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An integrative bioinformatics approach in microRNA data analytics of Alzheimer's disease

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ABSTRACT

Alzheimer's disease (AD) is the most common type of dementia clinically recognized by cognitive function impairment. Recently, the blood-based biomarkers relating to AD have been intensively investigated due to the minimum invasiveness and relatively low cost in the collection of blood samples compared to the cerebrospinal fluid in the brain. In line with this, the study of the deregulation of microRNA (miRNA) levels in the blood of AD patients is also rising. In this study, data analysis was performed on the miRNA expression profiling dataset using an integrative bioinformatics approach. K-nearest neighbor imputation and quantile normalization were carried out as the data pre-processing step to remove outliers and reduce bias in the dataset. Differential expression analysis was performed to identify 10 significant dysregulated miRNAs. Subsequently, 16 pathways were determined to be involved by the selected 10 miRNA signatures, and 7 genes were predicted as the common target genes. The roles of these target genes in AD were substantiated through a review of the existing literature. Expansion of the current work on a larger scale of data analysis is needed to further validate and understand the mechanism of miRNAs in AD development.

Keywords: Alzheimer's disease; microRNA; differential expression analysis; data analytics; bioinformatics

1. INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease that is clinically recognized by cognitive impairment (National Collaborating Centre for Mental Health [UK], 2007; Mucke, 2009). It is the most common type of dementia, a general term used to describe abnormal changes in the brain. The prevalence of AD is often related to aging, with a higher risk for older individuals. The advancement in health care and medical technology has brought an increase in life expectancy worldwide, resulting in the expansion of an aging population globally. According to the World Alzheimer Report 2015, it is estimated that 74.7 million and 131.5 million people will be living with dementia in the years 2030 and 2050 (Prince et al., 2015). It is reported that three people will develop dementia every three seconds.

Technologies such as qRT-PCR, microarray, and next-generation sequencing are applied in miRNA expression profiling (Roden et al., 2015). Differential expression analysis is performed on the expression profiles to study the differences in expression levels of miRNAs in the specific condition (Soneson and Delorenzi, 2013). Differential expression analysis can be done by statistical analysis or machine learning approaches. Pathway analysis is then carried out to analyze and identify the relationship between the groups of genes as well as the biological role of the candidate gene.

MicroRNAs (miRNAs) play a post-transcriptional role in the regulation of gene expression. Significant miRNAs can distinguish between AD and healthy controls, giving the potential to support AD diagnosis (Keller et al., 2016). However, the collection of cerebrospinal fluid is an invasive procedure with potential side effects. Blood gene expression data are useful for predicting AD classification and have been shown to be consistent with the observations from brain tissue-based studies (Lee and Lee, 2020).

More recent studies are focusing on the roles of miRNA in AD (Wang et al., 2019). Several studies have related miRNAs to the pathogenesis of neurodegenerative disease and reported that the miRNAs are essential for neuronal function and survival (Delay et al., 2012). For example, miR-9 is one of the most frequently altered miRNAs and is downregulated in AD brains. Circulating miRNAs are among the most promising candidates for easily accessible and non-invasive biomarkers for AD diagnosis (Sturmberg et al., 2015). Over the years, it has been found that miR-101, miR-20a, and miR-17 play important roles in AD pathogenesis (Kumar and Reddy, 2016; Liu et al., 2022). On the other hand, the level of miR-107 has been found to be correlated with AD (Fransquet and Ryan, 2018; Kumar and Reddy, 2016; Liu et al., 2022; Takousis et al., 2019). Besides, the suppression of miR-203 was found to subsequently alleviate cognitive function (Liu et al., 2022). miRNAs post-transcriptionally regulate gene expression by repressing protein synthesis and have a broad influence over AD pathogenesis. Hence, identifying miRNAs that perform aberrantly can provide new insights into the whole biological pathway and phenotype of AD.

In general, this study involved data pre-processing, differential expression analysis, and pathway analysis on the miRNA expression profiles. The raw read counts of miRNA expression were pre-processed by *k*NN imputation and normalized using the quantile normalization method. Differential expression analysis was performed using Welch's t-test to identify the differentially expressed miRNAs based on statistical and fold change cut-offs. Next, pathway analysis was performed. By comparing the results from the pathway analysis, the common significant pathways and target genes that are related to AD were identified. The identified target genes may serve as potential biomarkers that could be beneficial in therapeutic approaches to AD.

