (Supplementary Table S9) with a mean absolute Pearson correlation coefficient of 0.902, which is markedly higher than the mean absolute Pearson correlation coefficients for neurons treated with APOE ε3 (0.613) or for untreated neurons (0.571) (Supplementary Tables S10 and S11 for the pairwise Pearson correlation coefficient matrices for APOE ε3-treated neurons and for untreated neurons, respectively). These results confirm the co-expression of these six genes detected in the violet module.

The mRNA expression of 12 genes in the complete graph of light cyan module of AD APOE ε4 non-carriers displayed similar or opposite time-dependent patterns in primary cultured neurons treated with APOE ε3 (Figure 3E). The pairwise Pearson correlation coefficient matrix showed positive or negative correlations between most gene pairs (Supplementary Table S12), with a mean absolute Pearson correlation coefficient of 0.834. These results validated the co-expression of genes in the complete graph of the light cyan module. The co-expression patterns were much weaker in neurons treated with APOE ε4 and in untreated neurons (Figure 3D, Supplementary Table S13 for the pairwise Pearson correlation coefficient matrix for APOE ε4-treated neurons and Figure 3F, Supplementary Table S14 for untreated neurons, the mean Pearson correlation coefficients were 0.616 and 0.629, respectively).
FIGURE 2 | Graphic visualization of modules and complete graphs identified in AD APOE ε4 carriers and non-carriers. (A–C) Graphic visualization of the violet (A), and dark magenta (B) modules for AD APOE ε4 carriers and the light cyan module (C) for AD APOE ε4 non-carriers with hub genes. Nodes were filled with different intermediate colors varying from red to ivory white, where red represents high intramodular connectivity and ivory white represents low connectivity. Edges were weighted by the strengths of the connections between two nodes, for which shorter edges correspond to stronger relationships and vice versa. (D) Complete graph of the top six hub genes in the violet module of AD APOE ε4 carriers. (E) Complete graph of the top sixteen hub genes in the light cyan module of AD APOE ε4 non-carriers.
**FIGURE 3** | Time-dependent mRNA expression of genes within the complete graphs of the violet and light cyan modules. (A–C) The mRNA expression of six genes within the complete graph of the violet module of AD APOE ε4 carriers. The legends of the six genes are shown on the right. (D–F) The mRNA expression of twelve genes within the complete graph of the light cyan module of AD APOE ε4 non-carriers. The legends of the 12 genes are shown on the right.

The primary cultured neurons were treated with recombinant human APOE ε4 (A,D) or ε3 (B,E) at the concentrations of 5 µg/ml or without APOE treatment (C,F) on DIV19 and collected for quantitative RT-PCR after 24, 48, and 72 h of treatment. The relative mRNA levels were calculated by a comparative Cp value. The data are presented as the mean ± SD from at least three independent experiments.

**Differential Biological Processes Enriched in Specific Modules**

Co-expressed genes in a module may directly interact with each other or take part in the same biological processes and signaling pathways. Hub genes within each module may play pivotal roles in module function. Thus, IPA was employed for further analysis.

As shown in **Figure 4A**, the genes of the violet module identified in AD APOE ε4 carriers were enriched in hereditary disorders, neurological diseases, psychological disorders, and nervous system development and function (Supplementary Table S15). The first two hub genes, **ISOC1** and **ENO3**, were enriched in the first annotation, hereditary disorders. These two hub genes and another hub gene **GNB3** were enriched in neurological diseases (Supplementary Table S16). The primarily enriched signaling pathways were acetyl-CoA biosynthesis III (from citrate), NAD biosynthesis III, NAD salvage pathway III and NAD biosynthesis from 2-amino-3-carboxymuconate semialdehyde (**Figure 4B**: Supplementary Table S17), which are mainly energy metabolism-associated signaling pathways. Two relatively important energy metabolism-associated signaling pathways, gluconeogenesis I and glycolysis I, were enriched by the hub gene **ENO3** (Supplementary Table S17). Genes of the dark magenta module were mostly enriched in hereditary disorders, neurological diseases, psychological disorders, and G protein-coupled receptors (GPCRs) and second messenger-associated signaling pathways of GPCRs (**Figures 4C,D**, see Supplementary Tables S18–S20 for all enriched IPA terms). The protein level of the hub gene, **GAD2**, which catalyzes glutamate to γ-aminobutyric acid, has been reported to be reduced in some cerebral regions of AD patients (Schwab et al., 2013).

