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Identification of Potential Therapeutic Targets of Alzheimer's Disease By Weighted Gene Co-Expression Network Analysis

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Objective Alzheimer's disease (AD) is the most common cause of dementia. The pathophysiology of the disease mostly remains unearthed, thereby challenging drug development for AD. This study aims to screen high throughput gene expression data using weighted co-expression network analysis (WGCNA) to explore the potential therapeutic targets.

Methods The dataset of GSE36980 was obtained from the Gene Expression Omnibus (GEO) database. Normalization, quality control, filtration, and soft-threshold calculation were carried out before clustering the co-expressed genes into different modules. Furthermore, the correlation coefficients between the modules and clinical traits were computed to identify the key modules. Gene ontology and pathway enrichment analyses were performed on the key module genes. The STRING database was used to construct the protein-protein interaction (PPI) networks, which were further analyzed by Cytoscape app (MCODE). Finally, validation of hub genes was conducted by external GEO datasets of GSE 1297 and GSE 28146.

Results Co-expressed genes were clustered into 27 modules, among which 6 modules were identified as the key module relating to AD occurrence. These key modules are primarily involved in chemical synaptic transmission (GO:0007268), the tricarboxylic acid (TCA) cycle and respiratory electron transport (R-HSA-1428517). *WDR47*, *OXCT1*, *C3orf14*, *ATP6V1A*, *SLC25A14*, *NAPB* were found as the hub genes and their expression were validated by external datasets.

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Conclusions Through modules co-expression network analyses and PPI network analyses, we identified the hub genes of AD, including *WDR47*, *OXCT1*, *C3orf14*, *ATP6V1A*, *SLC25A14* and *NAPB*. Among them, three hub genes (*ATP6V1A*, *SLC25A14*, *OXCT1*) might contribute to AD pathogenesis through pathway of TCA cycle.

ALZHEIMER'S disease (AD) is the most common cause of dementia, leading to impaired cognition, memory, language, and dysfunctions in daily activities. The pathological characterization includes formation of neurofibrillary tangles and amyloid plaques in brain.^[1, 2] Familial AD, linked with APP or PS1 mutation, involves less than 1% of the AD population. The sporadic AD accounts for over 99% of the cases, although the etiology largely remains unknown. Moreover, based on the current theories, no therapy can effectively prevent the occurrences of AD.^[3] Therefore, the exact underlying mechanism of the onset of AD and its molecular basis remain to be fully elucidated.

Weighted gene co-expression network analysis (WGCNA)^[4] is a systematic biology procedure to explore clusters (modules) of highly correlated genes between different groups of samples, and to analyze the association of these modules with clinical traits to illustrate the trait-related key modules. Lastly, intra-modular analysis and network visualization could be performed to identify the key genes within modules, which could further facilitate the discovery of novel therapeutic targets or candidate biomarkers.

In this study, we conducted WGCNA on GSE36980 of AD and normal controls. In each group, three tissue samples of different parts of brain were studied. Initially, 27 modules were elucidated by constructing the weighted co-expression network. Computation of the correlation coefficients between the modules and clinical traits, six key modules were identified. Intramodular analysis was executed; gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were then conducted. After network hub genes screening and hub genes validation, we determined three hub genes (*ATP6V1A*, *SLC25A14*, *OXCT1*) that may be involved in AD by regulating tricarboxylic acid (TCA) cycle, and these hub genes may be explored as the potential targets for AD therapy.

MATERIALS AND METHODS

Data source

Based on the platform of GPL6244 Affymetrix Human

Gene 1.0 ST Array, the mRNA microarray dataset of GSE36980 and corresponding metadata were downloaded from the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>). GSE36980 included three tissue samples of frontal cortex, hippocampus, and temporal cortex from postmortem brains (*Homo sapiens*) of overall 32 AD donor and 47 normal controls.

