Proteomics reveals ribosome associated proteins as potential biomarkers of Alzheimer's disease

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Abstract

Accumulated evidences confirmed that amyloid-β (Aβ) deposition, tau phosphorylation, acetylcholine decrease, etc, all contributed to the pathogenesis of Alzheimer’s disease (AD). However, the underlying mechanism remains unclear, and novel AD biomarkers are urgently needed to be discovered. Herein, APP/PS1 double transgenic mice (AD mice) were employed, and the differentially expressed (DE) proteins of mice hippocampus were identified and analyzed by 4D label Free quantitative proteomics technology and parallel reaction monitoring (PRM). The results indicated that the hippocampus of AD mice showed significant Aβ deposition. Compared to that of wild type mice, 29 proteins were up-regulated and 25 proteins were down-regulated in the AD mice group. Gene Ontology (GO) enrichment analysis of BP showed that DE proteins were mainly enriched in Ribosomal large subunit biogenesis. MF results showed that DE proteins were mainly enriched in 5.8S rRNA binding and Structural constituent of ribosome. CC results showed that DE proteins were mainly enriched in Polysomal ribosome, Cytosolic large ribosomal subunit, Cytosolic ribosome, Large ribosomal subunit, etc. KEGG results showed that DE proteins were mainly enriched in the Ribosome signaling pathway. The main target proteins were Rpl18, Rpl17, Rpl19, Rpl24, Rpl35, and Rpl6, which all showed significant differentially expression compared with the wild type mice. The PRM verification results were consistent with the results of 4D Label-free quantitative proteomics. Taken together, our findings revealed that Rpl18, Rpl17, Rpl19, Rpl24, Rpl35 and Rpl6 as AD biomarkers may be the potential targets for drugs design.

1 Introduction

China has the most significant number of dementia patients in the world. With the advent of population aging in China, dementia has brought a heavy social and economic burden to public health in China and even the world [1]. As the most common type of dementia, Alzheimer's disease (AD) is a neurodegenerative disease characterized by progressive cognitive dysfunction. Epidemiological surveys showed that AD had become the third most important cause of health in the elderly after Cardiac-cerebrovascular diseases and tumors, and the mortality rate is increasing yearly [2]. The pathogenesis of AD remain unclear, so it is imperative to study the complex pathogenesis of AD. In vitro cell models and animal models are essential tools for studying the pathogenesis of AD. Due to the short experimental period, easy culture, infinite passage, and economic efficiency, some scholars have used cell models in AD research. Primary cells provide a model of outgrowth that closely corresponds to neurons in situ, but the need to continually prepare new cultures and the potential for variability, especially limitations such as the inability to simulate real microenvironments, make it unlikely they will be widely used for screening [3]. Presently, the pathogenesis and therapy of AD disease were mainly conducted by kinds of animal models, and natural aging, transgenic, and physical and chemical methods were all selected in basic research.

The APP/PS1 double transgenic mice were developed by gene targeting technology and showed severe learning and memory decline, cognitive dysfunction, neuronal degeneration, and other pathological characteristics at six months [4–6]. The Aβ and senile plaques (SP) of AD pathological features are
primarily studied in this mouse; the extracellular deposition of Aβ to form senile plaques is the characteristic pathological change of AD, and inhibition of Aβ production and promotion of Aβ clearance are the main strategies to prevent and control AD [7]. The hippocampus is part of the limbic system and undergoes functional and structural changes such as long-term potentiation and synaptic remodeling that are critical in learning and memory [8–9], the hippocampus is the first site eroded by AD pathological changes, and it is affected by Aβ and neuronal tangles in the early stage of AD and mild cognitive impairment [10]. Based on this, the 10-month-old APP/PS1 double transgenic mice were selected as the Model for the study of Aβ amyloid deposition, 4D Label-Free quantitative proteomics and targeted proteomics were used as research methods, and bioinformatics analysis to screen the potential pathogenesis of AD, to provide a theoretical basis for AD experimental and clinical research.

2 Materials and Methods

2.1 Experimental animals

Nanjing Junke Bioengineering Co., LTD. (SCXK (Jiang Su province) 2021-0013) provided 12 male APP/PS1 transgenic mice weighing (26.78 ± 1.11) g, aged 10 months, and 12 wild-type C57 mice weighing (25.60 ± 1.27) g, aged ten months, respectively. The breeding material and sterile water were fed freely at room temperature with alternating light and dark (12 h-12 h). After adaptive feeding for one week, the experiment was conducted and all experimental protocols were approved by the Animal Experiment Ethics Committee of Changchun University of Chinese Medicine. The animal experiment protocol of the project conforms to the principles of animal protection, animal welfare, and ethics. It complies with the relevant regulations of the National Ethical Committee for Laboratory Animal Welfare.

