Exploring Biomarkers Related to Autophagy in Alzheimer's Disease Based on Pathway Crosstalk Analysis

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Research Article

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Posted Date: August 10th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-784797/v1>

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Exploring biomarkers related to autophagy in Alzheimer's disease based on pathway crosstalk analysis

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Abstract

The pathological mechanism of Alzheimer's disease (AD) involves multiple pathways, and the crosstalk between autophagy and other pathways plays an increasingly prominent role in AD. However, current methods are primarily based on single-gene analysis or a single signal pathway to find therapeutic targets for AD, which are somewhat limited. The aim of our study is to identify autophagy-related biomarkers in AD based on the crosstalk between autophagy and other pathways. The pathway analysis method (PAGI) was applied to find the feature mRNAs involved in the crosstalk between autophagy and many other AD-related pathways. Then, the weighted gene co-expression network analysis (WGCNA) was used to construct a co-expression module of feature mRNAs and differential lncRNAs. Finally, clinical information was used to screen the biomarkers related to the prognosis of AD in the co-expressed gene modules. The experiment finally identified 8 mRNAs and 2 lncRNAs (TLN1, ARRB1, FZD4, AKT1, JMJD7-PLA2G4B, STAT5A, SMAD7, ZNF274; AC113349.1, AC015878.2) as biomarkers of AD, and they all interact directly or indirectly with autophagy. In summary, we provide an effective method for extracting autophagy-related biomarkers based on pathway crosstalk in AD. This method enriches the therapeutic targets of AD and provides new insights into the molecular mechanism of autophagy in AD.

Keywords: Alzheimer's disease (AD); Autophagy; Biomarkers; Pathway crosstalk; Weighted gene co-expression network analysis (WGCNA); Pathway analysis method based on global influence (PAGI)

1 Introduction

Alzheimer's disease (AD) is one of the common forms of dementia, and its typical pathological features are the presence of amyloid (A β) deposition, hyperphosphorylated Tau protein aggregation, and neurofibrillary tangles in the brain [1]. Although the pathological relevance is known, the exact pathogenesis of AD is still poorly understood. Research in recent years has discovered and explained many signal pathways related to AD, among which the vital role of the autophagy pathway in AD is becoming more and more prominent [2]. Autophagy is a crucial regulator of the production of A β and Tau proteins, A β and Tau proteins can also induce autophagy to promote its clearance through the mTOR pathway or in an independent manner [3]. In addition, there are interactions between autophagy and other related signaling pathways, which provide new possibilities for exploring the pathogenesis and therapeutic targets of AD.

With the innovation of RNA sequencing technology and bioinformatics analysis, the identification of biomarkers and functional pathways in AD has developed rapidly [4]. In addition to mRNA, long non-coding RNA (lncRNA) and microRNA (miRNA) have also been found to be involved in the pathological progress of AD, including the induction of autophagy to promote the clearance of A β or Tau protein, the inhibition of neuroinflammation, and other biological processes [5][6]. Studying the molecular mechanism of these three RNAs (mRNA, lncRNA, and miRNA) and their interaction with autophagy will provide promising AD diagnosis and treatment [7][8]. Although these RNAs play a crucial role in AD, most of the current methods for excavating them are based on bioinformatics analysis and competition with endogenous RNA hypotheses, which may ignore their interconnection with autophagy or crosstalk between pathways. In excavating disease-causing genes, the above methods may lead to the failure to identify more meaningful biomarkers. Therefore, it is necessary to find new effective strategies.

With the accumulation of high-throughput genome-wide expression data, researchers can systematically study the functional relationship of single or several genes in diseases [9]. It is well known that genes do not function in isolation but work together within various metabolic, regulatory, and signaling pathways. Furthermore, increasing evidence shows that pathway-based methods are generally superior to gene-based counterparts [10]. Methods based on pathway analysis can explain complex biological processes and biological significance. AD is a multifactorial disease involving multiple cell signaling pathways, so crosstalk within and between pathways is inevitable. Because the number and combinations of transduceable signals are limited, crosstalk between pathways can create novel input/output combinations. Having more input/output combinations increases the possible ways that signaling information can flow within the cell, allowing more diverse phenotypes. Thus, genes generated by crosstalk play an essential role in the generation and development of disease [2][11][12]. Several recent techniques have used path topology information to identify dysfunctional paths. The PAGI algorithm is based on gene networks, looking for dysregulated pathways in diseases by considering the internal effects of pathways and crosstalk between pathways, which provides the possibility to explore AD biomarkers based on pathway crosstalk [13].

Studies have shown that lncRNA can directly mediate crosstalk in pathways by cooperating with coding genes that play an important role in diseases [14]. In addition, lncRNAs and mRNAs can compete with each other through miRNAs response elements to regulate AD progress [15]. Based on the interaction of autophagy with the RNA mentioned above and other signal pathways in AD, it is expected to be a new idea to explore the feature of autophagy-related biomarkers in AD and their pathogenic mechanisms in diseases from the perspective of pathway crosstalk.

