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Genome-Wide Profiling of miRNA and mRNA Expression in Alzheimer's Disease

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Study Design A
Data Collection B
Statistical Analysis C
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Background: Our study aimed to identify key differentially expressed genes (DEGs) and miRNAs (DEmiRNAs) which can serve as potential biomarkers for diagnosis and therapy of Alzheimer's disease (AD).

Material/Methods: We performed miRNA and mRNA integrated analysis (MMIA) to identify DEGs and DEmiRNAs of AD. The AD-specific DEmiRNAs-targets interaction network was contrasted. Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis were performed. Q-RT-PCR was used to verify the expression of selected DEGs and DEmiRNAs.

Results: We conducted MMIA of AD based on 1 miRNA dataset and 3 mRNA datasets derived from the Gene Expression Omnibus (GEO) database; 1759 DEGs and 12 DEmiRNAs were obtained. DEGs of AD were significantly enriched in Huntington's disease and AD. LRP1, CDK5R1, PLCβ2, NDUFA4, and DLG4 were 5 DEGs regulated by 4 DEmiRNAs, including miR-26b-5p, miR-26a-5p, miR-107, and miR-103a-3p. These 4 miRNAs were the top 4 miRNAs covering most DEGs. According to the qRT-PCR results, the expression of PLCβ2, NDUFA4, DLG4, miR-107, and miR-103a-3p was consistent with our integrated analysis.

Conclusions: We concluded that LRP1, CDK5R1, PLCβ2, NDUFA4, and DLG4 may play a role in AD regulated by miR-26b-5p, miR-26a-5p, miR-107, and miR-103a-3p. Our findings will contribute to identification of biomarkers and new strategies for drug design for AD treatment.

MeSH Keywords: Alzheimer Disease • Biological Markers • Gene Regulatory Networks

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Background

As the most common form of dementia, Alzheimer's disease (AD) is predicted to affect 11.8% of all people globally by 2050 [1]. Alzheimer's disease is a fatal neurodegenerative disorder which can be divided into early onset familial AD (EOAD) and late onset Alzheimer's disease (LOAD) [2]. More than 98% of AD cases are LOAD, which are "sporadic" with no apparent familial recurrence of the disease and typically appears in older individuals (age 65 years and over [3]. It is characterized by progressive neuronal degeneration, pathology of amyloid-β (Aβ) deposition (senile plaques, SP), and loss of synapses and formation of neurofibrillary tangles (NFTs) that consist mainly of filaments of hyper-phosphorylated tau [4].

The Aβ precursor protein (APP), apolipoprotein E (APOE), and cleavage of APP by β-secretase or β-site APP cleaving enzyme 1 (BACE1) are indicated to be associated with AD [5,6], and APOE4 (E4 allele of apolipoprotein E) is the only verified genetic risk factor for late- onset AD [4,7].

miRNAs are a class of small non-coding RNAs of 20–22 nucleotides in length, which regulates more than 60% of protein-coding genes [8] and miRNAs are associated with many neurodegenerative diseases, such as AD [9]. Consequently, the research on miRNAs function and the exploration of miRNA replacement therapy is rapidly growing. Researchers have used microarray or high-throughput sequencing technology to detect mRNA and miRNA expression in AD. At present, autopsy is still necessary to diagnose AD accurately [10]. In addition, there is no curative therapy for AD and the current drugs only have limited efficacies [4]. Therefore, identifying non-invasive, accurate, early, and reliable biomarkers is essential for diagnosis and treatment for AD. In this study, through integrated analysis, we aimed to obtain more accurate results than possible with individual study. Functional annotation of DEGs and AD-specific miRNA-target gene network was performed, seeking to identify key DEGs and DEmiRNAs in AD to discover new diagnostic biomarkers for AD.

Material and Methods

Microarray expression profiling in GEO

The Gene Expression Omnibus (GEO) is the largest database of high-throughput gene expression data, developed and maintained by the National Center for Biotechnology Information. The microarray datasets of AD were obtained from the GEO database (*http://www.ncbi.nlm.nih.gov/geo*). Microarray datasets in blood of AD patients and normal control (NC) group were selected and data with drug stimulation or transfection were excluded. The miRNA datasets were derived from the microarray experiments by Petra-Leidinger et al.

Data analysis

The raw data was preprocessed with background correction. We performed the normalization using the Linear Models for Microarray Data (Limma) package in R. Two-tailed Student's t-test was used to calculate individual P-values, and the Stouffer test was used to merge individual P-values. Multiple comparison correction was performed by Benjamini & Hochberg method to obtain FDR. We finally identified the DEGs with selected criterion of FDR <0.001.

miRNA-target prediction

We used 6 bioinformatic algorithms (RNA22, miRanda, miRDB, miRWalk, PICTAR2, and TargetScan) to predict the putative target genes of differentially expressed miRNAs (DEmiRNAs). The threshold for the targets of DEmiRNAs was those genes predicted by ≥4 algorithms. Moreover, verified target genes of DEmiRNAs were obtained by the online tools of miRWalk (*http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/*). MiRNAs down-regulated the expression of its target genes in post-transcription, and the target genes whose expressions were inversely correlated with corresponding miRNAs were selected as miRNA targets with high accuracy based on the obtained gene expression data. Using Cytoscape software (*http://www.cytoscape.org/*), the miRNA-target gene interaction networks were constructed.