2.2 Experimental reagents and instruments

DL-Dithiothreitol (DTT), iodoacetamide (IAM), tetraethylammonium bromide (TEAB), and Urea (8 M: after reconstitution with 16 mL high purity water) were purchased from Sigma-Aldrich Co., Ltd. (cat. Nos. D9779, A3221, 426288 and U4883, respectively). BCA Protein Assay Kit and Sodium pyruvate (98%, Reagent grade) were purchased from Beyotime Biotechnology, Inc. (cat. Nos. P0012 and ST1661-100g, respectively). Trypsin (Trypsin Gold, Mass Spectrometry Grade) was purchased from Promega Technology Co., Ltd. (cat. Nos. V5280). QE HF-X mass spectrometer (Thermo Scientific). Scientific EASY nLC1200 chromatography instrument (Thermo Scientific).

2.3 Immunofluorescence staining of Aβ

Brain tissue after dehydration by gradient alcohol was embedded in paraffin and cut into 4 µm sections. Sections were fixed by 4% paraformaldehyde, and incubated anti-Aβ primary antibody at 4°C overnight. Then, sections were washed and incubated the secondary antibodies for 2 h at room temperature. Aβ expression in hippocampal region was observed under a fluorescence microscope.

2.4 Hippocampal tissue extraction
APP/PS1 double transgenic mice and wild-type C57BL6 mice were randomly selected by the random number table method, isoflurane was used for anesthesia. The concentration of induction anesthesia was 3% and the concentration of maintenance anesthesia was 2%. After exposure and free heart, the left ventricular insertion perfusion needle is fixed, with frozen sterile 0.9% sodium chloride solution (4°C) with left ventricle perfusion, cut auricula dextra, Wait until the liver and lungs turn white and clear fluid flows from the atrium dextrum, The brain was decapitated. The hippocampus was isolated and frozen in a -80°C refrigerator.

2.5 Protein extraction and Trypsin digestion

Samples from −80 °C was added into a pre-cooled mortar, liquid nitrogen was added and ground into powder. Lysis buffer (8 M Urea, 1% protease inhibitor cocktail) was added into the powder, and sample was given sonication three times on ice using a high-intensity ultrasonic processor. Sample was centrifugated at 12000 g for 10 min at 4 °C, the supernatant was collected and the protein concentration was determined with a BCA Protein Assay Kit. For digestion, the protein solution was reduced with 5 mM dithiothreitol for 30 min at 56 °C and alkylated with 11 mM iodoacetamide for 15 min at room temperature in darkness. Then, sample was diluted by 100 mM TEAB to urea concentration less than 2 M. Trypsin was added for digestion and the sample were finally desalted by the C18 SPE column.

2.6 4D Mass Spectrometer

Peptides after digestion was dissolved in solvent A (0.1% formic acid and 2% acetonitrile in water), and separated by solvent B (6%-23% for 68 min, 23%-32% for 14 min, and 80% for 4 min) at a constant flow rate of 500 nL/min on a nanoElute UHPLC system. Peptides after separation was injected into a NSI ion source for ionization. The ion source voltage applied was 2.30 kV. Orbitrap Exploris™ 480 mass spectrometry (Primary mass spectrometry: scan range: 400–1200 m/z, scan resolution: 60,000; The secondary mass spectrometry: Scanning range: 110 m/z, scanning resolution: 15000); Data acquisition mode: data-dependent scan program; Automatic gain control (AGC): 100%, signal threshold: 5E4 ions/s, maximum injection time: Auto, dynamic exclusion time: 20 s.

2.7 Quantification of selected proteins via parallel reaction monitoring (RPM)

Chose twenty target proteins from different protein groups for parallel reaction monitoring (PRM) verification. Use the methods described previously to obtain the mobile phase composition, electrospray voltage, Orbitrap resolution, and mass spectrometry procedure. Run mobile phase at a constant flow rate of 1000 L/min in gradient mode as below: solvent A (0.1% formic acid in 2% acetonitrile); an increase in solvent B (0.1% formic acid in 90% acetonitrile) from 8–30% over 16 min, 30–40% in 6 min, 40–80% in 4 min, and then the flow was kept at 80% for the last 4 min. The flow rate is kept at 500 L/min.