2. MATERIALS AND METHODS

Figure 1 illustrates the workflow proposed in this study. The miRNA dataset was collected and followed by data pre-processing such as imputation and normalization. Next, differential expression analysis was performed to identify the differentially expressed miRNAs that were used in the pathway analysis. Finally, significant pathways and target genes related to AD were identified.

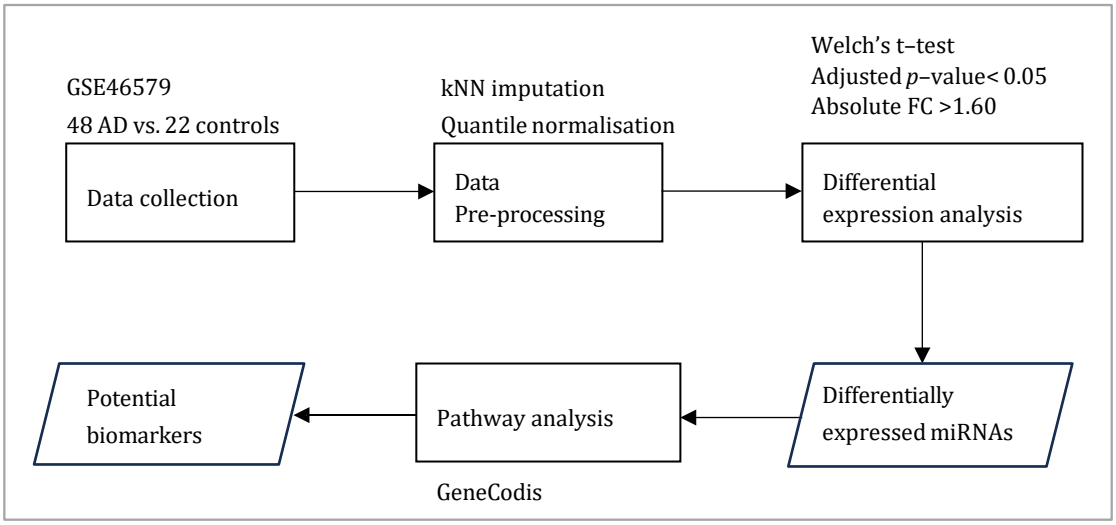

Figure 1. The proposed workflow

2.1 Materials

The dataset used in this study, with the accession number GSE46579 (Leidinger et al., 2013), was downloaded from the National Centre for Biotechnology Information Gene Expression Omnibus (NCBI GEO), which is an open-source database repository that stores the array- and sequence-based gene expression data. The miRNA dataset consisted of 140 unique mature miRNAs, which were collected from the blood samples of 48 AD patients and 22 healthy controls. The platform used to sequence the blood samples was Illumina HiSeq 2000.

All the pre-processing steps and differential expression analysis were conducted using the Python programming language. Several Python libraries were used for the data pre-processing such as Pandas and Scikit-learn. AnnData and diffxpy libraries were used to carry out the differential expression analysis. GeneCodis (https://genecodis.genyo.es/) (Carmona-Saez et al., 2007) was used in the pathway analysis as it searches for annotations that frequently co-occur in a set of genes from different sources, such as Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and gene ontology terms, and ranks them by statistical significance.

The identifier for each miRNA was based on the database miRbase (Leidinger et al., 2013) in the raw miRNA dataset. The identifiers in that database are in the form of hsa-mir-121, while the first three letters represent the organism. If there are distinct precursor sequences and genomic loci that express identical mature sequences, those miRNAs will get the names in the form of hsa-mir-121-1 and hsa-mir-121-2. The mature miRNA is signified by miR-121 while mir-121 refers to the miRNA gene and the predicted stem-loop portion of the primary transcript. However, let-7 and lin-4 are exceptions to the naming scheme, which are retained for historical reasons (Griffiths-Jones et al., 2006). The dataset was first filtered to remove the microRNAs that did not follow the naming scheme, such as brain-mir-192. Then, pre-processing of data was carried out.