Functional annotation

To analyze the function and the potential pathway of miRNA-target genes, functional annotation including Gene Ontology (GO) classification (molecular functions, biological processes and cellular component) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment was performed by using the online software GeneCodis (*http://genecodis.cnb.csic.es/analysis*). FDR<0.05 was defined as statistical significance.

QRT-PCR confirmation

The 6 blood samples were collected from 3 normal control subjects and 3 patients who were diagnosed as having AD. Informed written consent was obtained from all participants, and research protocols were approved by the Ethics Committee of our hospital.

Total RNA was extracted with Trizol reagent (Invitrogen, China). cDNA was generated immediately from 1 μg extracted RNA by using SuperScript® III Reverse Transcriptase (Invitrogen, China). With Power SYBR® Green PCR Master Mix (Applied Biosystems, USA), quantitative PCR was performed by using an ABI 7500 real-time PCR system. The miScript II RT Kit was used to obtain the reverse transcriptions of miRNAs, and the miScript SYBR

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Table 1. mRNA and miRNA expression datasets used in this study.

Data type	GEO ID	Platform	Samples (N: P)
mRNA	GSE63060	GPL6947 Illumina HumanHT-12 V3.0 expression beadchip	104: 145
mRNA	GSE63061	GPL10558 Illumina HumanHT-12 V4.0 expression beadchip	134: 139
mRNA	GSE18309	GPL570 Affymetrix Human Genome U133 Plus 2.0 Array	3: 3
miRNA	GSE46579	GPL11154 Illumina HiSeq 2000 (Homo sapiens)	22: 48

Table 2. Significantly enriched up- and down-regulated miRNA in AD [11].

miRNAs	Regulation	FDR	miRNAs	Regulation	FDR
hsa-miR-151a-3p	Up	7.29E-07	hsa-let-7d-3p	Up	0.000872
brain-mir-112	Up	7.29E-07	hsa-let-7f-5p	Down	1.01E-05
hsa-miR-1285-5p	Up	4.54E-06	hsa-miR-107	Down	0.000367
hsa-miR-5010-3p	Up	8.15E-05	hsa-miR-103a-3p	Down	0.000437
brain-mir-161	Up	0.003185	hsa-miR-532-5p	Down	0.015277
hsa-miR-26a-5p	Up	0.003185	hsa-miR-26b-5p	Down	0.044145

Green PCR Kit (Qiagen, Germany) was used to perform quantitative PCR. Relative gene expression was analyzed using $2^{-\Delta\Delta Ct}$ method. The human 18srRNA and U6 were used as endogenous controls for mRNA and miRNA expression in analysis.

Results

Differential expression analysis of genes in AD

Three gene expression datasets (GSE63060, GSE63061, GSE18309) and 1 miRNA expression dataset (GSE46579) was enrolled in this study (Table 1). Twelve DEmiRNAs between the AD and NC groups used in our study were created by Petra-Leidinger et al. [11] (Table 2). The panel included 5 down-regulated and 7 up-regulated miRNAs. By integrated analysis (FDR <0.001), 1759 genes displayed altered expression between AD and NC group, with 847 up-regulated genes and 912 down-regulated genes. The top 100 significantly up-regulated or down-regulated genes in AD compared to normal controls are displayed in a heat-map (Figure 1).

AD-specific DEmiRNA-target interaction network

In total, 784 miRNA-mRNA interaction pairs were obtained, in which 578 miRNA-mRNA pairs included 159 up-regulated miRNA-mRNA pairs and 419 down-regulated miRNA-mRNA pairs were predicted by more than 4 algorithms. Additionally, 37 up-regulated miRNA-mRNA pairs and 169 down-regulated miRNA-mRNA pairs were verified by experiments. The miRNA-mRNA regulatory network was constructed based on these miRNA-mRNA interaction pairs, which consisted of 10 miRNAs (miR-26b-5p, miR-103a-3p, miR-107, miR-26a-5p, let-7f-5p, miR-532-5p, miR-151a-3p, miR-5010-3p, miR-1285-5p, let-7d-3p) and 695 targets differently expressed (Figure 2). MiR-26b-5p (degree=107), miR-103a-3p (degree=88), miR-107 (degree=88) and miR-26a-5p (degree=80) were the top 4 DEmiRNAs covering most DEGs.

Functional annotation

After GO enrichment analysis (FDR <0.05, Table 3), the miRNA targets were significantly enriched in regulation of transcription (FDR=7.38E-05), interspecies interaction between organisms (FDR=0.000270215), protein binding (FDR=2.14E-26), and metal ion binding (FDR=2.50E-09). According to the KEGG pathway enrichment analysis (FDR <0.05), several pathways were significantly enriched, including the insulin signaling pathway (FDR=0.0262808), lysosome (FDR=0.0441909), Huntington's disease (FDR=0.0247485, Figure 3), and basal transcription factors (FDR=0.0216603, Table 4). In addition, we found 36 DEGs that were associated with Alzheimer's disease-related metabolic pathways (Table 5), among of which 4 genes including low-density lipoprotein receptor-related protein 1 (LRP1) , phospholipase C beta 2 (PLCβ2), NDUFA4, and Cyclin-dependent kinase activator (CDK5R1) were also the targets of DEmiRNAs (Figure 4).