In our research, we introduced a novel method to identify biomarkers related to autophagy in AD, which are involved in the crosstalk between autophagy and other related pathways and acted in synergy with various RNAs in the pathological biological process of AD. Firstly, the PAGI pathway analysis algorithm was applied to gene expression data to obtain pathways and crosstalk genes associated with AD. Secondly, the WGCNA algorithm was used to select candidate biomarkers with co-expression relationships, and genes in the same expression module may have similar biological functions [16]. Finally, the survival analysis was applied to candidate biomarkers, and biomarkers related to AD survival were extracted. Results showed that based on PAGI, more than 36 autophagy-related pathways dysregulated and crosstalk with each other in AD. Furthermore, 103 lncRNAs and 650 mRNAs related to autophagy with co-expression relationships

were identified by using WGCNA analysis. Next, 8 mRNAs and 2 lncRNAs were identified as autophagy-related biomarkers related to the prognosis of AD by survival analysis.

2 Materials and Methods

2.1 Data Source

The data used in this study comes from subjects of the Religious Order Study (ROS) or the Rush Memory and Aging Project (MAP), which are two prospective clinical-pathological cohort studies of aging and dementia. The two studies (collectively referred to as ROSMAP) share clinical and neuropathological standards, allowing joint data analysis [17][18].

The gene expression profile and clinical information needed in this research were obtained from the AMP-AD Knowledge Portal database (the raw count data can be obtained online at <https://www.synapse.org/#!Synapse:syn8691134>, the filtered raw count data can be obtained online at <https://www.synapse.org/#!Synapse:syn8456637>, and the clinical data was downloaded online at <https://www.synapse.org/#!Synapse:syn3191087>). According to clinical information, we screened 155 AD samples and 86 normal samples as the data for this study. We downloaded the autophagy gene file from the Human Autophagy Database (<http://www.autophagy.lu/>) for the annotation of autophagy genes on the pathway. The experimental flow chart of this paper is shown in Figure 1, and we will discuss each part in detail later.

Figure 1 framework of the experiment.

2.2 Methods

2.2.1 Data preprocessing and identification of differentially expressed lncRNAs

According to the human genome assembly GRCh38, the ensemble ID of the raw count data was switched to the gene symbol. According to the gene type annotation, the genes in the raw data were divided into 19,677 mRNAs and 14,259 lncRNAs. We only kept the highly expressed mRNA (obtained from filtered count data syn8456637, delete genes whose count is less than 1 CPM in at least 50% of the samples, and delete genes whose length and GC content are missing). Then, we performed TPM standardization and normalization on the filtered mRNA expression profile and used the SVA package to eliminate batch effects (sequencing batch of samples, 9 batches in total) in the standardized data.

The R package “DESeq2” was used to identify differentially expressed lncRNAs (DElncRNAs) [19]. Firstly, we used the DESeqDataSetFromMatrix function to convert the count matrix into a DESeqDataSet (dds) object. The formula of the design parameter of DESeqDataSetFromMatrix function is as follows: $\text{design} = \sim \text{batch} + \text{group}$, where batch represents the sequencing batch information of the sample (batch value is from 0 to 8), group is the grouping information of the sample (the samples were divided into diseased group and normal group). Then, we used the DESeq function to normalize (by calculating the size factor of each column of samples in the count matrix) dds and analyze the differential expression of lncRNAs in normal samples and diseased samples. The criteria for screening differentially expressed lncRNAs was that the p -value is less than 0.05, and a log2FC range of -0.5 to 0.5 was rejected.

2.2.2 Pathway analysis based on global influence (PAGI)

The PAGI algorithm is a pathway analysis method based on global influence, which identifies dysregulated pathways by considering both within-pathway effects and crosstalk between pathways. We used the PAGI algorithm to identify dysregulated pathways in AD. Principle of the PAGI algorithm:

Firstly, constructing the global gene-gene network. Based on the structural information and genetic relationship of each pathway in KEGG, the "iSubpathwayMiner" system is used to construct a global gene network that reflects the relationship between and within a pathway [20][21].

Secondly, calculating the global dysregulated score (GDS). A GDS was defined to measures the degree to which genes are affected by internal effects of pathways and crosstalk between pathways. The Random Walk with restart (RWR) algorithm captures global relationships within a network and can calculate the proximity of a node to a set of source nodes [22]. In the application process, the two-sample (diseased sample and normal sample) t-test was performed to evaluate the extent of differential expression (t-score). All genes represented in the gene expression profile were mapped to the global network as source nodes. To calculate GDS and reflect the global influence of the gene on the source nodes, the RWR algorithm was modified by combining the t-score and the global network topology. The revised algorithm formula is as follows:

$$p^{t+1} = (1 - r)Mp^t + rp^0 \quad (1)$$

where M is the column-normalized adjacency matrix of the global network graph G , $p^t = (p_1^t, p_2^t, \dots, p_n^t)'$ is the node vector at time t , and its i th element p_i^t represents the probability of

being at node i at time t , and n represents the number of all nodes (genes) in G . r is the restart probability, which controls the degree to which the random traverser returns to the source node in each iteration.