First, *k*-nearest neighbor (*k*NN) (Dudoit et al., 2002) was used to impute the missing values in the dataset in which the information of correlation among genes was used. It estimated the *k* nearest group of miRNAs that were similar to the missing target miRNA, then averaged those miRNAs to impute the missing value of the target gene. In this study, *k*=5 was used. Next, in the normalization step, the quantile normalization method was applied to the dataset. Quantile normalization was initially developed for gene expression microarrays, but nowadays it can be applied to various data types, including RNA-sequencing (Cloonan et al., 2008; Garmire and Subramaniam, 2012). Quantile normalization is a global transformation method by assumes the statistical distribution for each sample is the same. It takes the average distribution, which is obtained from the mean of each quantile across samples, as the reference and forces the observed distributions to be identical.

2.2 Differential expression analysis

Before conducting the differential expression analysis, the normalized data were input as an annotated data matrix by using the AnnData library as required for differential expression analysis. The data was annotated into two groups, which were AD and controls. In this study, the diffxpy library was used to conduct the differential expression analysis. Various statistical tests were provided from the library, and Welch's t-test was used in the study.

Welch's t-test is used when the variances of the two groups are not identical (Yaari et al., 2013). To model the difference in mean expression for miRNA i between two groups, treatments (T) and controls (C), we define:

$$t_i = \frac{\bar{E}_i^T - \bar{E}_i^C}{\sqrt{\frac{(s_i^T)^2}{N^T} + \frac{(s_i^C)^2}{N^C}}} \quad (1)$$

where $\bar{E}$ is the mean expression value of the miRNA, s is the standard deviation for the respective group on the miRNA, and N is the total number of the samples that belong to the particular group.

Fold-change (FC) is an essential threshold that is used to identify the differentially expressed miRNAs. In this study, the differentially expressed miRNAs were selected by using the cut-offs of adjusted *p*-value<0.05 and absolute arbitrary FC >1.6, which is equivalent to $\log_2$FC >0.678.

2.3 Pathway analysis

GeneCodis (Carmona-Saez et al., 2007) was used for the pathway analysis. Two types of pathway annotations were selected, which were the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and the Gene Ontology Biological Process (GO). Those pathways that have an adjusted *p*-value of less than 0.05 from the hypergeometric test are considerably significant. From the filtered significant pathways, the pathways from KEGG and GO were compared to determine the common significant pathways. Lastly, the significant target genes could be identified from the common significant pathways.

3. RESULTS AND DISCUSSION

The differentially expressed miRNAs were selected as shown in Table 1 by using the cut-offs of adjusted *p*-value<0.05 and absolute arbitrary FC >1.6 (which is equivalent to $\log_2$FC >0.678). A total of 11 differentially expressed miRNAs were selected.

Table 1. Significant miRNAs identified from the Welch's t-test with cut-offs of adjusted *p*-value<0.05 and absolute FC >1.6 (or $\log_2$FC >0.678)

Precursor	Mature sequence	*p*-value	adjusted *p*-value	$\log_2$FC
hsa-mir-378e	hsa-miR-378e	0.002631	0.028485	-0.82127
hsa-mir-4781	hsa-miR-4781-3p	2.67E-07	6.02E-05	-0.88574
hsa-mir-5001	hsa-miR-5001-3p	7.69E-05	0.005796	-0.74152
hsa-mir-378b	hsa-miR-378b	0.000679	0.013395	-0.91671
hsa-mir-330	hsa-miR-330-3p	0.002401	0.02838	-0.68803
hsa-mir-3127	hsa-miR-3127-3p	6.23E-06	0.000939	-0.98285
hsa-mir-5701-1*	hsa-miR-5701	0.00354	0.034788	-0.68454
hsa-mir-5701-2*	hsa-miR-5701	0.00354	0.034788	-0.68454
hsa-mir-4659a	hsa-miR-4659a-3p	0.001111	0.018591	-0.71012
hsa-mir-26b	hsa-miR-26b-3p	2.11E-05	0.002385	-0.70693
hsa-mir-1468	hsa-miR-1468	3.88E-08	1.75E-05	-0.9363

Note: The hsa-miR-5701-1 and hsa-miR-5701-2 are different precursors that produce the same mature miRNA sequence.

From the 11 identified differential miRNAs, there were two identical mature miRNA sequences, hsa-miR-5701-1 and hsa-miR-5701-2, which were produced from different precursors. Therefore, only 10 unique miRNA mature sequences were used for the pathway analysis.

The identifiers of the 10 unique miRNAs were used as input to GeneCodis. The functional enrichment analysis was performed based on the KEGG pathways and GO. The significant pathways were identified by an adjusted *p*-value<0.05 from the hypergeometric test.