Unlike the modules identified in AD APOE ε4 carriers, genes of the light cyan module identified in AD APOE ε4 non-carriers were enriched in some other types of diseases, such as immunological and cardiovascular diseases, in addition to the primarily enriched neurological diseases (**Figure 4E**, see also Supplementary Table S21). The top-ranked hub gene, **DUSP5**, was enriched in neurological diseases (Supplementary Table S22). Moreover, another hub gene, **BDNF**, has been widely reported to be related to AD (Tapia-Arancibia et al., 2008). Our analysis also demonstrated that approximately 66% of enriched genes found in either immunological or cardiovascular diseases were also found in neurological diseases (Supplementary Figure S1). However, genes within the two modules of AD APOE ε4 carriers were not highly enriched in immunological and cardiovascular diseases (Supplementary Tables S16 and S19). In addition, genes...
FIGURE 4 | Ingenuity pathway analysis (IPA) gene set enrichment analysis of the modules identified in AD APOE ε4 carriers and non-carriers. IPA-identified top five diseases and functions (A,C,E) and top five canonical pathways (B,D,F) of the violet (A,B) and dark magenta (C,D) modules of AD APOE ε4 carriers and of the light cyan module (E,F) of AD APOE ε4 non-carriers. The verticals indicate the significant thresholds set by IPA.

of the light cyan module were mainly enriched in the following signaling pathways: tRNA charging, endoplasmic reticulum (ER) stress pathway, TR/RXR (thyroid hormone receptor/retinoid X receptor) activation and TNFR1 (tumor necrosis factor receptor type 1) signaling (Figure 4F; Supplementary Table S23).

Module-Based Enrichment of Genetic Association Signals

Next, we examined whether the three sub-dataset-specific co-expression modules had genetic bases. The violet module in AD APOE ε4 carriers showed significant enrichment of signals from the GWAS dataset of Myers of the corresponding APOE ε4 carriers in the gene expression data \( P < 0.001 \) and \( P < 0.001 \) after false discovery rate (FDR) correction, Figure 5A]. Moreover, the significant enrichment of signals was replicated in the other two GWAS datasets of APOE ε4 carriers \( P = 0.014 \) for the data from GenADA and \( P = 0.034 \) for the data from ADNI, Figures 5B,C). The dark magenta module of AD APOE ε4 carriers only showed a significant enrichment of signals from the GenADA GWAS dataset of APOE ε4 carriers \( P = 0.015 \), Supplementary Figure S2). Comparative QQ plots showed that genes within violet and dark magenta modules, especially the violet module, deviated from the expected values even further compared with the black dots, which represent all genes (Figures 5D–F). The larger genomic dispersion factors \( \lambda_{\text{violet}} \) and \( \lambda_{\text{dark magenta}} \) further validated their genetic bases (Figures 5D–F). Furthermore, genes in these two modules showed significant enrichment of signals from the IGAP GWAS dataset, which is the biggest AD-associated GWAS dataset to date (FDR-corrected \( P \)-value \( < 1 \times 10^{-4} \) for the violet module and \( P \)-value \( = 0.009 \) for the dark magenta module, Supplementary Figure S3). The QQ plots also showed a deviation of genes from the expected values (Supplementary Figure S4). Moreover, almost all significant SNPs within the 20 kb gene boundaries of the violet module in APOE ε4 carriers were insignificant in non-carriers (Supplementary Table S24). The light cyan module in AD APOE ε4 non-carriers did not show a significant enrichment of signals.

Analysis of Possible Transcription Modulation of Complete Graphs in Specific Modules

Complete graphs were detected and validated in the violet module of AD APOE ε4 carriers and in the light cyan module
of AD APOE ε4 non-carriers, suggesting common transcription modulation. Thus, we first identified conserved promoter motif(s) by employing promoter homologous sequences of these genes from four species, Homo sapiens, Rattus norvegicus, Mus musculus, and Canis lupus familiaris. Two conserved promoter motifs with extremely low conservative P-values (P = 6.539 \times 10^{-122} for the 49-base motif and P = 1.003 \times 10^{-74} for the 14-base motif) were detected for the genes of the complete graph of the violet module of AD APOE ε4 carriers (Figures 6A,B), and an adenine-rich conserved promoter motif was identified with a P-value of 1.935 \times 10^{-125} among the genes of the complete graph of the light cyan module of AD APOE ε4 non-carriers (Figure 6C).

