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Exosomal Small RNA Sequencing and Analysis from Serum of Alzheimer's Disease Patients

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Abstract:
The pathological mechanisms of Alzheimer's disease (AD) encompass intricate systemic regulatory networks. Here, we investigated the potential involvement of exosomal small RNAs in AD progression by profiling their expression patterns in the peripheral blood of AD patients. Serum exosomes serve as crucial mediators of intercellular communication and carry microRNAs (miRNAs) whose regulatory roles in AD pathogenesis remain largely undefined. To explore this, we isolated serum exosomes from AD patients and healthy controls (HCs) and performed small RNA sequencing, which revealed 19 differentially expressed miRNAs. Subsequent bioinformatics analysis indicated that the predicted target genes of these miRNAs are significantly enriched in key pathological pathways associated with AD. Collectively, our findings contribute to the understanding of molecular alterations underlying AD and may offer valuable insights for the identification of novel diagnostic biomarkers and therapeutic targets.

Keywords: *Alzheimer's disease; exosomes; small RNA sequencing; miRNA*

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I. Background

Alzheimer's disease (AD), as an age-related neurodegenerative disorder, has seen its incidence rise with the intensifying global aging trend, posing a severe public health challenge. The disease is characterized clinically by progressive cognitive impairment, with typical pathological hallmarks including extracellular β-amyloid (Aβ) deposits forming senile plaques and intraneuronal hyperphosphorylated tau protein aggregations leading to neurofibrillary tangles[1-3]. These hallmark pathological changes collectively drive multi-level cascade pathological responses, including synaptic dysfunction, neuron loss, mitochondrial metabolic abnormalities, and persistent neuroinflammation, ultimately resulting in neural network dysfunction and progressive impairment of cognitive domains[4-6]. Collectively, these findings highlight the importance of shifting from a focus on isolated cellular lesions to a systemic perspective. Deciphering the intricate intercellular crosstalk and overarching regulatory networks is crucial for unraveling the full complexity of AD pathogenesis.

Notably, brain-derived exosomes are capable of traversing the blood-brain barrier (BBB) into the peripheral circulation, thereby offering a non-invasive means to assess the pathological processes of the central nervous system (CNS) through peripheral blood analysis. Studies have shown that exosomes and their bioactive contents (especially nucleic acid molecules such as miRNAs) play a crucial role in the pathogenesis of neurodegenerative diseases. By regulating the gene expression networks and signaling pathways of target cells, they actively participate in and drive disease progression, making them a central focus for studying their molecular mechanisms.

In the central nervous system (CNS), exosomes are key mediators of intercellular communication, actively secreted by diverse cell types including neurons, astrocytes, and microglia[7, 8]. These nanovesicles carry a diverse molecular cargo—comprising cell-specific proteins, lipids, and nucleic acids—and are subsequently internalized by neighboring or distant target cells via paracrine signaling or systemic circulation[9-12]. Through this intercellular transfer, exosomes play a dual role in both maintaining physiological homeostasis and propagating pathological signals. Notably, their ability to traverse the blood-brain barrier (BBB) into the peripheral bloodstream renders peripheral blood a valuable, non-invasive window into the pathological state of the CNS[13-15]. Accumulating evidence implicates exosomes and their bioactive cargo—particularly microRNAs (miRNAs)—in the pathogenesis of neurodegenerative diseases. By modulating gene expression networks and signaling pathways in recipient cells, exosomal miRNAs are actively involved in driving disease progression, positioning them as pivotal elements in understanding molecular mechanisms[16].Specifically, exosomal miRNAs have been shown to regulate key pathological hallmarks of Alzheimer's disease (AD), including amyloid-β (Aβ) production (e.g., by targeting APP and BACE1), Tau protein phosphorylation (e.g., through modulation of kinases such as GSK3β and CDK5), neuroinflammatory responses, and synaptic plasticity. For instance,

miR-15a influences Aβ production by targeting both APP and BACE1; miR-128 suppresses GSK-3β-mediated Tau hyperphosphorylation; and downregulation of the miR-29 family (e.g., miR-29c) correlates with elevated BACE1 protein levels in the brains of sporadic AD patients, underscoring its regulatory role in β-secretase activity. Collectively, these examples illustrate that exosome-derived miRNAs are intricately involved in AD progression by orchestrating multiple targets and interconnected pathways.