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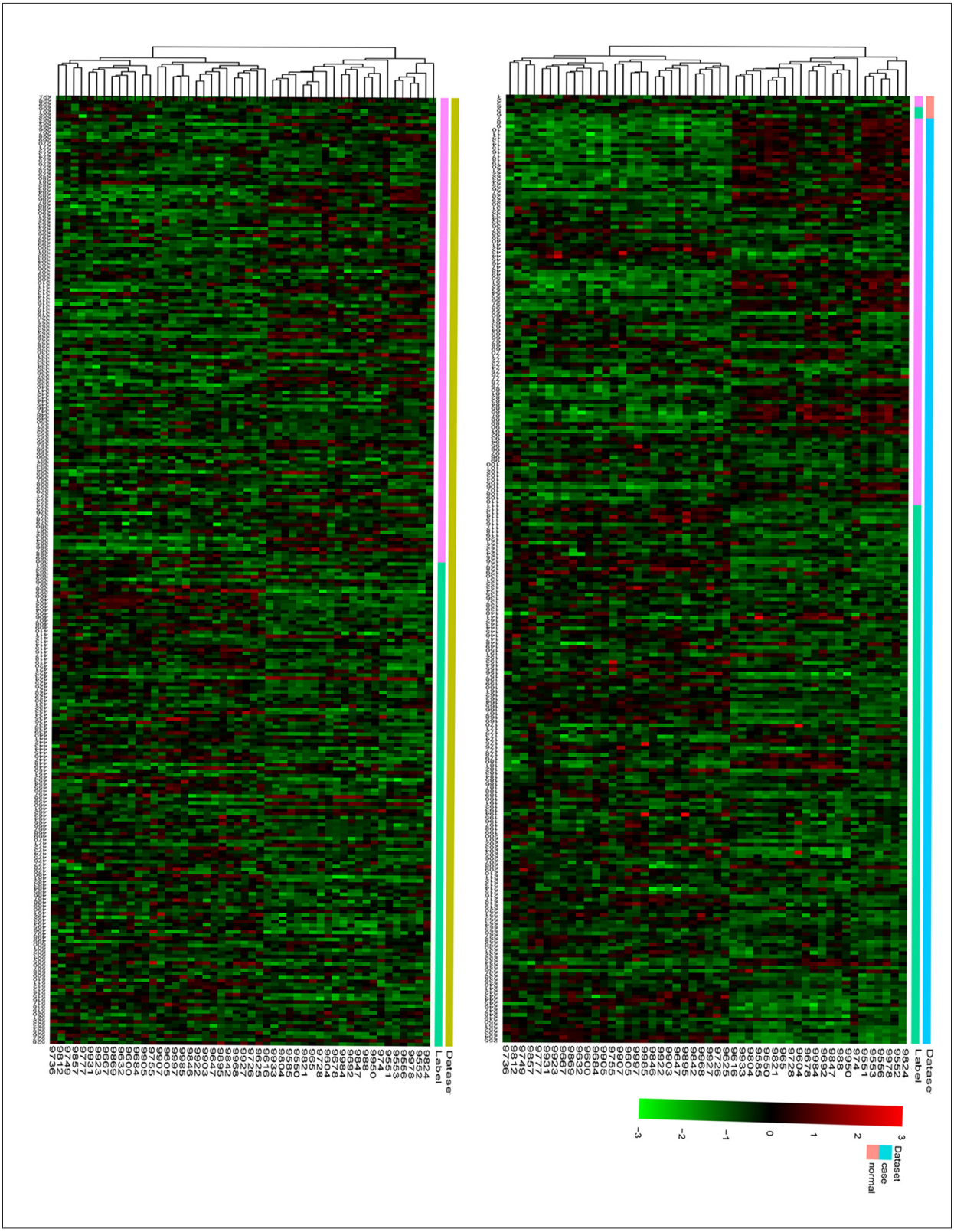

Figure 1. Heat-map image displaying the top 100 genes that were significantly up-regulated or down-regulated (P-value <0.05) in AD compared to normal controls.