To start this algorithm, the initial probability $p^0 = (p_1^0, p_2^0, \dots, p_n^0)'$ is normalized to the unit vector $p_i^0 = |t - score|_i / \sum |t - score|_i$. The higher the p_i^0 of gene i , the greater the degree of disturbance to other genes. p^t can reach a stable state p^∞ after multiple iterations, it can be used to measure the GDS of genes. The GDS of gene i can be assigned by the normalized p_i^∞ as: $GDS_i = (p_i^\infty - \min(p^\infty)) / (\max(p^\infty) - \min(p^\infty))$. Through this method, the GDS of each gene in the global network can be obtained.

Thirdly, identify dysfunctional pathways. The gene list $L = \{g_1, g_2, g_3, \dots, g_n\}$ consists of all genes in the expression profile sorted according to $t_j^{1+GDS_j}$, t_j represents $|t\text{-score}|$ of gene j , and GDS_j represents GDS of gene j . The dysfunction score of P path is calculated based on the information of its gene mapping in the L path, and is calculated by cumulative distribution functions (CDFs). The CDFs of Inp (genes in P) and $Notp$ (genes in L , not in P) are used to evaluate the fraction of genes in P weighted by their correlation ($t_j^{1+GDS_j}$), and the fraction of genes not in P present up to a given position i in L . The formula is as follows:

$$CDF_{Inp}(i) = \sum_{\substack{g_i \in P \\ j \leq i}} \frac{t_j^{1+GDS_j}}{N_R} \quad (2)$$

and

$$CDF_{Notp}(i) = \sum_{\substack{g_i \notin P \\ j \leq i}} \frac{1}{N_{NotP}} \quad (3)$$

where $N_R = \sum_{g_i \in P} t_j^{1+GDS_j}$; N_{NotP} represents the number of genes in L not in P . With the position i walking down the list L , the formula for calculating the dysfunction score of path P is as follows:

$$S_p = \max_{i \in L} \{CDF_{Inp}(i) - CDF_{Notp}(i)\} \quad (4)$$

Finally, the significantly dysregulated pathways in AD obtained according to the false discovery rate ($FDR < 0.01$) were used as candidate pathways. According to the autophagy gene annotation results of each candidate pathway and the literature review, the pathways that interacts with autophagy were selected as the feature pathways, and the mRNAs in the feature pathway were used as the feature mRNAs.

2.2.3 Construction of co-expression gene modules based on WGCNA analysis

The R package “WGCNA” was used to identify DElncRNAs and feature mRNAs that have a co-expression relationship. Therefore, the feature mRNAs and DElncRNAs serve as the input of WGCNA. Firstly, the absolute value of the Pearson correlation coefficient between genes was used to construct the correlation matrix ($S_{i,j}$, i and j indicate the i^{th} and j^{th} gene). To fit with the scale-free network, the threshold of the fitting index was set to 0.85 ($R^2 > 0.85$). When the fitting index reached 0.85, the β value (soft threshold) that maximized the average connectivity was selected to perform a power-law operation to convert the correlation matrix into an adjacency

matrix ($a_{i,j}$, $a_{i,j} = |S_{i,j}|^\beta$) [23]. Then, the pickSoftThreshold function was used to calculate the corresponding R^2 and average connectivity for different β values (the β values were set between 1 and 20). Next, we transformed the adjacency matrix into a topological overlap matrix (TOM). To classify genes with co-expression relationships into the same modules, we used the DynamicTreeCut algorithm to construct the average linkage hierarchical clustering dendrogram [24]. Finally, we calculated the module eigengenes, hierarchically clustered the modules, and merged similar modules [25].

2.2.4 KEGG enrichment analysis

In order to determine the function of mRNA-lncRNA co-expression gene modules obtained by WGCNA, [26] we used KEGG to perform enrichment analysis on each gene module. The filter condition of the enrichment pathway is that the p -value and the adjusted p -value (q -value) are both less than 0.05. Finally, according to the results of KEGG enrichment, the gene modules needed in this study were obtained, and the genes in the modules were used as AD candidate biomarkers.

2.2.5 Cox proportional hazards regression analysis

In order to obtain biomarkers related to the prognosis of AD, we used the R package “survival” to perform univariate and multivariate Cox regression analysis on the AD candidate biomarkers (genes in the turquoise module). Firstly, univariate Cox regression analysis was used to identify genes that are significantly related to the overall survival (OS) of patients with AD. The criterion for screening genes related to the OS of patients is that the p -value is less than 0.05. Then, we performed multivariate Cox regression analysis on the genes screened by univariate Cox regression analysis and constructed a prognostic-related model of AD. We used the stepwise selection of variables based on the lowest Akaike information criterion (AIC) to optimize the prognostic-related model of AD. And then, we calculated the risk score of each patient, which can be used to divide patients into a high-risk group and a low-risk group. The formula is as follows:

$$Risk\ Score = \sum_{k=1}^n coef(k) * x(k) \quad (5)$$

where coef(k) represent the Cox regression coefficient; x(k) represent the expressive value of each genes, n represents the number of genes.