Among the diverse repertoire of regulatory molecules carried by exosomes, small non-coding RNAs—particularly microRNAs (miRNAs)—serve as crucial "information codes." miRNAs are short-chain non-coding RNAs that typically mediate the degradation or translational repression of target mRNAs by binding to their 3'-untranslated regions (UTRs), thereby exerting post-transcriptional regulation of gene expression[17-19]. Notably, significant dysregulation of miRNA expression profiles has been observed not only in brain tissue and cerebrospinal fluid (CSF) of AD patients, but also in peripheral blood-derived exosomes[9, 20, 21]. These dysregulated miRNAs are extensively involved in core pathological processes of AD, including amyloid-β (Aβ) production and clearance (e.g., by targeting APP and BACE1), Tau protein phosphorylation (e.g., through modulation of kinases such as GSK3β and CDK5), neuroinflammatory responses, and synaptic plasticity[22]; For instance, miR-15a influences Aβ production by targeting both APP and BACE1; miR-128 suppresses GSK-3β-mediated Tau hyperphosphorylation[23]; and downregulation of the miR-29 family (e.g., miR-29c) correlates with elevated BACE1 protein levels in the brains of sporadic AD patients, underscoring its regulatory role in β-secretase activity[24, 25]. Collectively, these findings demonstrate that exosome-derived miRNAs are intricately involved in AD progression by orchestrating multiple targets and interconnected pathways.

While these studies have established the involvement of exosomal miRNAs in the central pathology of AD, systematic analyses of their expression profiles in the peripheral circulation of AD patients remain relatively limited. Consequently, their regulatory functions and mechanisms within systemic pathological networks are yet to be fully elucidated. To address this gap, the present study performed small RNA sequencing coupled with bioinformatics analysis on serum-derived exosomes from AD patients and healthy controls. We systematically screened for differentially expressed miRNAs and analyzed the regulatory networks of their target genes, aiming to provide new experimental evidence for unraveling the peripheral-central crosstalk mechanisms underlying AD pathogenesis.

II. Materials and Methods

2.1 Serum Sample Collection and Exosome Isolation

Serum samples were obtained from patients diagnosed with Alzheimer's disease (AD) at the Fifth People's Hospital of Zigong City, along with age-matched healthy controls. The study protocol was approved by the Institutional Ethics Committee of the Fifth People's Hospital of Zigong City (Approval No. 2023-101), and written informed consent was obtained from all participants or their legal representatives. For each subject, 4 mL of fasting whole blood was collected via venipuncture into EDTA-containing anticoagulant tubes. Within 2 hours of collection, blood samples were centrifuged at 1,500×g for 10 min at 4°C to separate the serum. The resulting supernatant was immediately aliquoted into sterile cryovials and stored at −80°C until further analysis.

Serum exosomes were isolated by differential ultracentrifugation. Briefly, thawed serum samples were centrifuged at 1,500×g for 15 min at 4°C, and the supernatant was subjected to an additional centrifugation at 1,500×g for 30 min at 4°C to thoroughly remove cellular debris and large contaminants. The clarified supernatant was then transferred to ultracentrifuge tubes and centrifuged at 100,000×g for 70 min at 4°C using an Optima™ ultracentrifuge (Beckman Coulter, USA). The supernatant was discarded, and the crude exosome pellet was gently resuspended in ice-cold phosphate-buffered saline (PBS). This suspension was subjected to a second ultracentrifugation step under identical conditions (100,000×g, 70 min, 4°C) to obtain purified exosomes. The final exosome pellet was resuspended in an appropriate volume of PBS, aliquoted, and stored at −80°C for subsequent analyses.