WGCNA analysis

R software (version 3.6.1) with WGCNA 1.67 package^[4] was utilized to process the dataset. Dataset normalization was performed by limma 3.38.3 package^[5] and then verified for outliers. T-distributed stochastic neighbor embedding (T-sne) and principal components analysis (PCA) were employed for quality control. Thereafter, the probes, with median expression less than 1/5 of the average level, were screened out. The standard deviation (SD) of the remaining gene expression was computed. For further analysis, the top 10,000 most variant genes were selected. To achieve a reasonable scale-free topology fit, the soft-thresholding power^[6, 7] was calculated initially and the adjacencies were evaluated. Next, the adjacency was transformed into Topological Overlap Matrix (TOM) to minimize the effects of noise. Finally, a dendrogram was constructed by hierarchical clustering, and Dynamic Tree Cut^[8] was performed to detect highly co-expressed genes (modules) according to their similarity in the TOM.

Identification of significant modules of clinical traits

Identification of the module-trait relationships was achieved by estimating the correlation between modules and clinical traits. Gene significance (GS) was analyzed to assess the correlation between module genes (eigengene) and clinical traits. Furthermore, the significant modules were located. The principal components of module gene expression were considered to be module membership (MM), which could measure the correlation of the module eigengene and the gene expression profiles. The genes with high GS and MM were investigated to identify the significant gene relevant

to development of AD. Module gene with GS and MM higher than 0.5 or lower than -0.5 were identified as the hub gene.

Intramodular and intermodular analysis

Intramodular analysis^[8] was performed on selected modules to explore the module biological functions, including Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, by Metascape^[9] online database. GS and MM analyses within module were carried out to identify hub genes with high GS and MM. Correlation of eigengene was examined to reveal the intermodular relationship.

Network construction

Network constructions were conducted by calculating the interconnection weight of paired genes in key modules in TOM. Module genes with weight above 0.08 were selected and visualized by Cytoscape (version 3.7.0).^[10]

Protein-protein interaction network construction

Genes in key modules were selected for Protein-protein interaction (PPI) network analysis by using the STRING database 11.0 (<https://string-db.org/>)^[11] and Cytoscape. To isolate clusters with high interconnected regions or protein complexes in the network, the Cytoscape app MCODE 1.5 (<http://apps.cytoscape.org/apps/mcode>) was used.

Hub genes validations

Two GEO datasets were downloaded from the GEO database for hub genes validations. The microarray dataset of GSE1297 contained hippocampus tissues from 9 normal controls and 22 AD patients, as well as the corresponding clinical information including MiniMental Status Examination (MMSE) score. The microarray dataset of GSE28146 contained hippocampus tissues from 8 normal controls and 22 AD patients who were classified as three stages: incipient, moderate, and severe. To further explore the potential role of hub genes in cognitive and clinical features, pearson correlations between hub genes expression and MMSE score were calculated.

Statistical analyses

Intramodular and intermodular analyses were performed according to pearson correlation among different modules using WGCNA 1.67 package,^[4] Pearson

correlation, one-way analysis of variance (ANOVA) or *t*-tests were performed to validate the hub gene among AD patients with different stage and controls in GSE1297 and GSE28146 datasets by using SPSS 20.0 software.

RESULTS

Key modules of Alzheimer's disease

After the normalization, quality control (supplementary **Figure S1**), and filtration, the top 10,000 genes with the highest SD (standard deviation) were selected for WGCNA. We chose 7 as the soft-thresholding power by PickSoftThreshold function calculation (**Figure 1A, 1B**). The dendrogram was constructed by setting minimum module size as 30 and Dynamic Tree Cut high as 0.25. There were 27 corresponding modules clustered (**Figure 1C, Table 1**). The genes which could not be attributed to any modules were categorized into the grey module.

Identification of the key modules and the key trait of AD

By the module-trait relationship heatmap based on the correlation between the modules and clinical traits (**Figure 2**), we identified 6 modules (darkturquoise, tan, black, salmon, grey60, and turquoise) as the key modules. According to the heatmap, the hippocampus had the highest correlation coefficient among 6 modules, indicating that the greatest alterations of gene expression between AD and controls were obtained in sample of hippocampus. Hence, the hippocampus was selected as a key trait of AD for further analyses.