Finally, the Kaplan-Meier (KM) curve and forest plot of multivariate Cox regression analysis were generated by R package “survival”. The KM curve was used to judge whether it exists a difference in survival between the high-risk group and the low-risk group. The forest plot was used to judge whether the risk score is an independent prognostic factor affecting OS. Then Time-dependent receiver operating characteristic (ROC) curve and the multi-index ROC curve were used to evaluate the accuracy of the prognostic-related model by R package “timeROC”.

3 Results

3.1 Data preprocessing results and DElncRNAs

After quality control and TPM standardization of mRNAs data, 13,556 mRNAs were obtained. Then the standardized data were adjusted in batches (9 batches in total) through the combat function of the SVA package, and the adjusted data were reserved as the input of the PAGI

algorithm.

In order to identify differentially expressed lncRNAs, we used the DESeq2 package to standardize the gene expression profile of lncRNAs and analyze differential expression. The lncRNAs with a p -value less than 0.05 and $|\log_2\text{FC}| > 0.5$ were extracted as statistically significant differential genes. Finally, 180 differentially expressed lncRNAs were obtained, of which 75 were up-regulated, and 105 were down-regulated. The top 30 differential genes (15 down, 15 up) were shown in the supplementary material (Online Resource 1).

3.2 Exploring autophagy-related pathways based on PAGI

In order to explore autophagy-related biomarkers in AD from the perspective of pathway crosstalk, we applied the mRNA expression profile of AD to the PAGI algorithm. According to the FDR value less than 0.01, a total of 94 pathways related to AD were screened out. Then, by consulting the literature and annotating the autophagy genes on each pathway, 36 autophagy-related pathways and crosstalk genes required for this study were screened out. A total of 1436 crosstalk genes (feature mRNAs) are included in the 36 pathways, which were one of the input data of the WGCNA algorithm. 10 out of the 36 pathways were confirmed to be related to autophagy in AD and were shown in Table 1, and information about the remaining pathways was shown in the supplementary materials (Online Resource 2). The 10 autophagy-related pathways (in Table 1) and their crosstalk genes were shown in Online Resource 3.

Table 1 10 pathways related to autophagy identified by the PAGI

Pathway Name	Pathway ID	Size	Pathway Score	Gene\%	Signal	Autophagy
Protein processing in endoplasmic reticulum	hsa04141	152	0.44823	0.308	0.359	27
Apoptosis	hsa04210	70	0.44783	0.152	0.231	17
Alzheimer's disease	hsa05010	132	0.42487	0.37	0.357	13
mTOR signaling pathway	hsa04150	46	0.48142	0.15	0.297	16
Insulin signaling pathway	hsa04910	119	0.51274	0.0637	0.262	16
Neurotrophin signaling pathway	hsa04722	114	0.54769	0.144	0.326	15
Type II diabetes mellitus	hsa04930	32	0.5506	0.0875	0.314	6
Endocytosis	hsa04144	174	0.42422	0.192	0.268	10
Wnt signaling pathway	hsa04310	123	0.4932	0.0972	0.252	5
Calcium signaling	hsa04020	133	0.57959	0.167	0.367	4

In Table 1, the first column is the pathway name based on PAGI algorithm screening, the second column is the pathway ID, and the third column 'Size' indicates the number of genes contained in the pathway, the fourth column 'Pathway Score' is the score after the pathway passes the PAGI algorithm, the fifth column 'Gene\%' is the percentage in the gene list before running enrichment peak, and the sixth column 'Signal' indicates the intensity of the enrichment signal, the seventh column is the number of autophagy genes included in the pathway.

It can be seen from Table 1 that the scores of these 10 pathways were all higher than 0.4, and the pathway score of Alzheimer's disease (hsa05010) pathway was 0.42487, which directly proved the effectiveness of the pathway selection through PAGI. Furthermore, the pathway score of the

mTOR signaling pathway (hsa04150) was 0.48142, which is currently one of the most promising targets for autophagy-related AD therapy [3]. Moreover, autophagy genes (232 in total) in the selected pathways all account for a high proportion, which provided a basis for extracting autophagy-related crosstalk genes.

3.3 WGCNA results

In order to obtain feature mRNAs (autophagy-related crosstalk genes) and lncRNAs with a co-expression relationship, we used the WGCNA algorithm to construct a co-expression module for it. Firstly, we obtained the count matrix composed of differentially expressed lncRNAs and autophagy-related crosstalk genes. Then, we used the `DESeqDataSetFromMatrix` function and `varianceStabilizingTransformation` function in DESeq2 to normalize the matrix. To construct the scale-free network, $\beta = 6$ ($R^2 = 0.89$) was set as the soft-thresholding parameter (Figure 2a). Here, the number of genes in each module was defined as at least 50. Next, 7 modules were identified based on DynamicTreeCut algorithm. Finally, based on the module eigengenes, the height of cut was 0.25 to merge similar modules (Figure 2b). And 6 co-expression modules were identified (Figure 2c), the 6 modules were shown in Table 2. From Table 2, we can see that the number of autophagy genes in the turquoise module is the largest among the 6 modules.