After isolation, the peptides are injected into the NSI ion source for ionization, followed by Q ExactiveTM Plus mass spectrometry for analysis. The operation is as follows: primary mass spectrometry scan range: 400–1000 m/z, resolution: 70,000, automatic gain control (AGC): 3E6, maximum ion implantation
time (MIT): 50 ms. Secondary mass spectrometry Orbitrap scan resolution: 17,500. AGC: 1E5, MIT: 160 ms. The peptide parameters were as below: protease was set to trypsin [KR/P], the maximum number of missed cut sites was set at 0 and peptide length was set at 7–25 amino acid residues. Cysteine alkylation was set as a fixed modification.

2.8 Database search

The resulting MS/MS data were processed with MaxQuant search engine (v.1.6.15.0), database: Mus_musculus_10090_SP_20210721.fasta. Tandem mass spectra were searched against the human SwissProt Database (20422 entries) connected with the inverse decoy database. Trypsin/P was specified as the cleavage enzyme allowing up to two missing cleavages. The mass tolerance for precursor ions was set as 20 ppm and 5 ppm in the first search and main search, meantime, the mass tolerance for fragment ions was set as 0.02 Da. The Carboxymethyl (C) was designated as a fixed modification, whereas Oxidation (M), Acetyl (N-terminus), Met-loss (M), Met-loss + acetyl (M), Deamidated (N, Q) were designated as variable modification. The false discovery rate was adjusted to < 1%.

2.9 Bioinformatics analysis

Principal components analysis (PCA) and relative standard deviation (RSD) were performed to assess variations in the data from the model and control groups. The thresholds of up-regulated and down-regulated proteins were set as fold change \( \geq 1.5 \) and fold change \( \leq 1/1.5 \), and a student's t-test was used to decide the number of up-regulated and down-regulated proteins in every contrast group. Subcellular localization analysis of differentially expressed protein was performed with WoLF PSORT software. Detailed protein annotations were performed by Gene Ontology (GO) (biological process, cellular component, molecular function) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. We also performed EuKaryotic Orthologous Groups (KOG) annotation to comprehend the potential functions of differentially expressed proteins employing the KOG database. Functional enrichment of the differentially expressed (DE) proteins against all identified proteins was calculated as follows:

\[
\text{Fold Enrichment} = \frac{\text{Mapping} \div \text{Background}}{\text{All Mapping} \div \text{All Background}}
\]

For each classification, a two-tailed Fisher's accurate test was employed to check the enrichment; a corrected \( p\)-value < 0.05 is considered as significant. The STRING database was applied to identify functional enrichments in the network, and the R package "networkD3" was applied to visualize interactions amongst proteins.

2.10 Statistical analysis

Use Mean ± Standard Deviation (Mean ± SD) to express the data with normal distribution. Use student's \( t \)-test to contrast the data between two different groups. Statistical analysis was performed with software
SPSS v26.0 (IBM Corporation, Armonk, NY, USA). *P values < 0.05* was considered as statistically significant.

### 3 Results

#### 3.1 Aβ deposition in AD mice

The deposition of Aβ can lead to neuronal cell loss and degeneration, which are the basis for the impairment of cognitive function in AD. Immunofluorescence staining showed significant Aβ deposition in hippocampal CA1 area (Fig. 1B) when compared to that of wild-type mice (Fig. 1A), as vividly convinced by the enhanced green fluorescence. The results indicated that Aβ deposition in hippocampal area can be detected in the 10-months APP/PS1 mice.

#### 3.2 Identification of proteins via profiling using 4D label-free analysis

Hippocampus was excised from the mice, 4D label-free, high-resolution LC-MS/MS analyses were conducted to quantitative proteomics studies (Fig. 2A). There were a total of 5707 proteins identified in the excised tissues from six mice, among them 4928 were quantifiable (Fig. 2B). After the database was searched, the quality control evaluation was carried out from the distribution of peptide length, peptide per protein, protein coverage distribution, and protein mass distribution to ensure that the quality of the results met the standard. The results showed that most of the peptides were distributed in the range of 7–20 amino acids, which was consistent with the general rule based upon the fragmentation mode of enzymatic hydrolysis and mass spectrometry (Fig. 3A); Most proteins were identified according to more than two peptides, which indicated the results have high accuracy and repeatability (Fig. 3B); The coverage of most proteins is below 30% (Fig. 3C); The molecular weight of identified proteins was uniformly distributed in different stages, which showed a well-proportioned distribution (Fig. 3D). PCA was applied to detect and visualize variations in the hippocampus between two different groups. The results indicated that both groups shared good similarity within the group, and the difference between the groups is obvious. (Fig. 4A). The RSD results also indicated the intra-group differences were small (Fig. 4B).