Intramodular analyses and intermodular analyses

Estimation of the hub genes with a high correlation to AD status was achieved by conducting GS and MM analyses. To explore the differential expression of the hippocampus between AD and controls, we screened the 6 modules (turquoise, darkturquoise, salmon, tan, black, and grey 60). In each module, module hub genes were screened by correlation coefficient (GS and MM) for further analyses (**Figure 3, A-F**; supplementary **Figure S2**), which provide unique insights into the biological mechanism of AD. Dendrogram of the eigengenes and heatmap (**Figure 4**) revealed high correlations of 3 modules (darkturquoise, tan, black) with the AD status, suggesting those module genes may exert relevant biological functions. On the other hand, 3 modules (yellow,

green, and blue) were also selected for GS and MM significant genes screening to explore differentially expressed genes among different tissues. **Figure 3 G-I** demonstrated high correlation in those modules, which indicate hug amount of genes were differentially expressed among three tissues, suggesting they may play various roles in AD pathogenesis.

Module genes GO and KEGG analysis

Genes in black, tan, and darkturquoise modules were subjected to GO functional and KEGG pathway enrichment analyses by using online database of Metascape, and the results were shown in **Figure 5**. Tan and darkturquoise modules were primarily associated with synaptic functions, including chemical synaptic transmission (GO:0007268) and transmission across chemical synapses (R-HSA-112315). Meanwhile, the black module had significant association with citric acid (TCA) cycle and respiratory electron transport (R-HSA-1428517), signaling by ROBO receptors (R-HSA-376176).

Identification of hub genes

Network analyses on darkturquoise, tan, and black modules selected the top 36 genes, and the hub genes in networks were further screened out by degree and visualized. *PCMT1*, *WDR47*, *OXCT1*, *C3orf14*, *ATP6V1A*, *SLC25A14*, and *NAPB* were screened out in darkturquoise, tan, and black modules (**Figure 6A**). *AGAP6*, *AGAP7P*, *ATXN7* in the grey60 module, *MIR326*, *KRTAP5-3*, *RNA5SP408*, *RNA5SP413* in the salmon module, and *RBFOX1*, *FRMPD4*, *DNM1*, and *LNK1* in the turquoise module were identified as hub genes (**Figure 6, B-D**).

PPI network analysis

For the genes in darkturquoise, tan, and black modules, the PPI network constructed by the STRING database were shown in **Figure 7**. The network MCODE scores of Darkturquoise, tan, and black modules were measured, and visualized by color scaling. *SLC32A1*, *GAD1*, etc., in the darkturquoise PPI network, *EPHA7* and *TCEB1* in the tan PPI network, and *RCHY1*, *TCEB1*, *FBXO4*, etc., in the black module PPI network showed high MCODE score and were screened out by PPI network nodes connectivity. Ten hub genes in the black module was identified by MCODE. The results of GO and KEGG enrichment analyses were presented in **Table 2**, which demonstrated a significant correlation of the key sub-network with the Class I major histo-

compatibility complex (MHC) mediated antigen processing (R-HSA-983169).

Hub genes validations

Expression of the hub gene in GSE 1297 and GSE 28146 datasets showed that *WDR47*, *OXCT1*, *C3orf14*, *ATP6V1A*, *SLC25A14*, *NAPB*, *RCHY1*, *EPHA7*, and *TCEB1* expressions were significantly different between controls and severe state AD patients, signifying their potential target roles in AD pathogenesis (**Figure 8**). The expressions of *OXCT1* ($r=0.547$, $P<0.01$), *C3orf14* ($r=0.564$, $P<0.001$) were significantly correlated to the MMSE scores. However, how these genes participate in AD pathogenesis have so far not been investigated.

DISCUSSION

AD is a complex chronic neurodegenerative disease, which is characterized by decline in memory, language, motivation, and problem-solving ability. Moreover, significant progress has been achieved in the classical biomedicine based on molecular biology, which merely focus on individual genes and proteins that forms the basis of experimental biology. Since the pathophysiology of this complex disease remains unknown, it may be achieved by the breakdown of a significant amount of correlated genes rather than individual genes. Therefore, the application of network-based analysis^[12] approaches could help to elucidate the dynamics of AD progression^[13] or the expression patterns across different regions of the brain.^[14] This study aims to understand the information captured by high-throughput experiments data which is far richer than a list of differentially expressed genes.

In this study, we conducted WGCNA on microarray datasets downloaded from the GEO database which contained the frontal cortex, hippocampus, and temporal cortex from AD patients and non-AD donors. The GSE36980 samples expression was heterogeneous between the 3 tissues and homogeneous between AD patients and non-AD patients (**Figure 1**). After normalization and filtering of the data, the top 10,000 genes with the highest SD were selected to prevent high correlation produced by genes without notable variance. Following gene co-expression network construction, 27 modules were clustered and identified.