KEGG pathway enrichment analysis was performed on the genes of each module, and the turquoise module was finally determined as the research object according to the analysis results. The turquoise module includes 103 lncRNAs and 650 mRNAs. 101 of the 650 mRNAs are located in the Alzheimer's disease pathway (369 genes), and 45 are autophagy genes (232 genes), as shown in Figure 3. Pathway enrichment analysis of gene modules was implemented by David (<https://david.ncifcrf.gov/>) [27]. The pathway enrichment results of the turquoise module were shown in Figure 4. David obtained the first 20 pathways with a p -value less than 0.05, including Alzheimer's disease pathway, Pathways of neurodegeneration-multiple diseases, and PI3K-Akt signaling pathway [28]. The discovery of the above pathways directly proved the significance of the turquoise module as a research object. In addition, MAPK signaling pathway [29], Protein processing in endoplasmic reticulum [30], Calcium signaling pathway [31], Focal adhesion [32], Insulin signaling pathway [33], Neurotrophin signaling pathway [34], Regulation of actin cytoskeleton [35], Endocytosis [36] and Ras signaling pathway [37], these 9 pathways are related to AD and have crosstalk with autophagy which was supported by kinds of literature. Through the analysis of the above pathways, 15 out of the 20 pathways are related to AD, and 13 are related to autophagy. The above results proved the feasibility of the genes in the turquoise module as candidate biomarkers related to autophagy in AD.

Figure 2 Network construction of co-expressed genes. (a) Analysis of the scale independence and mean connectivity for various soft-threshold powers. (b) The cluster dendrogram of module eigengenes. (c) Dendrogram clustered based on a dissimilarity measure (1-TOM).

Table 2 6 co-expressed gene modules obtained by WGCNA

Module	blue	green	grey	red	turquoise	yellow
Number of lncRNAs	16	3	53	4	104	3
Number of mRNAs	470	102	27	82	650	102
Number of autophagy genes	38	13	1	4	45	8

In Table 2, the first row is the name of the 6 co-expression modules, and each column represents the number of lncRNA, mRNA, and autophagy genes contained in the module.

Figure 3 Venn diagram of crosstalk genes, autophagy genes, and genes of Alzheimer's disease pathway. In Figure 3, blue represents 369 genes in the Alzheimer's disease pathway, yellow represents 650 characteristic mRNAs in the module, and the green represents 232 autophagy genes, and the overlapping portions represent the same genes on the two pathways.

From Figure 3, the crosstalk genes in the turquoise module overlap more with the genes in autophagy and AD, which not only indicates that the co-expression module we selected has a strong correlation with AD and autophagy but also proves that our idea of exploring autophagy-related biomarkers in AD based on pathway crosstalk is feasible.

Figure 4 The results of pathway enrichment. Figure 4 shows the pathway information obtained after pathway enrichment analysis of genes in the turquoise module. The horizontal axis is the number of genes in the pathway, and the vertical axis is the pathway list. Red to blue indicates the q -value (adjusted p -value).

3.4 Establishment of prognostic risk model of biomarkers

The clinical data downloaded from the AMP-AD database were sorted and screened. Then the survival time and clinical characteristics (braaksc: braak stage, ceradsc: assessment of neuritic plaques, dcfdx_lv: clinical cognitive diagnosis summary at last visit) of 82 patients were obtained (shown in the Online Resource 4). Based on the results of WGCNA and KEGG, we selected the genes in the turquoise module (104 lncRNAs and 650 mRNAs) as the input for univariate Cox regression analysis. According to the *p*-value (mRNA with a *p*-value less than 0.02, lncRNA with a *p*-value less than 0.05), we screened 23 genes (21 mRNAs and 2 lncRNAs) that were significantly related to the prognosis of AD. Subsequently, we performed multivariate COX analysis on 23 prognostic-related genes obtained from univariate Cox regression analysis. Afterward, according to the lowest AIC value, the prognostic risk model of 8-mRNA and 2-lncRNA (TLN1, ARRB1, FZD4, AKT1, JMJD7-PLA2G4B, STAT5A, SMAD7, ZNF274; AC113349.1, AC015878.2) was constructed.