#### 3.3 Identification of differentially expressed (DE) proteins in Alzheimer's disease

Through analyzing and comparing the expression patterns of DE proteins in both groups to identify DE proteins involved in the potential pathogenesis of Alzheimer’s disease. The threshold of 1.5-fold and *P values < 0.05* were set to perform significance analysis. The results indicated 54 DE proteins were in the Model vs. Control groups. 29 proteins of them were up-regulated, and the others were down-regulated (Fig. 2C). Then we used a clustered heatmap to visually show the relative expression level of DE proteins.
in each sample, the results showed that these 54 DE proteins might have similar functions and partake in certain relevant metabolic processes or signaling pathways (Fig. 2D). Details about the 54 DE proteins, including protein accession numbers, descriptions, gene names, fold changes, and \textit{P-value}, have been presented in Table 1.
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</table>

### 3.4 Functional analysis of the global proteome in Alzheimer’s disease

To explicate the potential functional significance of the changes observed in DE proteins in model vs. control groups, we performed a functional classification based upon Subcellular localization, GO terms, and a KOG analysis. The Subcellular localization annotation results showed that most proteins were concentrated in the "Nucleus", "Cytoplasm" and "Plasma membrane". The number of proteins was 21, 11, and 9 DE proteins, respectively (Fig. 4C). The GO analysis results showed a high proportion of DE proteins involved in different Biological Processes. The top five GO terms were "Cellular process", "Biological regulation", "Metabolic process", "Localization" and "Multicellular organismal process". These processes involved 39, 34, 29, 26, and 22 DE proteins, respectively (Fig. 4D). The DE proteins’ functional classification based upon the KOG analysis has been presented in (Fig. 4E). Notably, most DE proteins could be categorized into "Signal transduction mechanisms," "Translation, ribosomal structure, and biogenesis.". After combining the GO and KOG analysis of functional classification, we found that “Ribosomal structure and biogenesis” possibly play critical roles in Alzheimer’s disease.

### 3.5 Bioinformatics analysis of DE proteins and selection of potential target proteins

Our team conducted analyses of GO classification and KEGG pathway enrichment for DE proteins in every comparison group (signification \( P \text{ value} \) was calculated with Fisher's exact test), aiming to get to know if DE proteins have significant enrichment trend in certain functional types. The functional classification and pathways of DE proteins with significantly enriched were presented in a bubble diagram. BP enrichment analysis results showed that: the DE proteins in “Negative regulation of hemostasis”, “Negative regulation of coagulation”, “Ribosomal large subunit biogenesis”, and “Blood Coagulation were significantly enriched”. The main proteins are Rsl1d1, Rpl24, Rpl35, Rpl6, Fgg, Serpine2, App, Psen1, etc. (Fig. 5A); MF enrichment results showed that: the main DE proteins were significantly enriched in “Nucleic acid binding”, “DNA binding”, “Structural constituent of ribosome”, “5.8S rRNA binding and Chromatin DNA Binding”. The main proteins are Rpl18, Rsl1d1, Rpl24, Rpl19, Rpl35, Rpl6, Alb, H1-0, H1-2, H1-4, App, etc. (Fig. 5B); CC enrichment analysis results shows that DE proteins were mainly significantly enriched in “Polysomal ribosome”, “Cytosolic large ribosomal subunit”, “Cytosolic ribosome”, \( P \text{ value} \).
“Large ribosomal subunit”, “Ribosomal Subunit” and “Ribosome”. The main proteins are Rpl18, Rpl24, Rpl19, Rpl35, Rpl6, Hba, etc. (Fig. 5C). KEGG enrichment analysis results showed that the DE proteins were mainly concentrated in the “Ribosomal signaling pathway”. The main proteins were Rpl18, Rpl17, Rpl19, Rpl24, Rpl35, Rpl6, etc. (Fig. 5D).

Next, we visualized the protein-protein interaction (PPI) based upon confidence score > 0.7 (means high confidence) for DE proteins. After our analysis, the top 50 proteins with the closest interaction relationship were shown in the PPI interaction visualization. According to the results, Rpl18, Rpl17, Rpl19, Rpl24, Rpl35, and Rpl6 had close interaction relationships which were related to the “Ribosomal signaling pathway” (Fig. 6A). Combined with the expression results of BP\MF\CC\KEGG enrichment analysis and PPI interaction visualization, it is suggested that ribosomal-related proteins Rpl18, Rpl17, Rpl19, Rpl24, Rpl35, and Rpl6 may be related to the potential pathogenesis of Alzheimer’s disease. See the detailed expression trend in Fig. 6B.