In module-trait relationship heatmap, the first column of trait illustrated significant correlation of 3 modules (darkturquoise, tan, and black) with AD. By

Figure 1. Construction of co-expression network.

(A) Analysis of network topology for various soft-thresholding powers. In this case, we chose the power of 6 which is the lowest power for the scale-free topology index reach 0.80. (B) Analysis of the mean connectivity showed the soft threshold of 7 was the fittest power value. (C) The clustering dendrogram of genes in GSE36980. The 27 colors below represents the corresponding co-expression modules.

Table 1. Modules and the number of genes in each module

Module	Gene number	Module	Gene number	Module	Gene number	Module	Gene number
grey	813	turquoise	1852	blue	971	brown	947
yellow	943	green	691	red	683	black	421
pink	402	magenta	396	purple	357	greenyellow	281
tan	179	salmon	152	cyan	146	midnightblue	144
lightcyan	87	grey60	74	lightgreen	69	lightyellow	62
royalblue	59	darkred	53	darkgreen	52	darkturquoise	50
darkgrey	45	orange	39	darkorange	32		

comparing the three tissue traits separately, the probable contribution of hippocampus genes expression pattern to the onset of AD was identified. Furthermore, the salmon module exhibited a high correlation solely with Hippocampus.

PCMT1 was identified as a hub gene through network visualization in the current study. A previous study^[15] demonstrated that the deficiency of *PCMT1* led to progressive epileptic disease or progressive neurodegeneration.^[16] Similarly, the role of *PCMT1* as a

negative regulator in A β peptide formations was also documented. Increased β -amyloid production was associated with the knock-down of protein *PCMT1*.^[17]

DNA methylation is a key factor in regulating synaptic plasticity, therefore affects learning and memory.^[18,19] Meanwhile, *C3orf14* was demonstrated as a methylation regulated gene in methylation arrays research,^[20] which was also identified by WGCNA and validated by external data in the current study. However, there is no published study pertaining to its potential

Figure 2. Module-trait relationships heatmap of the expression matrix.

Traits including AD occurrence, age, sex, tissues from frontal cortex, hippocampus, and temporal cortex, and combination of tissue with AD occurrence were analysed to explore the correlation with module genes expression. Each cell was the correlation coefficients (color coded) with the P value in parentheses. The modules correlated to the AD occurrence were taken for further analysis. FC, frontal cortex; TC, temporal cortex; HI, hippocampus. *combining the AD occurrence and the tissue together to explore expression change in tissues between AD and non-AD.

role in AD development.

On the other hand, the black module portrayed prominent application in citric acid (TCA) cycle (R-HSA-1428517), respiratory electron transport, mitochondria functions, and metabolism. Deficiencies of mitochondria functions and TCA cycle-related metabolites in AD models were highlighted in various research activities^[21,22] and in mitochondria-targeted therapy.^[23-25] The administration of TCA cycle-related metabolites could alleviate AD cognitive deficiency.^[26,27] Therefore, the results in our study suggested that the black module hub genes may be involved in the TCA cycle and act as potential targets for AD therapy.

ATPase H⁺ transporting V1 subunit A (ATP6V1A),

a multisubunit enzyme that mediates acidification of eukaryotic intracellular organelles, is also associated with synaptic vesicle proton gradient generation in brain, energy metabolism, and ATP synthesis. ATP6V1A was screened out by co-expression network construction of black module with high network connectivity. Meanwhile, the black module was significantly related to the TCA cycle and mitochondria functions. The external dataset also validated this finding. Therefore, we inferred that ATP6V1A may play an important role in AD. Research by Fassio found that mutations in ATP6V1A attributed to the onset of developmental encephalopathy with epilepsy, which suggested its role in regulation of neuronal development.^[28]

Figure 3. GS and MM scatterplot and hub genes screening in the selected key modules

(A) Black module, (B) darkturquoise module, (C) grey60 module, (D) salmon module, (E) tan module, and (F) turquoise module were the key modules which were significantly correlated to AD occurrence. (G) Blue module, (H) green module, and (I) yellow module were the selected key modules which can reveal differential expressed genes among different tissues (hippocampus, temporal cortex, frontal cortex). GS and MM were taken absolute values to locate the hub genes in the upper right corner of each plot. The key module were selected for hub genes screening based on the GS and MM value. GS, gene significance; MM, module membership.