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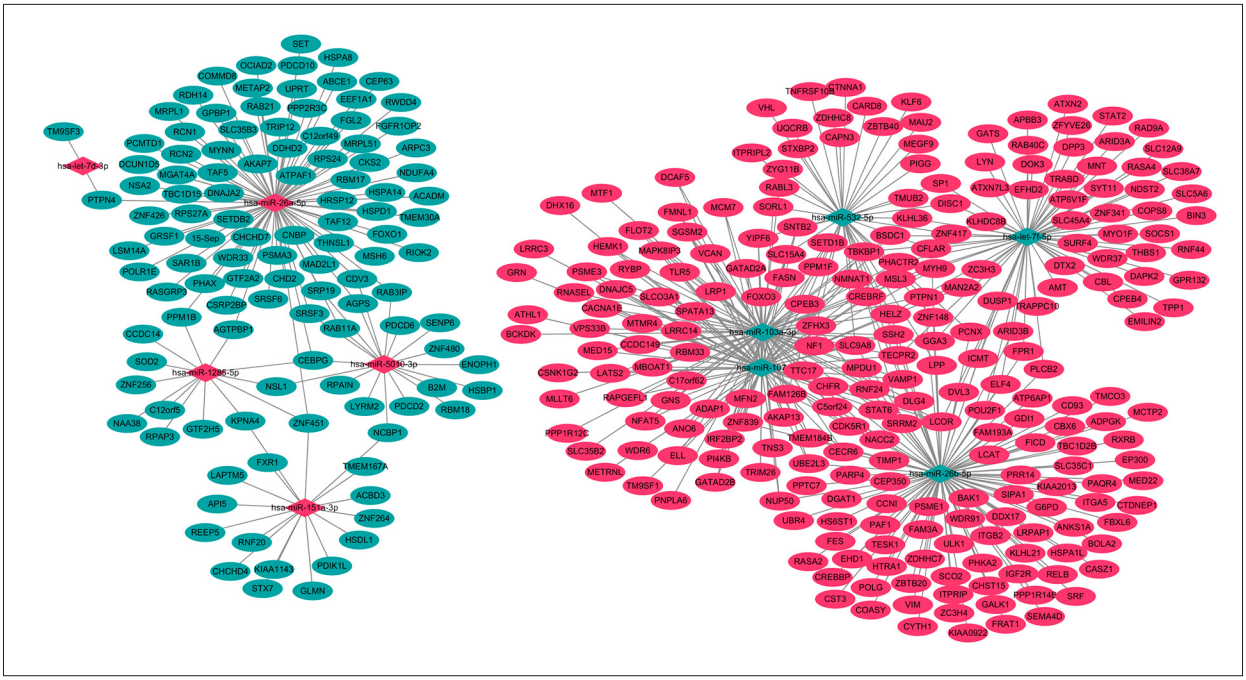

Figure 2. AD-specific DEmiRNA-mRNA interaction network. The green rectangle represents down-regulation of miRNAs, and the red ellipse represents the up-regulation of target genes. The arrow lines indicate miRNAs-targets pairs with negative correlations.

Table 3. Top 15 most significantly enriched GO terms in AD.

GO ID	GO Term	Count	P-value	FDR
Biological process				
GO: 0006355	regulation of transcription, DNA-dependent (BP)	42	6.75E-08	7.38E-05
GO: 0044419	interspecies interaction between organisms (BP)	16	4.94E-07	0.000270215
GO: 0042981	regulation of apoptotic process (BP)	12	2.07E-06	0.000754514
GO: 0016192	vesicle-mediated transport (BP)	11	6.76E-06	0.0018482
GO: 0006366	transcription from RNA polymerase II promoter (BP)	13	1.66E-05	0.00302682
GO: 0001889	liver development (BP)	7	1.45E-05	0.00316647
GO: 0046580	negative regulation of Ras protein signal transduction (BP)	4	3.28E-05	0.00512894
GO: 0008219	cell death (BP)	9	4.40E-05	0.00600976
GO: 0016573	histone acetylation (BP)	4	5.12E-05	0.00621195
GO: 0035556	intracellular signal transduction (BP)	12	0.000100293	0.00913503
GO: 0016070	RNA metabolic process (BP)	11	9.84E-05	0.00978072
GO: 0042493	response to drug (BP)	12	9.42E-05	0.0102999
GO: 0006470	protein dephosphorylation (BP)	7	0.000126898	0.0106692
GO: 0000209	protein polyubiquitination (BP)	7	0.000142801	0.0111487
GO: 0015939	pantothenate metabolic process (BP)	3	0.000179128	0.0130525

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Table 3 continued. Top 15 most significantly enriched GO terms in AD.