### 3.6 Validation of DE Proteins by PRM

PRM verification was performed with the remaining brain tissue of Model group and Control group. PRM was quantified according to the peak area. In the design of this experiment, more than two unique peptides were used to quantify every protein. PRM quantification was conducted for the target proteins and the results showed that the six DE proteins, viz. Rpl6, Rpl18, Rpl17, Rpl19, Rpl24, and Rpl35 showed trends similar to those observed with quantitative proteomics, and all results were statistically significant (as shown in Table 2).
### Table 2
Relative expression levels of selected proteins measured via parallel reaction monitoring (PRM)

<table>
<thead>
<tr>
<th>Protein accession</th>
<th>Gene name</th>
<th>Model/Control Ratio PRM</th>
<th>Model/Control P value</th>
<th>Model/Control Ratio PRO</th>
<th>Model/Control Trend consistency</th>
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<tr>
<td>P01942</td>
<td>Hba</td>
<td>0.37</td>
<td>1.73e-03</td>
<td>0.41</td>
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<td>P02088</td>
<td>Hbb-b1</td>
<td>0.39</td>
<td>5.94e-03</td>
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<tr>
<td>P07724</td>
<td>Alb</td>
<td>0.45</td>
<td>1.31e-02</td>
<td>0.49</td>
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<tr>
<td>P10922</td>
<td>H1-0</td>
<td>4.51</td>
<td>2.84e-02</td>
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<tr>
<td>P12023</td>
<td>App</td>
<td>2.58</td>
<td>4.70e-04</td>
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<tr>
<td>P35980</td>
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<td>5.17e-02</td>
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<tr>
<td>P43274</td>
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<td>P47911</td>
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<td>P62806</td>
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<tr>
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<td>P50571</td>
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</table>

### 4 Discussion

With the increasing degree of global aging, AD has become one of the most intractable medical and social problems in mankind. An epidemiological survey showed that there were about 15.07 million dementia patients over 60 years old in China, including 9.83 million AD patients[11]. Data show that the number of AD patients in China is expected to reach 25 million by 2050, which will become an important constraint factor affecting the development of families and society[12]. Despite hundreds of years of research, the exact pathogenesis of AD is still unclear. At present, the "Beta-amyloid cascade theory" is widely recognized as the initial factor leading to the complex pathological events of AD, which is the core
factor of the occurrence and development of AD[13, 14]. In recent years, NIA-AA proposed biomarkers Aβ deposition (A), pathological Tau (T), and neurodegeneration (N) as the new diagnostic criteria for AD, emphasizing that Aβ positivity is the first and necessary condition, and it is included in the disease spectrum of AD[15]. Some scholars believe that Aβ can induce apoptosis, stimulate inflammatory cascade, produce oxidative stress, lead to mitochondrial dysfunction, and aggravate the pathological process of AD[16]. Therefore, it is necessary to study the deposition of Aβ amyloid protein.

4.1 Selection of APP/PS1 double transgenic model mice

With the continuous development of transgenic technology, the AD transgenic animal model has gradually matured. APP transgenic animal model is based on APP gene mutation, overexpression or mutation of APP gene leads to the deposition of amyloid beta, which is the central part of AD pathological changes, now has become an ideal model to study the pathogenesis of AD. APP/PS1 transgenic mice were modified with APP/PS gene and belonged to C57BL/6J strain. At about 10 months of age, they developed plaques; gliosis; synaptic and neuronal loss; cerebral vascular amyloidosis; spatial and learning disabilities[17]. As A precursor protein of Aβ, APP affects the production of Aβ. The overexpression of Aβ protein can form senile plaques (SP) and affect the pathological process of AD. APP gene is located on chromosome 21q11.2-22.2, which consists of 18 exons and 17 introns. Its gene product is translated from the APP gene by selective splicing. APP has at least six splicing forms, of which 695, 751 and 770 amino acid residues are the main forms. In the hippocampal gyrus and cortex of AD patients, the ratio of APP695/APP751 was abnormal and positively correlated with the number of plaques. APP mutation can lead to the production of neurotoxic Aβ42 and trigger a variety of pathological mechanisms, which promote apoptosis or death of nerve cells and eventually lead to AD[18]. The PS1 gene is located on chromosome 14 and consists of 10 exons. PS-1 gene is on chromosome 14 and the PS-2 gene is on chromosome 1 jointly control the formation of the γ-secretase complex (APP splice enzyme), which plays an important role in the process of Aβ production. PS1 mutation causes loss of the hydrophilic ring domain of the protein which was encoded by PS1, affects the structural stability of γ-secretase complex, the downstream APP cleavage process, and increases the production of Aβ42, which is one of the main causes of familial AD.