Then, common human TFs of two motifs in the violet module were identified (Table 1). Interestingly, two TFs, ZNF263 and ESR1, were shared by the two motifs, and SNPs within ESR1 have been widely reported to be associated with AD (Mattila et al., 2000; Corbo et al., 2006). RREB1, a TF of 49-base motif, which can potentiate the transcriptional activity of NeuroD1/β2 (Ray et al., 2003), has been reported to be related with AD and potentially with the NFκB gene (Pasluosta et al., 2011). Another neuro-associated TF of 49-base motif, named REST, which can repress neuronal genes in non-neuronal tissues (Yeo et al., 2005), has been demonstrated to both silence and repress neuronal genes (Greenway et al., 2007). SNPs proximal to TP63, a TF of 14-base motif, which is present in the aging human hippocampus (Yang and Wang, 1994), are reported to be associated with brain morphometric measures of AD (Shen et al., 2010). Another TF of 14-base motif, named EGR1, up-regulates the PSEN2 gene in neuronal cells (Renbaum et al., 2003). However, currently no highly scored human TFs could be predicted for the motif identified in the light cyan module of AD APOE ε4 non-carriers.

Furthermore, we investigated whether the mRNAs of these genes in the complete graph could be regulated by some common miRNAs. For the six genes in the violet module of AD APOE ε4 carriers, hsa-miR-194, hsa-miR-199a-5p, hsa-miR-199b-5p, hsa-miR-30a, hsa-miR-30d, and hsa-miR-30e were indicated to bind with at least four of the five hub genes except GNB3 (Supplementary Table S25). hsa-miR-194 has been reported to be down-regulated in white matter of AD patients and to be negatively associated with neurofibrillary tangles in gray matter and in neuritic plaques and neurofibrillary tangles in white matter of AD patients (Wang et al., 2011). miRNAs in the hsa-miR-199 family have been shown to target genes involved in neurodegenerative diseases (Roshan et al., 2009). Five members of the miR-30 family are up-regulated in AD patients (Leidinger et al., 2013). However, currently no highly
scored common miRNAs were predicted for the genes in the complete graph of the light cyan module of AD APOE ε4 non-carriers.

**Correlated Modules between AD and PD**

Parkinson’s disease is also a neurodegenerative disorder with Lewy bodies deposited in neurons. Approximately 41.3% of PD patients are complicated with dementia according to a large population-based investigation (Mayeux et al., 1992). Pathologically, many PD patients show senile plaques and fibrillary tangles within the cerebral cortex (Boller et al., 1980), and many AD patients display Lewy bodies in cortical and subcortical regions (Hansen et al., 1990). BD is associated with an increased risk of dementia (Wu et al., 2013). Some symptoms and neuropathology of AD and BD overlap, such as brain atrophy, cognitive impairment, and emotional disturbances (Rao et al., 2012), however, no plaques and fibrillary tangles have been reported to be characteristic of BD. Thus, to further validate the identified modules, we applied the WGCNA analysis to these two neurological diseases to investigate whether the three identified co-expression modules were related to these diseases.

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**FIGURE 6 | Conserved promoter motifs of genes within the complete graphs of the violet and light cyan modules.** WebLogo representations of conserved promoter motifs in the violet module (A,B) of AD APOE ε4 carriers and in the light cyan module (C) of AD APOE ε4 non-carriers. The height of a letter at a particular position is proportional to the frequency of that nucleotide substitution in that position.