Two result files, each from KEGG and GO, were downloaded in tsv format and converted to .xls format. There were 78 significant pathways found in KEGG, and 369 significant biological processes were identified in GO. Each significant pathway from KEGG as well as from GO was filtered by masking the cancer-related terms and excluding those without any supported findings that suggested it was related to AD. After the filtration step, there were 46 pathways and 162 pathways left for KEGG and GO, respectively.

Next, the filtered pathways from KEGG and GO were compared to determine the common significant pathways. There were 16 common significant pathways identified from both KEGG and GO, which were AGE-RAGE signaling pathway, VEGF signaling pathway, neurotrophin signaling pathway, estrogen signaling pathway, endocytosis, focal adhesion, MAPK signaling pathway, oxidative phosphorylation, adherens junction, glial cell proliferation, Fc gamma R-mediated phagocytosis, axon guidance, cell cycle, cellular senescence, cytokine production involved in inflammatory response, and HIF-1 signaling pathway.

Next, the target genes for each pathway were identified from the common significant pathways that were determined earlier. A total of 11 target genes (9 target genes from KEGG and 9 target genes from GO) were identified from the sixteen common significant pathways listed above. The results of the target genes identified are listed in Table 2. From these 11 target genes, 7 common target genes were identified, as shown in the Venn diagram (Figure 2).

Finally, the 7 common genes identified were input to GeneCodis to visualize the gene-annotation cluster network. Figure 3 and Figure 4 show the top 10 significant pathways for KEGG and GO generated from GeneCodis, respectively.

Table 2. Target genes of pathways from KEGG and GO

Terms	Target genes	
	KEGG	GO
AGE-RAGE signalling pathway	CDC42, VEGFA	CDC42, VEGFA, NTRK3
VEGF signaling pathway	CDC42, VEGFA	CDC42, VEGFA
Neurotrophin signaling pathway	CDC42, NTRK3	NTRK3
Estrogen signaling pathway	SP1, ESR1	ESR1
Endocytosis	CDC42, SH3GL2	CDC42, SH3GL2
Focal adhesion	CDC42, VEGFA	VEGFA
MAPK signalling pathway	CDC42, VEGFA	CDC42, VEGFA, NTRK3
Oxidative phosphorylation	COX2	COX2
Adherens junction	CDC42	CDC42
Glial cell proliferation	E2F1	E2F1
Fc gamma R-mediated phagocytosis	CDC42	CDC42
Axon guidance	CDC42	VEGFA
Cell cycle	E2F1	E2F1
Cellular senescence	E2F1	PDCD4
Cytokine production involved in inflammatory response	EGR2	PDCD4
HIF-1 signaling pathway	VEGFA	VEGFA, NKX3-1, E2F1