GO ID	GO Term	Count	P-value	FDR
Molecular function				
GO: 0005515	protein binding (MF)	130	4.93E-29	2.14E-26
GO: 0046872	metal ion binding (MF)	71	1.15E-11	2.50E-09
GO: 0000166	nucleotide binding (MF)	53	4.56E-09	6.59E-07
GO: 0003700	sequence-specific DNA binding transcription factor activity (MF)	28	3.96E-07	4.30E-05
GO: 0008270	zinc ion binding (MF)	45	6.18E-07	5.37E-05
GO: 0003677	DNA binding (MF)	42	1.05E-06	7.63E-05
GO: 0016740	transferase activity (MF)	20	1.04E-05	0.000646436
GO: 0005524	ATP binding (MF)	34	2.21E-05	0.00119919
GO: 0005509	calcium ion binding (MF)	20	2.91E-05	0.00140122
GO: 0001948	glycoprotein binding (MF)	5	6.10E-05	0.00264899
GO: 0008134	transcription factor binding (MF)	11	0.000128895	0.00372935
GO: 0004402	histone acetyltransferase activity (MF)	5	9.73E-05	0.00383997
GO: 0003723	RNA binding (MF)	18	0.000126578	0.00392391
GO: 0016787	hydrolase activity (MF)	24	0.000114083	0.00412599
GO: 0003713	transcription coactivator activity (MF)	10	0.00012437	0.00415205
Cellular component				
GO: 0005737	cytoplasm (CC)	130	5.21E-22	1.37E-19
GO: 0005634	nucleus (CC)	130	5.23E-21	6.88E-19
GO: 0005739	mitochondrion (CC)	42	1.14E-09	7.49E-08
GO: 0005622	intracellular (CC)	52	9.02E-10	7.91E-08
GO: 0005829	cytosol (CC)	54	2.55E-09	1.34E-07
GO: 0016020	membrane (CC)	81	9.40E-09	4.12E-07
GO: 0005654	nucleoplasm (CC)	28	3.39E-07	1.11E-05
GO: 0005730	nucleolus (CC)	38	3.27E-07	1.23E-05
GO: 0000139	Golgi membrane (CC)	15	4.59E-05	0.00134136
GO: 0005624	membrane fraction (CC)	16	0.00015706	0.00413068
GO: 0005783	endoplasmic reticulum (CC)	24	0.000176871	0.00422883
GO: 0005794	Golgi apparatus (CC)	23	0.000239488	0.00524877
GO: 0008537	proteasome activator complex (CC)	2	0.000329029	0.00618105
GO: 0031235	intrinsic to internal side of plasma membrane (CC)	3	0.000305668	0.00618389
GO: 0030529	ribonucleoprotein complex (CC)	7	0.000516178	0.00905032

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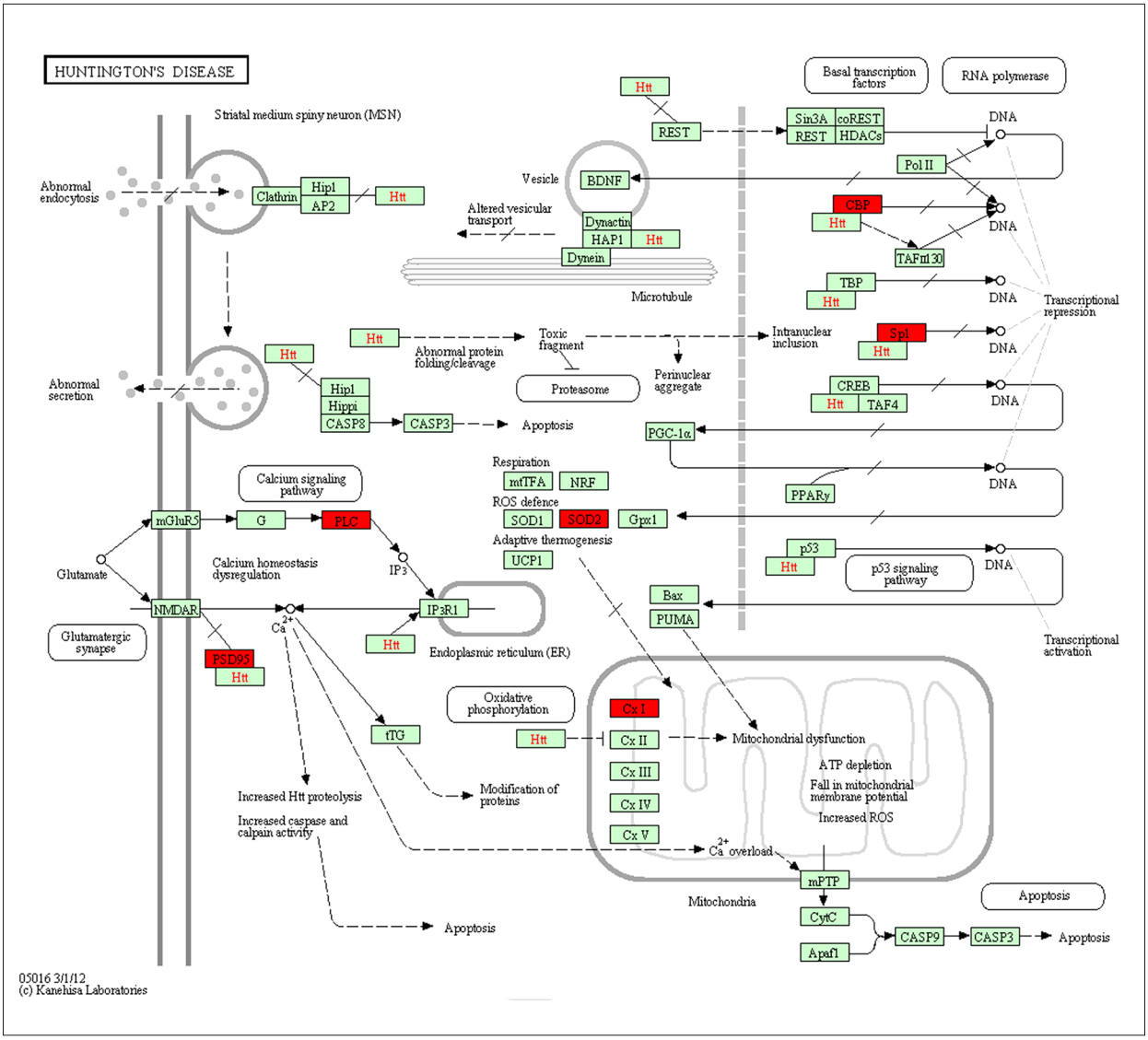