Hippocampus is the most specific part in the course of AD[19], and the first part to change in the early stage of AD[20]. This was consistent with our quantitative proteomics observation that the expression of APP and PSEN was significantly upregulated and the deposition of Aβ amyloid protein in brain region. Alzheimer's disease (AD) is a common neurodegenerative disease with insidious progression. At present, there is no effective treatment. Therefore, early diagnosis and identification are particularly important for patients with AD. Previous studies have indicated that in AD patients, neuropathic lesions of the olfactory cortical structures in the hippocampus are present, Such as reduced neurite connections and loss of neurons. Therefore, some researchers at home and abroad have concluded that the olfactory cortex and hippocampal atrophy may be important sensitive indicators for the early diagnosis of AD[21]. Studies have shown that the hippocampal volume of AD patients is smaller than that of mild cognitive impairment patients and normal subjects, Hippocampal volume size and MMSE score were a
significantly positive correlation. In addition, the more obvious hippocampal atrophy is in AD patients, the more severe the degree of cognitive impairment is. Therefore, the hippocampus has a certain reference value in the early diagnosis of AD[21–23]. Therefore, the study of the hippocampus of the APP/PS1 double transgenic rat model is the first choice to study the potential pathogenic mechanism of Aβ and SP in the pathological characteristics of AD[24].

4.2 4D-Label Free quantitative proteomics and PRM results analysis

Quantitative proteomics is a commonly used technology in the study of screening early diagnostic markers of related diseases. Compared with traditional 3D proteomics, 4D label-free quantitative proteomics has the characteristics of rapid, high-throughput and high sensitivity, which is conducive to the early diagnosis of diseases and the screening of candidate markers, has great clinical value and research significance[25, 26]. PRM is a targeted verification proteomics technology based on mass spectrometry, which is a perfect alternative to Western blot and Elisa. The advantage is it can overcome the difficulty of antibody preparation. At the same time, the verification rate has been greatly improved[27].

In this study, the hippocampus of APP/PS1 double transgenic mice was used as the research object, and 4D label-free quantitative proteomics and targeted proteomics technology were used as the research means. Hippocampus was excised from the mice and subjected to 4D label-free and high-resolution LC-MS/MS analyses for quantitative proteomics studies. A total of 4928 were quantifiable. After the database was searched, the quality control evaluation was carried out from the distribution of peptide length, peptide per protein, protein coverage distribution, and protein mass distribution to ensure that the quality of the results met the standard. Then PCA and RSD were performed to detect and visualize variations in the hippocampus between the two comparison groups. The PCA results showed that both groups shared good similarities within the group, and the difference between the groups is obvious. The RSD results also indicated the intra-group differences were small. If a fold change ≥ 1.5 and ≤ 1/1.5 were set as the thresholds of up-regulated and down-regulated proteins, respectively. In the model group vs. the control group, twenty-nine proteins were up-regulated and twenty-five proteins were down-regulated. The fifty-four DE proteins were further enriched and analyzed, we performed a functional classification based on Subcellular localization, GO terms, and a KOG analysis, we found that ribosomal structure and biogenesis possibly play critical roles in AD.

Next, we conducted GO classification and KEGG pathway enrichment analyses for DE proteins in each comparison group, aiming to find out whether differentially expressed proteins have significant enrichment trends in certain functional types. Then, we visualized the protein-protein interaction (PPI) based on a confidence score > 0.7 (high confidence) for DE proteins. At last, we used PRM to validate the target Proteins. Combined with the results of BP\MF\CC\KEGG enrichment analysis, PPI interaction visualization, and PRM validation, it is suggested that ribosomal-related proteins Rpl18, Rpl17, Rpl19, Rpl24, Rpl35, and Rpl6 may be related to the potential pathogenesis of AD.
4.3 Ribosomal proteins and Alzheimer’s disease

Ribosomes are mainly synthesized in the nucleolus (the largest subnuclear organelle), which is a membraneless and highly dynamic structure. Nucleolus components include rRNA, rDNA, and ribonucleoprotein. A nucleolus is a place for rRNA gene storage, rRNA synthesis and processing, and assembly of ribosomal subunits. In biological cells, ribosomes act as factories that move along mRNA templates to perform protein synthesis functions. The number of ribosomes in eukaryotic cells can reach 106. Ribosomes are complex enzyme systems composed of Ribosomal RNA (rRNA) and Ribosomal protein (Rp). Only within this overall structure can individual enzymes or proteins have catalytic activities and jointly undertake the task of protein biosynthesis. In eukaryotes, the 60S large subunit and the 40S small subunit assemble together to form the mature ribosome, the large subunit was composed of 5S, 5.8S, 28S rRNA, and 49 RPs, and the small subunit was composed of 18S rRNA and 33 RPs. The large ribosomal subunit is responsible for carrying Aminoacyl-tRNA (AA-tRNA), peptide bond formation, and binding of AA-tRNA to peptide chains. The A site, P site, and transpeptidase center are also located in the large subunit. The small ribosomal subunit is responsible for the specific recognition of mRNAs, such as the recognition of the initiation part and the interaction between codons and anticodons. The mRNA binding site is also on the small subunit.