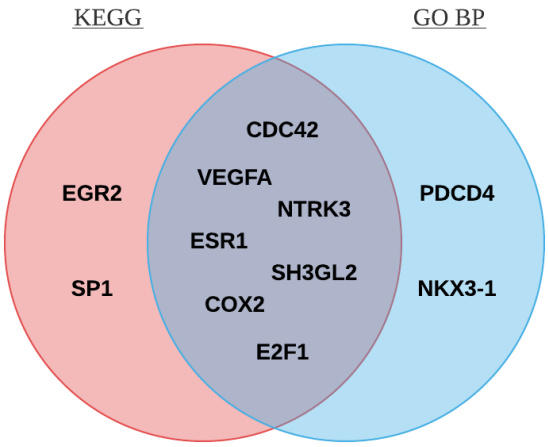

Figure 2. Venn diagram of target genes between KEGG and GO

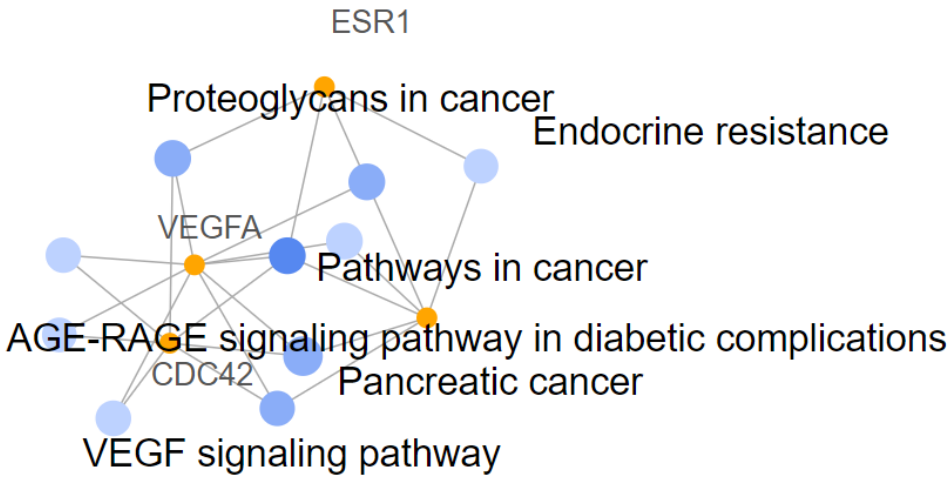

Figure 3. The top ten significant pathways for KEGG from GeneCodis

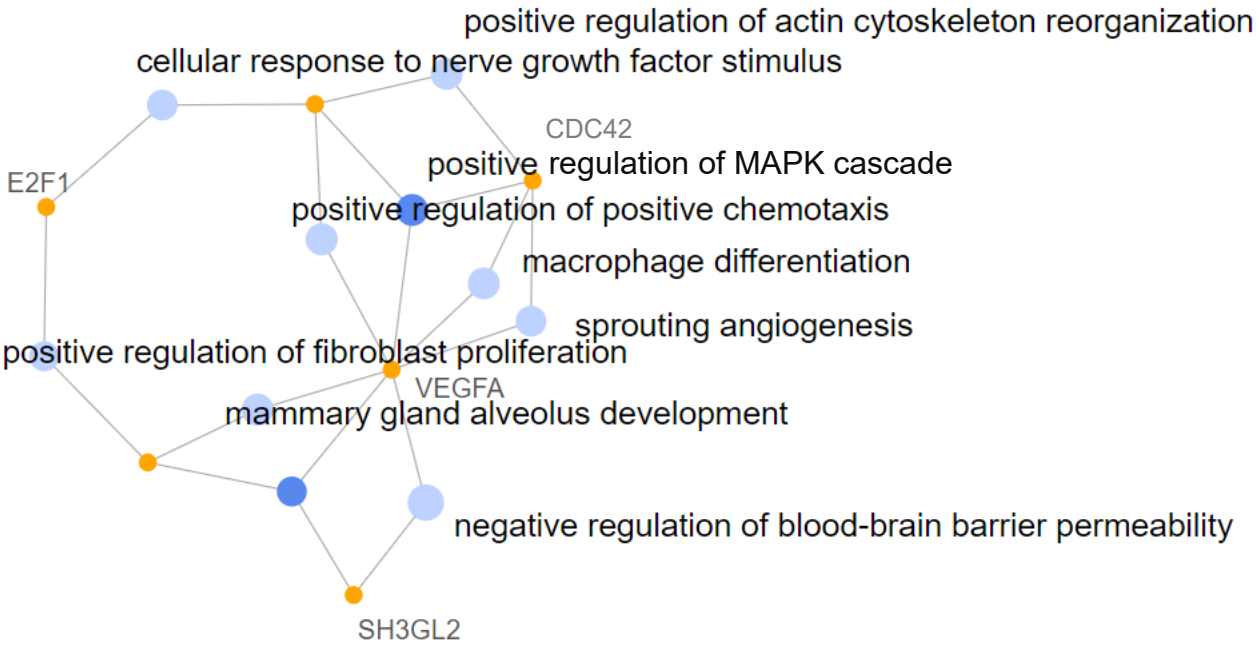

Figure 4. The top ten significant pathways for GO from GeneCodis

4. DISCUSSION

In this study, 10 differentially expressed miRNAs were identified from differential expression analysis. The significance of the 10 miRNAs identified was related to their functions in AD or other types of neurogenerative diseases. Table 3 lists the 10 identified miRNAs with related functions.

Seven common target genes were identified from the results shown in Figure 4. The common target genes were Cdc42, VEGFA, NTRK3, ESR1, SH3GL2, COX2, and E2F1. Table 4 lists the functions related to AD for each of the target genes.