Figure 3. Pathway of Huntington's disease enriched in target DEGs of DEmiRNAs of AD. The red rectangles represent the elements regulated by the target DEGs of DEmiRNAs that are enriched in Huntington's disease.

Table 4. Most significantly enriched KEGG pathways for differentially expressed target genes.

KEGG ID	KEGG term	Count	FDR	Genes
hsa04910	Insulinsignaling pathway	7	0.0262808	FOXO1, PTPN1, PHKA2, FLOT2, SOCS1, FASN, CBL
hsa04142	Lysosome	6	0.0441909	GGA3, TPP1, ATP6AP1, GNS, IGF2R, LAPTM5
hsa05016	Huntington's disease	7	0.0247485	DLG4, PLCβ2, EP300, NDUFA4, SOD2, CREBBP, SP1
hsa03022	Basal transcription factors	4	0.0216603	GTF2H5, GTF2A2, TAF12, TAF5
hsa04010	MAPK signaling pathway	10	0.0366068	PPM1B, RASGRP3, CACNA1E, DUSP1, RELB, RASA2, SRF, MAPK8IP3, NF1, HSPA8
hsa03040	Spliceosome	7	0.0382035	SRSF6, RBM17, DHX16, NCBP1, NAA38, SRSF3, HSPA8

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Table 5. Differently expressed genes enriched in pathway of Alzheimer's disease.

KEGG ID	KEGG term	Count	FDR	Genes
hsa05010	Alzheimer's disease	36	1.38E-12	COX4I1, NDUFA8, ATP5J, NDUFAB1, ITPR3, COX7C, NDUFV2, ATP5F1, ATP5D, NDUFS4, COX6A1, FAS, LRP1, COX5B, PLCB2, ATP5H, NDUFB3, NAE1, NDUFA4, NCSTN, NDUFB6, UQCRC2, ATP5C1, NDUFA9, COX7A2, COX7B, UQCRB, NDUFA1, CDK5R1, NDUFB2, COX6C, ATP5O, NDUFB5, NDUFS3, UQCRH, UQCRQ