A total of 82 patients with AD were divided into high-risk (N=41) and low-risk (N=41) group according to the median risk score. It can be seen from the KM curve that the OS rate of high-risk patients was significantly lower compared with low-risk patients within five years (Figure 5a). Multivariate Cox regression analysis revealed that the risk score of prognostic risk model (HR=1.658, *p*<0.001) was an independent prognostic factor affecting the OS of patients with AD (Figure 5b). A time-dependent ROC curve was generated, and the area under the ROC curve (AUC) was calculated to assess the predictive ability of the model. The 1-, 2-, and 3-year AUCs were 0.709, 0.761, and 0.713 (Figure 6a). The Multi-index ROC curve showed that the AUC value of the risk score based on the prognostic risk model was greater than 0.7 (AUC=0.709), which was more significant than other clinical prognostic indicators, such as braaksc, ceradsc and dcfdx_lv (Figure 6b).

Figure 5 Cox proportional hazard regression analysis of mRNAs and lncRNAs. (a) Kaplan–Meier curve to compare OS of high-risk with low-risk samples (*p*<0.001); (b) Forest plot of multivariate independent prognostic analysis. The square on the horizontal line shows the hazard ratio (HR), and the horizontal line represents the 95% confidence interval.

Figure 6 Receiver operating characteristic curves of the risk model. (a) Time-dependent ROC curve analysis of the risk score model for predicting 1-, 2-, 3-year OS. (b) Multi-index ROC curve. The curve area is used to assess the accuracy of the risk model (model AUC=0.709).

3.5 Prediction results of miRNA and target genes

In order to verify and enrich the biomarkers related to autophagy, we performed miRNA and target genes prediction on 10 genes obtained from the prognostic analysis. 200 miRNAs (shown in the Online Resource 5) related to prognostic genes were obtained from the MiRcode database [38]. Many miRNAs in the miRNA results have been proven in the literature to play an important role in AD. For example, [39] microRNA miR-212 interacts with PDCD4 to induce neurotoxicity in AD through the PI3K/AKT signaling pathway. miR-219-5p inhibits Tau phosphorylation by targeting TTBK1 in AD [40]. By analyzing the miRNAs related to biomarkers, it can be seen that the 10 biomarkers finally obtained in the experiment are potential targets for exploring the pathogenesis of AD.

Then, we used miRTarBase, miRDB, and TargetScan databases to predict the target genes of the obtained miRNA, and the target genes supported by the three databases are reserved as target genes related to prognostic biomarkers. 200 miRNAs were put into three databases for predicting target genes, and obtained 1269 target genes. By performing KEGG pathway enrichment analysis on target genes, the first 20 pathways with a p -value less than 0.05 were selected, as shown in Figure 7. From Figure 7, we can see that the MAPK signaling pathway, Neurotrophic signaling pathway, Autophagy pathway, and many other pathways related to AD, which also proved the effectiveness of the biomarkers we found.

Figure 7 Pathway enrichment results of target genes related to prognostic biomarkers. In Figure 7, the horizontal axis is the number of genes in the pathway, the vertical axis is the pathway list, and red to blue indicates the size of the q -value (adjusted p -value).

4 Discussion

4.1 Extract autophagy-related paths based on pathway crosstalk

A variety of signal pathways in AD are involved in the generation and development of diseases, and crosstalk is inevitable between these signal pathways. Crosstalk between pathways provides a novel combination of nonlinear response dysfunction. Furthermore, genes generated by crosstalk play an essential role in the generation and development of the disease. As the primary regulator of the production and clearance of A β and Tau protein in AD, it has recently been discovered that the effect of autophagy on AD is related to its interaction with various signaling pathways and known AD biomarkers [2][5]. Therefore, exploring the autophagy-related biomarkers in AD from the perspective of pathway crosstalk has become the direction of our exploration.

Due to the internal effects of pathways and crosstalk between pathways, dysregulated genes may cause changes in the expression characteristics of nearby genes, which may eventually lead to dysfunction of the pathways of nearby genes. The dysregulated genes and pathways are important factors affecting diseases. Pagi algorithm can identify dysfunctional pathways based on gene networks by considering the overall impact of crosstalk inside and outside the pathway. [13] Through the Pagi algorithm, we obtained a total of 94 pathways related to AD. Then by consulting the literature and annotating the autophagy genes of each pathway, 36 pathways and crosstalk genes on the pathways needed for this study were screened out. Among these 36 pathways, the hsa05010 (Alzheimer's disease) pathway is included, which directly proves the

accuracy of the PAGA algorithm. The remaining 35 pathways all interact with autophagy pathways. For example, [41] hsa04150 (mTOR signaling pathway) pathway is a key regulator of autophagy and can increase Tau levels and phosphorylation. hsa04910 (Insulin signaling pathway), hsa04141 (Protein processing in endoplasmic reticulum), hsa04722 (Neurotrophin signaling pathway), hsa04062 (Chemokine signaling pathway), hsa04020 (Calcium signaling pathway), hsa04310 (Wnt signaling pathway), hsa04930 (Type II diabetes mellitus) and other pathways that interact with autophagy are supported by the literature [2]. The above results provide a basis for screening crosstalk genes related to autophagy in AD.