**TABLE 1 | Human TFs predicted for the two conserved promoter motifs in the violet module.**

<table>
<thead>
<tr>
<th>Motif</th>
<th>TF name</th>
<th>Class</th>
<th>Family</th>
<th>Score</th>
<th>Percent score</th>
</tr>
</thead>
<tbody>
<tr>
<td>49-base motif</td>
<td>ZNF263</td>
<td>Zinc-coordinating</td>
<td>ββα-zinc finger</td>
<td>36.811</td>
<td>87.646</td>
</tr>
<tr>
<td></td>
<td>RREB1</td>
<td>Zinc-coordinating</td>
<td>ββα-zinc finger</td>
<td>33.664</td>
<td>84.161</td>
</tr>
<tr>
<td></td>
<td>ESR1</td>
<td>Zinc-coordinating</td>
<td>Hormone-nuclear receptor</td>
<td>33.618</td>
<td>84.045</td>
</tr>
<tr>
<td></td>
<td>REST</td>
<td>Zinc-coordinating</td>
<td>ββα-zinc finger</td>
<td>32.833</td>
<td>78.173</td>
</tr>
<tr>
<td></td>
<td>PAX5</td>
<td>Helix-turn-helix</td>
<td>Homeo</td>
<td>32.654</td>
<td>85.931</td>
</tr>
<tr>
<td>14-base motif</td>
<td>SP2</td>
<td>Zinc-coordinating</td>
<td>ββα-zinc finger</td>
<td>24.495</td>
<td>87.483</td>
</tr>
<tr>
<td></td>
<td>ESR1</td>
<td>Zinc-coordinating</td>
<td>ββα-zinc finger</td>
<td>23.501</td>
<td>83.933</td>
</tr>
<tr>
<td></td>
<td>TP63</td>
<td>Zinc-coordinating</td>
<td>Loop-sheet-helix</td>
<td>23.084</td>
<td>82.443</td>
</tr>
<tr>
<td></td>
<td>ZNF263</td>
<td>Zinc-coordinating</td>
<td>ββα-zinc finger</td>
<td>23.082</td>
<td>82.435</td>
</tr>
<tr>
<td></td>
<td>EGR1</td>
<td>Zinc-coordinating</td>
<td>ββα-zinc finger</td>
<td>22.879</td>
<td>81.711</td>
</tr>
</tbody>
</table>

Top five predicted human TFs of the 49-base motif and 14-base motif from complete graph of violet module of AD APOE ε4 carriers.
DISCUSSION

Unlike the analysis at the single-gene level, which can be influenced by co-expressed genes and thus give biased information, WGCNA, a systems biology approach which identifies co-expression gene modules, may better uncover the pathogenic and pathological mechanisms of complex diseases. In the present study, we identified two specific co-expression modules (violet and dark magenta) in AD APOE ε4 carriers and one specific module (light cyan) in AD APOE ε4 non-carriers. The most highly connected hub gene clusters (complete graph) were further identified in the AD APOE ε4 carriers. These experimental data verified the results of the WGCNA and further demonstrated the specificity and existence of the identified gene clusters.

Our analysis demonstrated that the genes in both the violet and dark magenta modules of AD APOE ε4 carriers were strongly enriched in hereditary disorders, which was listed as the first among the diseases and functions of the IPA annotations. More importantly, a further analysis of enrichment with GWAS signals demonstrated that the expression patterns of these two modules had genetic bases in APOE ε4 carriers. Moreover, the significant SNPs of genes within both modules could not be replicated in APOE ε4 non-carriers. All these results demonstrated the genetic basis of APOE ε4 carriers for the development of AD.

The most highly connected hub gene cluster in the violet module was demonstrated to contain two phylogenetically conserved promoter motifs which could be targeted by some common TFs. Moreover, the mRNAs of these hub genes could be co-regulated by some common miRNAs. It is still unknown how the co-regulating feature in transcription is associated with the co-regulating feature in translation, however, the predicted conserved promoter motifs and common miRNAs suggest that some regulatory elements within these genes are phylogenetically conserved. This is consistent to the finding that the violet module has a genetic basis. Interestingly, three hub genes (ENO3, ISOC1 and GNB3) in this cluster were enriched in the first annotation (hereditary disorder) and/or the second annotation (neurological disease) of diseases and functions. Moreover, they were three highly ranked hub genes of the violet module and some SNPs around them were APOE ε4 carrier-specific (Supplementary Tables S3 and S24). One SNP, rs5443 within GNB3, has been

Transcriptomic data from three brain regions—Brodmann area 9 (BA9) of the prefrontal cortex, the putamen (PT) and the entire substantia nigra (SN) of PD patients were available for analysis. Among the detected PD-specific co-expression modules, only the purple module detected in BA9 of the prefrontal cortex which is the most PD-specific module (Supplementary Figure S5) was significantly correlated with the light cyan module of AD APOE ε4 non-carriers with an extremely low P-value (Figure 7A, \( P = 9.522 \times 10^{-24} \), Supplementary Table S26 for detailed overlapped genes). Two of the overlapping genes, DUSP5 and TBC1D8, were hub genes of the light cyan module, and DUSP5 was the top hub gene of the light cyan module (Supplementary Table S5). Furthermore, many annotations were the same between the two modules. The top diseases and functions and canonical pathways enriched in the light cyan module were also enriched in the purple module (Figures 7B,C). However, no BD-specific modules were found to be related to the three modules identified in AD (data not shown).
reported to correlate with increased APP expression (Bullido et al., 2004). In addition, this SNP is located within one of the ten most interesting genetic linkage regions related to AD (Guerreiro et al., 2012). Thus, we propose that these three genes could play important roles in AD APOE ε4 carriers with a genetic basis and also in a co-regulated manner.