Figure 4. Eigengene dendrogram and eigengene adjacency heatmap

(A) Eigengene dendrogram representing the correlation among different modules and the clinical traits. (B) Correlation between each two modules was presented by color in the matrix according the color scale.

Figure 5. GO and KEGG enrichment analysis results of the black, tan and darkturquoise modules by Matescape database.

Figure 6. Network analyses and hub genes visualization.

(A) Hub genes in darkturquoise, tan and black modules. (B), (C) and (D) showed hub genes in grey60, salmon, and turquoise modules, respectively. Node size correlates to the connectivity degree of genes.

However, there had been no study uncovering the potential function of this gene in AD.

SLC25A14 is a mitochondrial uncoupling protein

with a great abundance in brain.^[29] Meanwhile, Anitha *et al.*^[30] found that the reduction in *SLC25A14* expression was associated with mitochondrial dysfunction in

Figure 7. Visualization of protein-protein interaction (PPI) network analyses on genes of the darkturquoise, tan and black modules.

(A) The entire PPI network of the darkturquoise module. (B) The PPI network of genes in the Tan modules. Two hub network clusters sorted by MCODE scores were also shown. (C) The entire PPI network and network analysis of the black module. Two hub network clusters sorted by MCODE scores, and the sub-network of 10 hub genes with the highest connectivity degree in the PPI network of black module were also shown. Note: the nodes were sorted by MCODE scores according to the color scale bar.

Table 2. GO and KEGG analyses of the sub-network cluster in genes PPI network of black module[§]

Pathways	Description	Gene number	%	Log10(P)
R-HSA-983169	Class I MHC mediated antigen processing & presentation	13	61.90	-18.41
R-HSA-156827	L13a-mediated translational silencing of Ceruloplasmin expression	6	28.57	-9.38
GO:0032212	positive regulation of telomere maintenance via telomerase	3	14.29	-5.48
GO:0000209	protein polyubiquitination	5	23.81	-5.24
GO:0031396	regulation of protein ubiquitination	4	19.05	-4.58
GO:0097193	intrinsic apoptotic signaling pathway	4	19.05	-3.99
GO:0071478	cellular response to radiation	3	14.29	-3.23
GO:0051701	interaction with host	3	14.29	-3.11

[§]Pathways were sorted according to the *P* value in ascending order. GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Figure 8. Validation of the hub genes in darkturquoise, tan and black modules with AD and its states using datasets of GSE1297 and GSE28146.

(A) *OXCT1* expression value and MMSE score correlation analysis and comparison between groups. (B) *C3orf14* expression value and MMSE score correlation analysis and comparison between groups. (C) *WDR47*, (D) *SLC25A14*, (E) *ATP6V1A*, (F) *NAPB*, (G) *EPHA7*, (H) *RCHY1*, (I) *TCEB1*, each gene expression compared among the controls and the incipient, moderate, and severe state of AD patients. * $P<0.05$, ** $P<0.01$.

the autism spectrum disorders, presuming its role in the regulation of mitochondria functions in brain. A homodimeric mitochondrial matrix enzyme encoded by the gene *OXCT1* plays a central role in extrahepatic ketone body catabolism.^[31] It is also obtained as a hub gene in the black module. Above all, we uncovered three hub genes (*ATP6V1A*, *SLC25A14*, *OXCT1*) that may be involved in AD occurrence through pathway of TCA cycle or regulating mitochondria functions. To our best knowledge, no study had reported the potential function of these genes in AD.

In summary, we performed WGCNA on microar-

ray data and found six modules that were highly correlated with AD occurrence. Finally, we identified three hub genes (*ATP6V1A*, *SLC25A14*, *OXCT1*) that may be involved in AD pathogenesis through regulating TCA cycle and may serve as therapeutic targets for AD.

Conflict of interests

The authors declared no conflicting interests.

Supplementary materials

Figure S1 and Figure S2.

Available online at <http://cmsj.cams.cn/EN/10.24920/003695>.

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