4.2 Molecular biology analysis of autophagy-related biomarkers

In this study, we finally identified 10 autophagy-related biomarkers (TLN1, ARRB1, FZD4, AKT1, JMJD7-PLA2G4B, STAT5A, SMAD7, ZNF274; AC113349.1, AC015878.2) in AD. AKT1 is one of the three subtypes (AKT1, AKT2, and AKT3) closely related to AKT kinase and is involved in biological processes such as metabolism, cell survival, and growth [42]. Survival factors can inhibit nerve cell apoptosis by activating AKT1 in a unique way of transcription. Abnormal neuronal death is one of the main causes of neurodegenerative diseases, so proper promotion of AKT1 activation may play a protective effect on AD [43]. Experiments have shown that reactive oxygen species (ROS) signals reduce Akt1-mTOR signal transduction by mediating Akt1 oxidative modification and ultimately lead to synaptic dysfunction in AD. Therefore, promoting Akt1-mTOR signaling in synapses may provide new targets for AD treatment [44]. Studies have shown that the activation of AKT1 can inhibit GSK3 and promote mTORC1, which is conducive to the upstream of A β protein and normal Tau protein phosphorylation (Tau protein is the main component of nerve fiber entanglement in AD) [45]. ARRB1 (Arrestin Beta 1) is an adaptor protein that has the function of regulating G protein-coupled receptor (GPCR) signal transduction and transport [46]. In AD, GPCR participates in Tau phosphorylation through various downstream kinases (such as GSK-3 β , CDK-5, and ERK signaling cascade). It is known from the literature that ARRB1 can participate in Tau phosphorylation by regulating GPCR signals, thereby causing dementia or affecting the development of AD [47]. The reduction of ARRB1 can inhibit the expression of LC3B, Atg7, and Beclin-1 (LC3B, Atg7, and Beclin-1 are the key to autophagy activation), while the knockout of ARRB1 can eliminate the autophagy process. In addition, normal autophagy activation helps to eliminate A β accumulation in early AD and inhibits A β -induced neurodegeneration. The above results show that ARRB1 can play a neuroprotective effect on AD by activating autophagy [48][49][50]. FZD4 (Frizzled 4 receptor) is a member of the frizzled gene family, which encodes the seven-transmembrane domain (7-TM) protein (7-TM is the symbol of GPCR protein) [51]. Recent studies have shown that calcium disorders play an important role in the pathology and physiology of AD (regulating many signal pathways related to cell survival, transcription, and apoptosis), and the secondary messengers produced by GPCRs can regulate intracellular calcium homeostasis [52]. The receptor of FZD4 can be coupled with the β -catenin (CTNNB1) signal transduction pathway, which leads to the inhibition of GSK-3 kinase, nuclear accumulation of β -catenin, and the activation of Wnt target genes [53]. Studies have shown that the mTOR signaling pathway can inhibit the activation of GSK3 β and increase the expression of β -catenin by improving the expression level of Wnt3a, which helps to improve the pathology of AD [54]. JMJD7-PLA2G4B represents naturally-occurring readthrough transcription between the neighboring jumonji domain containing 7 (JMJD7) and phospholipase A2, group IVB

(cytosolic) (PLA2G4B) genes. JMJD7-PLA2G4B encodes fusion proteins that share an amino acid sequence with each individual gene product (including a partial JmjC domain and downstream C2 and phospholipase A2 domains). Phospholipase A2 plays a key role in the pathogenesis of A β -induced, and experiments have shown that reducing phospholipase A2 can reduce cognitive deficits in Alzheimer's disease mouse models [55][56]. The results from GeneCards (<https://www.genecards.org/>) show that JMJD7-PLA2G4B is involved in the VEGF signaling pathway [57], the GnRH signaling pathway [58] and the vascular smooth muscle contraction pathway [59]. These pathways are related to AD, which are supported by kinds of literature.

The protein encoded by SMAD7 is a nuclear protein that binds the E3 ubiquitin ligase SMURF2. The combined complex interacts with the TGF- β 1 receptor (TGFBR1), leading to the degradation of the encoded protein and TGFBR1 [60]. TGF- β 1 can reduce synaptic dysfunction and memory loss in AD mouse models by activating PI3K/Akt/Wnt/ β -catenin signaling [61]. In addition, studies have found that TGF- β 1 is related to A β accumulation and microglia clearing A β in AD mouse models, which shows the harmful and beneficial effects of TGF- β 1 on AD [62]. Therefore SMAD7 can become a potential target of AD pathology by targeting TGF- β 1. The protein encoded by STAT5A (Signal transducer and activator of transcription 5A) is a member of the STAT transcription factor family. STAT5A plays a key role in the response of various cytokines (such as IL-2, IL-3, IL-7) and hormones, so it is essential for regulating the body's immune and nervous system functions [63]. STAT5A has been shown to have disturbances in AD [64], and it is related to common DNA methylation changes in AD brain regions. Literature [65] pointed out that STAT5A can inhibit mitochondrial gene expression by directly binding to mitochondrial DNA (mtDNA). Mitochondrial dysfunction has become an established sign of neurodegeneration [66]. TLN1 (Talin 1) encodes a cytoskeletal protein that is concentrated in areas of cell-substratum and cell-cell contacts. TLN1 is the key mediator for the connection between the integrin adhesion receptor and the actin cytoskeleton, and TLN1 binds to F-actin to target the protein to focal adhesion [67]. The early pathogenesis of AD is synaptic dysfunction driven by A β , and the actin cytoskeleton is a key element to maintain the synaptic structure and synaptic plasticity [68]. The literature [69] shows that the adhesion of focal adhesions to A β fibers can mediate A β signaling and cell death in AD. From the above kinds of literature, we can know that TLN1 plays an important role in the pathogenesis of AD. ZNF274 (Zinc Finger Protein 274) encodes zinc finger protein, which can interact with neurotrophin receptor p75 (NTR) and participate in programmed cell death [70]. p75 (NTR) plays an important role in regulating A β metabolism in the brain, and its expression is changed in the brain of AD patients [71]. Therefore, ZNF274 can affect the survival of neuronal cells and the metabolism of A β by interacting with p75 (NTR), thereby participating in the pathological progress of AD.