Genes within the violet module of AD APOE ε4 carriers were mainly enriched in energy metabolism-associated signaling pathways. Energy metabolism and the expression of energy metabolism-associated genes are reported to be decreased in AD patients (Liang et al., 2008). However, the decrease in energy metabolism is even worse in APOE ε4 carriers who manifest it before AD development (Reiman et al., 2005; Wolf et al., 2013). Acetyl-CoA is a key intermediate in energy metabolism and also a key precursor for acetylcholine synthesis. It is almost exclusively synthesized in mitochondria from the pyruvate dehydrogenase complex (PDHC) reaction in the brain. It has been found that the activities of PDHC and choline acetyl transferase for acetylcholine synthesis are strongly suppressed in the brain cortexes of AD APOE ε4 carriers (Gibson et al., 2000; Bubber et al., 2005). Moreover, decreased acetyl-CoA for acetylcholine synthesis is responsible for decreased transmitter functions in cholinergic neurons of AD patients (Sztutowicz et al., 2013). Furthermore, in mice expressing human APOE ε4, evoked hippocampal acetylcholine release is reduced (Dolejsi et al., 2016). Thus, our findings explain the pathological differences between AD APOE ε4 carriers and non-carriers.

Genes within the dark magenta module of AD APOE ε4 carriers were mainly enriched in GPCRs and their second messengers-associated signaling pathways. Actually, many GPCRs have been validated to take part in the pathological process of AD, such as muscarinic acetylcholine receptors (M receptors) and metabotropic glutamate receptors (Albasanz et al., 2005; Wess et al., 2007). M2 and M4 receptors seem to inhibit the soluble amino-terminal ectodomain of APP (sAPPα) release and potentially aggravate Aβ generation (Farber et al., 1995). Deletion of M1 receptors is responsible for increased amyloid pathology (Davis et al., 2010). However, some specific phenomena are observed in APOE ε4 carriers. The density of M2 receptor is specifically higher in some cerebral regions of healthy APOE ε4 carriers compared to non-carriers (Cohen et al., 2003). Facilitating brain noradrenergic and vasopressinergic activities improves cognitive function only in AD APOE ε4 carriers (Richard et al., 1997). Defects in GPCRs, especially M receptors, have been widely reported in AD; however, the related signaling pathways were specifically enriched in AD APOE ε4 carriers. Thus, we hypothesize that alterations in GPCRs could play critical pathogenic and pathological roles in APOE ε4 carriers.

The genes in the light cyan module identified in AD APOE ε4 non-carriers were enriched not only in neurological diseases but also in some other types of diseases, such as immunological and cardiovascular diseases with high rankings. Furthermore, many genes were enriched in all of these diseases. However, these phenomena were not observed in AD APOE ε4 carriers. The involvement of the immune system in the pathogenesis of AD has long been demonstrated (Akiyama, 1994). Cardiovascular diseases have been reported to share some common risk factors and common signaling pathways with AD (Martins et al., 2006). Moreover, this module was enriched in various signaling pathways. tRNA charging is part of protein synthesis, TR/RXR regulates gene expression, and TNFR1 is a receptor for TNFα which is a potent proinflammatory cytokine. ER stress, retinoid signaling and TNFR1 are reported to be associated with AD (Corcoran et al., 2004; de la Monte et al., 2012; Laske et al., 2013). ER stress could enhance the production of Aβ (Liu et al., 2014). ER stress is also involved in tauopathy (Sakagami et al., 2013). Activation of retinoid acid receptors and RXR can up-regulate α-secretase ADAM10 (Tippmann et al., 2009). RXR can form heterodimers with peroxisome proliferator-activated receptor γ (PPARγ), and their combined activation cooperatively enhances the microglial uptake of Aβ (Yamanaka et al., 2012). TR and RXR can up-regulate the mRNA of an AD-associated gene (Ishida et al., 2013). TNFR1 is up-regulated in AD brains and is required in Aβ-induced neuronal apoptosis and cognitive impairment (Li et al., 2004; Cheng et al., 2010; Lourenco et al., 2013). However, these signaling pathways are not limited to AD. They are also involved in many pathological processes of various diseases such as cardiovascular diseases, immunological diseases, PD, and inflammation (Yoshida, 2007; Van Hauwermeiren et al., 2011; Nizamutdinova et al., 2013). Thus, these data indicate that the development of AD in APOE ε4 non-carriers may not stem from unique pathogenic processes, but rather, AD shares some common pathways or develops concomitantly with other types of diseases. However, our findings do not preclude the probable effects of APOE ε4 on these signaling pathways, such as pathways of the immune systems, which have been previously demonstrated (Keene et al., 2011).