The findings of this study were compared with similar previous studies involved in circulating miRNA in AD. A summary of the comparison is listed in Table 5. There are two main limitations of this study: First, only one dataset was used in this study, which consisted of 48 AD patients and 22 controls. Second, the proposed biomarkers were compared with previous studies only and were not validated through any analytical or clinical validation in the laboratory.

Table 3. Significance of selected 10 miRNAs

miRNAs	Functions	References
hsa-miR-378e	Downregulated in ALS, its overexpression inhibits glycolysis and promotes cell apoptosis, which indicates its therapeutic effect in glioma.	Kovanda et al., 2018; Ding et al., 2019
hsa-miR-4781-3p	Upregulated in AD	Sproviero et al., 2021
hsa-miR-5001-3p	Upregulated in AD	Kumar and Reddy, 2016
hsa-miR-378b	Downregulated known miRNA in cerebrospinal fluid-derived exosomes	Hou et al., 2019
hsa-miR-330-3p	Exert protective effects on Aβ production, oxidative stress and mitochondrial dysfunction by targeting VAV1 via the MAPK signaling pathway	Zhou et al., 2018
hsa-miR-3127-3p	Upregulated in AD	Leidinger et al., 2013
hsa-miR-5701	Induced mitochondrial dysfunction and a defect in autophagy flux in PD	Prajapati et al., 2018
hsa-miR-4659a-3p	Negatively regulated GNAQ, TMTC2, and BEND2 with multiple miRNAs in PD	Liu et al., 1996
hsa-miR-26b-3p	Deregulated early in AD brain, nearly 20 years before the onset of clinical symptoms (upregulated in the brain while downregulated in the blood)	Swarbrick et al., 2019
hsa-miR-1468	Upregulated in AD	Satoh et al., 2015

Table 4. Functions related to AD in target genes

Genes	Functions related to AD
Cdc42	- Regulation of actin cytoskeleton dynamics and spine formation - Increased level of Cdc42 in the frontal cortex of AD
VEGFA	- Co-accumulated with beta-amyloid deposits in AD brains - Up-and down-regulated in the brain, blood CSF of AD
NTRK3	- Activate neuronal survival pathways - Decreased NTRK3 expression found in AD, PD, and Huntington's disease
ESR1	- Decreased tau hyperphosphorylation - High ESR1 expression in the nucleus basalis of Meynert in AD
SH3GL2	- Increased endophilin A1 -> Increased JNK activation -> Neurons die
COX-2	- Regulated neurotoxicity - Increased COX-2 in AD brain
E2F1	- Increased immunoreactivity of E2F1 and ppRb in affected cortical brain region in AD

Table 5. Comparison with previous circulating miRNA AD studies

Reference	Methods	Findings
Kumar et al., 2013	Differential expression analysis, QIAGEN Ingenuity Pathway Analysis	7 signature miRNAs
Leidinger et al., 2013	Differential expression analysis, RT-qPCR	12 signature miRNAs
Satoh et al., 2015	Differential expression analysis & KEGG	27 signature miRNAs
Sproviero et al., 2021	Differential expression analysis, KEGG & GO	1 common miRNA was found in comparison between 4 different types of neurodegenerative diseases (including AD). Not able to perform pathway analysis in AD due to a limited number of deregulated miRNAs.
Current study	Differential expression analysis, KEGG & GO	10 signature miRNAs and 7 common target genes

5. CONCLUSION

In this study, the miRNA expression profiles of Alzheimer's disease patients and healthy controls were analyzed by an integrative bioinformatics data analysis approach. A total of 10 differentially expressed miRNAs were identified (hsa-miR-378e, hsa-miR-4781-3p, hsa-miR-5001-3p, hsa-miR-378b, hsa-miR-330-3p, hsa-miR-3127-3p, hsa-miR-5701, hsa-miR-4659a-3p, hsa-miR-26b-3p, hsa-miR-1468). Sixteen common significant pathways were identified from the pathway analysis, and their functions related to AD and other neurogenerative diseases were discussed. Next, seven common target genes were identified from the common significant pathways, including Cdc42, VEGFA, NTRK3, ESR1, SH3GL2, COX-2, and E2F1. In summary, the identified miRNAs and target genes could be potential biomarkers that would be beneficial towards therapeutic approaches in AD, and their roles in regulating the

expression, splicing, and post-translational modification of proteins that are related to AD pathology could be further investigated.

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