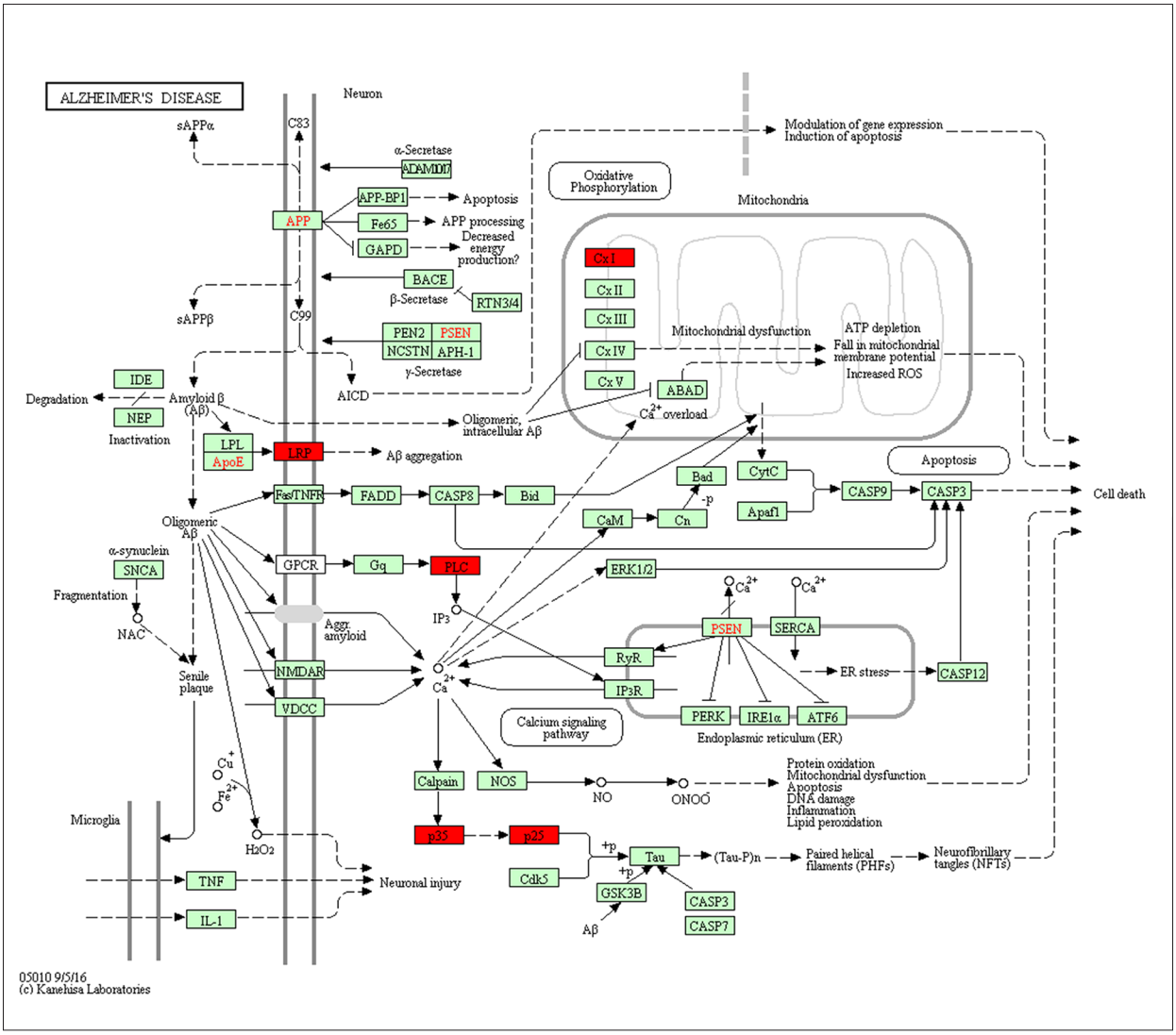

Figure 4. Pathway of AD enriched in target DEGs of DEmiRNAs of AD. The red rectangles represent the elements regulated by target DEGs of DEmiRNAs enriched in AD.

QRT-PCR confirmation

To verify the expression of integrated analysis, we selected 4 DEmiRNAs (miR-26b-5p, miR-26a-5p, miR-107, and miR-103a-3p) and 5 targets LRP1, CDK5R1, PLCβ2, NDUFA4, and discs large MAGUK scaffold protein 4 (DLG4). Based on the qRT-PCR results, the expression of LRP1, CDK5R1, and NDUFA4 were down-regulated, while the other 5 were up-regulated in AD compared to NC. The expression of miR-26b-5p, miR-26a-5p, LRP1, and CDK5R1 was inconsistent with results of our integrated analysis (Figure 5).

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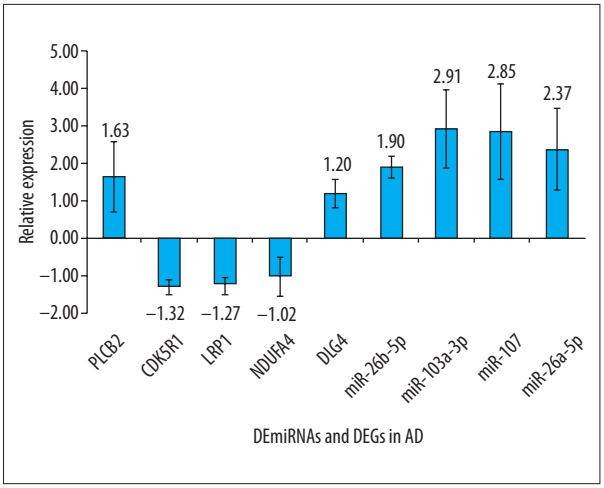

Figure 5. QRT-PCR results of DEmiRNAs and target DEGs in AD.

Discussion

As the principal cognitive problem in humans, the incidence rate of AD increased significantly with the aging population. Since there is no cure for it, finding novel strategies of treatment for AD has long been a central goal. In this study, we performed miRNA and mRNA integrated analysis (MMIA), and obtained 1759 DEGs and 12 DEmiRNAs in blood of AD patients compared to NC. According to the functional annotation and AD-specific DEmiRNA-target interaction network, 5 DEGs (LRP1, PLCβ2, NDUFA4, CDK5R1, and DLG4) upon the regulation of 4 DEmiRNAs (miR-26b-5p, miR-26a-5p, miR-107, and miR-103a-3p) were associated with AD.

Neuritic plaques (NP) and neurofibrillary tangles (NFT) in the brain are 2 major hallmarks of AD [12]. LRP1 is an APOE receptor in the brain. APOE can regulate the free cholesterol levels of neurons via LRP1, thereby mediating the APOE4 effect of NFT formation. Additionally, altered cholesterol metabolism significantly contributes to neuronal damage and to progression of AD. Hence, LRP1 may play a key role in the pathogenesis of AD by regulating cholesterol metabolism and NFT formation. Up-regulated LRP1 has been found in neurons in the hippocampus of AD patients [13,14]. According to our integrated analysis, LRP1 was up-regulated in the blood of AD patients. However, LRP1 was down-regulated in blood of patients with AD in our q-RT-PCR expression confirmation. The precise role of LRP1 in AD needs further research.