The promoter and enhancer in lncRNA are the binding sites of transcription factor (TF) and can locally regulate the lncRNA expression [72][73]. According to the results of lncRNA AC113349.1 and AC015878.2 in GeneCards, their promoters or enhancers share many common transcription factors (such as ZBTB20, USF1, STAT3). Although there is no literature to prove the role of these two lncRNAs in AD, their common transcription factors STAT3 and USF1 play an important role in AD [74][75]. Therefore, in exploring the pathogenesis of AD, two lncRNAs have important potential.

Our survival analysis results showed that the overall predictive value and risk value of these

10 genes are high, and the KEGG pathway enrichment analysis results of their target genes shown that all genes are involved in AD-related pathways, especially in the autophagy process. In addition, some miRNAs related to these 10 genes have also been confirmed to be related to the pathogenesis of AD. All the above evidence supported these 10 genes as candidate targets for AD diagnosis and treatment.

5 Conclusion

AD is a multi-factor disease involving multiple signaling pathways, and current methods for exploring new therapeutic targets need to be further enriched. In addition, considering the core role of autophagy in AD and its interaction with other signaling pathways, this article provides a new method for mining autophagy-related biomarkers in AD. At present, the molecular mechanism of autophagy in AD has not been explored from the perspective of pathway crosstalk. This article used the DESeq2 package to screen out differentially expressed lncRNAs. The PAGI algorithm was used to explore the pathways related to AD, and the crosstalk pathways related to autophagy were screened through the number of autophagy genes in the pathways and literature. The mRNAs on the crosstalk pathways related to autophagy were reserved as feature mRNAs. Then, using the WGCNA algorithm to extract the co-expression module of feature mRNAs and lncRNAs. Next, we applied clinical data to the genes in the co-expression module to obtain prognostic-related biomarkers. Based on survival time and literature, the role of the extracted biomarkers in AD was confirmed. Finally, 8 mRNAs and 2 lncRNAs (TLN1, ARRB1, FZD4, AKT1, JMJD7-PLA2G4B, STAT5A, SMAD7, ZNF274; AC113349.1, AC015878.2) were identified as AD biomarkers, and the effectiveness of the new method was verified. In addition, miRNAs related to biomarkers further enrich AD treatment targets.

ETHICAL STATEMENT

Ethics Approval and Consent to Participate

This article does not contain any studies with human participants or animals performed by any of the authors; therefore, the ethical approval and consent to participate are not applicable.

Consent for publication

The authors have consented to publication of this article.

Availability of data and materials

The gene expression profile and clinical information needed in this research were obtained from the AMP-AD Knowledge Portal database (the raw count data can be obtained online at <https://www.synapse.org/#!Synapse:syn8691134>, the filtered raw count data can be obtained online at <https://www.synapse.org/#!Synapse:syn8456637>, and the clinical data was downloaded online at <https://www.synapse.org/#!Synapse:syn3191087>).

Competing interests

The authors declare that they have no competing interests.

Funding

The Natural Science Foundation of Shanghai (No.18ZR1417200) and the National Natural Science Foundation of China (No.61803257).

Authors' contributions

Conception and design of the research: Fang Qian and Wei Kong. Acquisition, analysis, and interpretation of data: Fang Qian, Shuaiqun Wang, and Wei Kong. Statistical analysis: Fang Qian. Drafting the manuscript: Fang Qian. Manuscript revision for important intellectual content: Wei Kong. All authors have read and approved the manuscript.

Acknowledgements

This work was supported by the Natural Science Foundation of Shanghai (No. 18ZR1417200) and the National Natural Science Foundation of China (No. 61803257).

We thank the patients and their families for their selfless donation to further understanding Alzheimer's disease. The results published here are in whole or in part based on data obtained from the AD Knowledge Portal (<https://adknowledgeportal.org>). Additional phenotypic data can be requested at www.radc.rush.edu.

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