To further validate our results, we selected PD and BD, two brain diseases with some common clinical or pathological relationships with AD. Pathologically PD and AD are very similar, as both types of patients exhibit senile plaques and fibrillary tangles. Cognitive deficits in PD are attributed to the prefrontal cortex (Fuster, 2000), and PT and SN are responsible for extrapyramidal signs (Rinne et al., 1989). Our results demonstrated that the purple module detected in B9 of the prefrontal cortex of PD patients was highly correlated with the light cyan module of AD APOE ε4 non-carriers, and such a correlation was not observed in PT and SN, suggesting the relationship of cognitive deficits between AD and PD. Thus, our data not only provide the evidence of the validity of the light cyan module but also demonstrate a relationship between PD and AD, especially the close relationship between PD and AD of APOE ε4 non-carriers. However, our data are not consistent with a previous study, which failed to detect any overlapping genes between AD and PD (Talwar et al., 2014). Because we demonstrated the difference between AD APOE ε4 carriers and non-carriers and because no risk genes were found in PD to play such a significant role as APOE ε4 in AD, the disparity could be attributed to the fact that the authors of the previous study did not stratify the AD samples according to APOE status. Although BD also shares some common characteristics with AD, especially at a later stage during disease progression, few studies have reported overlapping genes between AD and BD. In our study,
disease-specific modules displayed no correlations between BD and AD. Thus, we propose that the clinical and pathological overlap between AD and BD could be due to downstream cascades from pathogenic factors of each disease. However, our study does not preclude the possible harmful effects of APOE ε4 on PD and BD which have been reported even though APOE ε4 is unlikely a strong risk factor for PD and BD (Soeira-de-Souza et al., 2010; Monsell et al., 2014).

The three modules identified did not show any evidence of correlation with aging (data not shown), the commonly acknowledged risk factor for AD. Infants carrying APOE ε4 have different temporal cortex structures compared to APOE ε4 non-carriers (Knickmeyer et al., 2014), and a similar difference was also observed in adults (Hua et al., 2008). Multiple approaches utilizing mice and humans have demonstrated that APOE ε4 can affect normal brain function even very early in life (DiBattista et al., 2016). Thus, the identified modules may exert roles in an age-independent manner which may take effect or promote disease progression at an early age.

In summary, the present study demonstrated that AD APOE ε4 carriers involve more genetic factors and that particular biological processes may exert pathogenic effects, whereas AD APOE ε4 non-carriers share more common pathways with other types of diseases, and AD may develop accompanying these diseases. Although some of the identified hub genes, signaling pathways, TFs and miRNAs are known to be AD-associated, our data further demonstrated that they are likely specifically associated with AD APOE ε4 carriers or non-carriers. Moreover, our study may help to uncover the function of poorly characterized hub gene(s), as WGCNA may reveal the functions of genes if they are in a module highly enriched in a particular biological process (Chen et al., 2013). Our study provides new insights into the pathogenic and pathological mechanisms other than those of APOE4, underlying the different characteristics of AD APOE ε4 carriers and non-carriers, which may open a new avenue for the further investigation of AD and might promote the differential treatment of AD based on APOE ε4 status.

AUTHOR CONTRIBUTIONS
SJ drafted the manuscript and contributed to the analytical approach and to the presentation and interpretation of the results. LT contributed to the experimental study and results analysis. NZ contributed to the experimental study. WY contributed to the analytical approach and discussion. YQ conceived of the study and contributed to the assembly and interpretation of the data, manuscript writing and the final approval of manuscript. H-ZC contributed to the critical review of manuscript, the supervision of the study and final approval of manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnagi.2016.00171

REFERENCES


Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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