Ribosomal RNA (rRNA) is the most abundant type of RNA in cells, accounting for 82% of the total RNA. It is also the type of RNA with the largest molecular weight. Combined with proteins, it can form ribosomes, whose function is to synthesize amino acids to peptide chains under the guidance of mRNA. Just only rRNA itself cannot perform its function, but also needs to combine with a variety of proteins to form ribosomes, which serve as the "assemble machine" for protein biosynthesis. In humans, rRNA genes are located in the short arm's proximal centromere of chromosomes 13, 14, 15, 21, 22. The exception is the 5S RNA gene, which is located in the nuclear plasma. The rRNA gene was transcribed to produce precursor 47S rRNA, which was modified and processed to form 28S, 18S and 5.8S rRNA. These rRNAs are assembled with ribosomal proteins to form large and small subunits, which are then exported from the nucleus. Finally, mature ribosomes are generated in the cytoplasm. Rp is an important component of the ribosome, which participates in ribosome assembly and stabilizes rRNA structure to improve translation efficiency and accuracy. Studies have shown that Rp not only participates in protein synthesis but also plays an important role in the cell cycle, cell division, cell apoptosis and DNA damage repair. Ribosome synthesis is rigorous and orderly, and damage to any step may affect protein synthesis, therefore, strictly controlling the expression of the Rp and rRNA for normal cell physiology function is very important.

Ribosomes are the main sites for the synthesis of nascent peptides. Compared with mature proteins, partially folded nascent peptides are metastable and more prone to misfolding. When the protein is misfolded, it loses its normal function, clogging cellular processes and creating toxic aggregates. Protein aggregation has been linked to a variety of aging-related diseases, like Alzheimer’s disease. Since the speed of ribosomes varies depending on their location during translational elongation, elongation deceleration leads to ribosome collisions and degradation of nascent peptides and
transcripts[38–41], destruction of translation dynamics or co-translation processing, and reduction of cell fitness, leading to aggregation of nascent proteins and folding of co-translated proteins[42–44], eventually leading to neurological degeneration[45–47].

Yingchao Li et al[48] through hippocampal, cortical proteomics and bioinformatic analysis indicated that aging is relevant to abnormal expression of proteins related to the ribosome (RPL4, RPS3). The researches show that dozens of transcripts encoding ribosome biogenesis and protein synthesis machinery components were specifically down-regulated with age at the translational level, consistent with the decline in protein synthesis with age[49]. Similarly, researchers also discovered that a large amount of ribosomal proteins had different expression with older age. Especially, all 60S and 40S ribosomal proteins were entirely reduced in aging muscle; except 60S ribosomal proteins RPL12 and RPL3, which were overrepresented in aging muscle[50]. These findings show that ribosomal protein expression decreases with age. However, in this research, the ribosomal protein expression found in the AD samples was elevated, we speculate that the increase was not due to age, but due to the pathological factors of AD. Masayoshi et.al[51] by employing comprehensive and accurate quantitative proteomics in Alzheimer's disease (AD) to explore the AD molecular mechanism. The results indicate that of the 29 ribosomal proteins that were quantified, 28 (RPLP0, RPL4, RPL6, RPL7A, RPL8, RPL10A, RPL11, RPL12, RPL14, RPL15, RPL18, RPL23, RPL27, RPL27A, RPL31, RPL35A, RPS2, RPS3, RPS3A, RPS4X, RPS7, RPS8, RPS14, RPS16, RPS20, RPS24, RPS25 and RPSA) were significantly upregulated in AD patients. This is in preliminary agreement with our experimental results. Therefore, they put forward that the increase of ribosome function is a common phenomenon in Alzheimer's disease and drug treatment of the disease is positively correlated with the inhibition of ribosome biosynthesis[51]. Especially, Donepezil, which is clinically used AD drugs, has also been reported to inhibit ribosome biosynthesis[52]. Ribosomal protein L6 (Rpl6) is a large subunit of ribosomal constituent proteins, and abnormal expression is related to cell damage and cell proliferation[53]. RPL6 encodes 60S ribosomal subunit that plays a vital role in oxidative phosphorylation, synaptic transmission, and neuronal signaling in Parkinson Disease (PD)[54]. When a DNA double-strand break occurs, Rpl6 is recruited to the DNA damage site, enhanced by interaction with histone H2a, and participated in the DNA damage response (DDR)[55]. H2a was also differentially expressed in this study, suggesting that Alzheimer's disease may be pathogenic by affecting the DNA damage response.