CDK5R1 encodes p35s, which is necessary for activation of CDK5. P35s can be cleaved into its more stable and truncated form, termed p25, and increased production of p25 enhances the activity of CDK5 [15]. With abnormal hyperphosphorylation of tau by activated CDK5, the numbers of NFTs which mainly composed of hyperphosphorylated microtubule associated protein tau was increased. Thus, the up-regulated CDK5R1 may increase the risk of AD by elevating the activation of CDK5, hyperphosphorylation of tau, and the formation of NFTs [16]. In our study, CDK5R1 was up-regulated in AD, which was shown to have the same pattern in a previous study [17]. In addition, increased p25 is associated with up-regulation of Aβ and accumulation of intra-neuronal Aβ, which are other major traits of AD [18].

NDUFA4 encodes a protein in the respiratory chain of mitochondria [19,20]. It has both NADH dehydrogenase activity and oxidoreductase activity [21]. Deficiency of complex I was found in various tissues of patients with neurodegenerative diseases like AD and Parkinson's disease [22]. In our study, NDUFA4 was down-regulated in AD, and it may be associated with the pathogenesis of AD via regulation in the respiratory chain of mitochondria.

DLG4 (also known as PSD95) is associated with anchoring glutamate receptors into the postsynaptic membrane [23]. Abnormally expressed DLG4 presumably leads to disturbance of glutamatergic neurotransmission. In addition, DLG4 is involved in regulating amyloid-β deposition [23]. Up-regulated DLG4 was detected in AD in our study, indicating its important role in AD, and up-regulated DLG4 might serve as a biomarker for AD.

Both extracellular and intracellular elevation of $Ca2^{+}$ are associated with the neurodegeneration in AD [24]. PLC (phosphoinositide-specific phospholipase C) is a major pathway activated by elevated intracellular calcium. As a member of the PLC family, PLCβ2 is activated by G proteins, and generates calcium signals in cells [25]. In our study, PLCβ2 was up-regulated in AD, which may have an important role in the progression of AD. Based on this, we may be able to develop a new therapeutic strategy.

According to the KEGG enrichment analysis, NDUFA4, PLCβ2, and DLG4 were enriched in another neurodegenerative disorder, Huntington's disease, emphasizing the importance of these 3 genes in AD as well. Moreover, PLCβ2, CDK5R1, NDUFA4, and LRP1 were found to be enriched in the pathway of AD, which supports our findings.

Rod-like structures were found in AD patients, which consisted of actin and the actin-binding protein cofilin [26]. Both miR103 and miR-107 have inhibitory effect on the translation of cofilin, so reduced miR103 and miR-107 may induce AD by exerting promotive effects on the level of protein cofilin and formation of rod-like structures. In addition, a correlation between NP counts, NFT counts, and decreased miR-107 expression was discovered [27]. Down-regulated MiR-107 was detected in the temporal cortical gray matter of humans in the early progression of AD and down-regulated miR-103a-3p has

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been detected in AD [27], which is consistent with our results in blood of AD patients. An AD-related gene, BACE1, is one of targets of both miR-107 and miR-103a-3p [28]. Up-regulated BACE1 has been found in AD patients with down-regulation of miR-107 [28,29]. MiR-107 and miR-103a-3p were 2 of top 4 DEmiRNAs covering most DEGs in AD, and BACE1, LRP1, CDK5R1, and DLG4 are all common targets of miR-107 and miR-103a-3p. Hence, we inferred that miR-107 and miR-103a-3p may play key roles in the progression of AD by regulating the expression of BACE1 and these 3 DEGs.

MiR-26a-5p and miR-26b-5p were the other 2 of top 4 DEmiRNAs covering most DEGs in AD. Our q-RT-PCR results showed that both miR-26a-5p and miR-26b-5p were up-regulated in AD. Previous studies also detected up-regulation of miR-26a-5p in AD by the NGS screening experiment [11]. Dysregulation of miR-26a and miR-26b was found in the peripheral blood mononuclear cells of Parkinson's disease [30] and AD patients [11]. Additionally, NDUFA4 is one of targets of miR-26a-5p. Both CDK5R1 and DLG4 are targets of miR-26b-5p. Hence, we suspected that both miR-26a and miR-26b were mediators of AD. We concluded that miR-26a-5p and miR-26b-5p play roles in AD by regulating NDUFA4, CDK5R1, and DLG4. However, the expression of these 2 DEmiRNAs in AD in our integrated analysis was inconsistent with our q-RT-PCR results. Studies with larger sample sizes are needed.

Conclusions

Our findings identified 5 DEGs (PLCβ2, CDK5R1, NDUFA4, DLG4, and LRP1) in blood of patients with AD compared to NC. Moreover, miR-107 and miR-103a-3p may play roles in the pathological process of AD by targeting LRP1, CDK5R1, and DLG4. MiR-26b-5p was also an up-regulator of AD by targeting CDK5R1 and DLG4. MiR-26a-5p was involved in AD by regulating NDUFA4. Thus, the DEmiRNAs and the corresponding targets obtained in our study will be potential biomarkers, and provide evidence for new strategies for design of drugs for AD treatment.

Conflict of interest

The authors declare that they have no conflict of interest.

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