The present experimental data indicated that ribosomal proteins Rpl18, Rpl17, Rpl19, Rpl24, Rpl35, and Rpl6 were significantly up-regulated in the hippocampus of APP/PS1 mice, which may be a potential pathogenic mechanism. However, the underlying regulatory association mechanism of ribosomal proteins remains unclear. And how to cause or aggravate AD and the sequence of occurrence with AD still need to be further explored. In addition, abundant cerebral blood supply is a decisive factor for maintaining normal function of the brain region. Previous studies have shown that hippocampal blood flow is positively correlated with cognitive function, and the hippocampus is particularly sensitive to hypoperfusion. Studies also shown that the ribosome protein in the hippocampus of AD patients is significantly upregulated, but it is only found in the brain capillary region, not in the brain parenchyma region. Therefore, the relationship between ribosomal proteins and brain capillaries needs further
investigation in the future. In addition, each subregion of hippocampus encodes different memory information, which is related to various factors such as Aβ deposition and Tau protein hyperphosphorylation. However, the research on the hippocampal subregion is still in the stage of basic research, which can be further studied in the future.

5 Conclusion

Ribosomal proteins Rpl18, Rpl17, Rpl19, Rpl24, Rpl35, and Rpl6 were screened as possible potential pathogenic mechanisms by quantitative proteomics and targeted proteomics for the first time. It may induce the ribosome to produce metastable new peptides, which cause misfolding and block cellular processes, produce toxic aggregates, and cause AD.

Declarations

6.1 Funding

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6.2 Competing Interests

There are no conflicts of interest related to this research.

6.3 Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on request.

6.4 Author Contributions

Baoliang Sun, Mingquan Li and Cundong Fan designed the study.

Lina Feng, Jingyi Sun, Qiang Shi, Jie Cui, Ling Xia, Xiaoyan Fu and Yajun Hou drafted the manuscript and edited and revised it critically for important intellectual content. Lina Feng and Cundong Fan contributed
to the quality assessment, data analysis, and interpretation of the data. All authors contributed to the article and approved the submitted version.

6.5 Ethics approval:

The whole process of animal experiments passed the ethical review of the Ethics Committee of Changchun University of Traditional Chinese Medicine (No.2021195 Changchun, China).

6.6 Consent to participate:

Not applicable.

6.7 Consent to publish:

Not applicable.

References


**Figures**
Figure 1

Immunofluorescent staining. (A): Control Group; (B): Model Group; Arrow: Aβ plaque accumulation.
Figure 2

Identification and analysis of the proteome via the 4D label-free technique. (A): The systematic workflow for quantitative profiling of the mice hippocampus global proteome. (B): Summary of results from the liquid chromatography-tandem mass spectrometry (LC-MS/MS) database search. (C): Volcano plots depicting fold change (based on log1.5 Fold change, x-axis) and statistical significance (−log10 P value, y-axis) of proteins in the hippocampus of the mice. (D): Heatmap of differentially expressed proteins in the hippocampus of the mice.
Figure 3

Data quality control results. (A): Peptide length; (B): Peptides per protein: In quantification, a protein corresponding to multiple specific peptides is beneficial to increase the accuracy and credibility of quantitative results; (C): Protein coverage distribution: In the shotgun mass spectrometry method, peptides with higher abundance are preferentially scanned. Therefore, the protein coverage was positively correlated with the abundance in the sample; (D): Protein mass distribution.
Figure 4

Sample repeatability test; Functional classification of differentially expressed (DE) proteins using Subcellular localization, Gene Ontology (GO), and EuKaryotic Orthologous Groups (KOG) analysis. (A): Principal Component Analysis (PCA), the degree of clustering among samples represents the difference between samples. (B): Relative Standard Deviation (RSD), the smaller the overall RSD value, the better the quantitative repeatability. (C): Subcellular localization annotation. (D): Functional classification of DE proteins by GO analysis. (E): Functional classification of DE proteins by KOG analysis.
Figure 5

Functional enrichment of differentially expressed (DE) proteins by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. (A-C): GO categories (Biological Process, Molecular Function, and Cellular Components) of DE proteins in mice hippocampus in the control and control groups. (D): KEGG analysis of DE proteins in mice hippocampus in the control and control groups.
Figure 6

Protein-protein Interaction (PPI) network diagram of DE proteins and Target protein expression changes. (A): Protein-protein Interaction network (green: down-regulated protein, red: up-regulated protein; The darker the color, the bigger the difference). (B): Target protein expression changes (* $P < 0.05$; ** $p < 0.01$; **** $p < 0.001$).