2.2 Western Blotting

Isolated exosomes were lysed in RIPA lysis buffer (Beyotime, China) supplemented with protease inhibitor cocktail (Roche, Switzerland) to extract total protein. Protein concentrations were determined using the Bicinchoninic Acid (BCA) Protein Assay Kit (Beyotime, China) according to the manufacturer's instructions. Equal amounts of protein (20 μg per lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto polyvinylidene fluoride (PVDF) membranes (0.45 μm pore size, Sangon Biotech, China). The membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature. Following blocking, membranes were incubated overnight at 4°C with primary antibodies against exosomal markers CD63 (1:1000, Sangon Biotech, China) and the negative marker calnexin (1:1000, Sangon Biotech, China). After three washes with TBST (10 min each), the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (1:5000, Sangon Biotech, China) for 1 h at room temperature. Following three additional

washes with TBST, protein bands were visualized using an enhanced chemiluminescence (ECL) detection system (E-BLOT, USA).

2.3 Transmission Electron Microscopy (TEM)

For morphological characterization, 10 μL of purified exosome suspension was dropped onto a Formvar-carbon-coated copper grid and allowed to adsorb for 10 min at room temperature. The grid was then gently rinsed with sterile deionized water and negatively stained with 2% (w/v) uranyl acetate solution for 1 min. After air-drying at room temperature, the samples were observed under a transmission electron microscope (Hitachi, Tokyo, Japan) operating at an accelerating voltage of 80 kV. Representative images were captured for analysis.

2.5 Serum Exosome Small RNA Library Construction and Sequencing

After total RNA extraction from exosomes, the integrity and concentration were assessed using the Agilent Bioanalyzer 2100 system and a small RNA kit. Qualified samples should display a typical 18–36 nt small RNA main peak to be used for subsequent library construction. Small RNA sequencing libraries were constructed using the Illumina TruSeq Small RNA Library Prep Kit, with specific steps including: sequential ligation of 3' and 5' adapters using T4 RNA ligase 2; cDNA synthesis by reverse transcription; limited-cycle PCR amplification introducing sample-specific indexes. The purified libraries were quantified using the Qubit PicoGreen dsDNA Assay Kit. Finally, qualified libraries were sequenced on the Illumina NovaSeq 6000 platform for single-end 50 bp reads, with each sample generating an average of no less than 10 million raw reads.

Raw sequencing data quality was evaluated using FastQC. Subsequent quality control processing was performed using fastp, including trimming N bases at both ends, filtering reads with quality scores below Q20, and removing adapter sequences. To eliminate non-miRNA small RNA interference, quality-controlled reads were aligned to the Rfam database using the Bowtie short sequence alignment tool, removing rRNA, tRNA, snoRNA, and other non-coding RNAs. Remaining reads were analyzed for miRNA identification and quantification using the miRDeep2 software. Expression levels were normalized for intergroup comparisons. Differentially expressed miRNAs were screened using DESeq2 analysis to identify statistically significant expression changes.

2.5 RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) Analysis

Total miRNA was extracted from exosomes using the Exosome RNA Purification Kit (EZBioseiece, China). Subsequently, RNA was reverse transcribed into cDNA using a reverse transcription kit. The expression level of mature miR-103a-3p was detected by real-time fluorescent quantitative PCR using the 2×Coloe SYBR Green qPCR Master Mix kit (EZBioseiece, China). U6 was used as the internal reference control for miRNA. The relative gene expression levels were calculated using the $2-\Delta\Delta Ct$ method.

2.6 Differential miRNA Target Gene and Functional Enrichment Analysis

The target genes of differentially expressed microRNAs (DEMs) were predicted using miRanda. Subsequently, the predicted target genes were mapped to the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases to annotate their potential biological functions.

2.7 Statistical Analysis

All statistical analyses were performed using GraphPad Prism 10.0 and R software (version number to be supplemented). Data are presented as mean ± standard error of the mean (SEM). Comparisons between two groups were conducted using a two-tailed unpaired Student's t-test; comparisons among multiple groups were performed using one-way analysis of variance (ANOVA). A p-value of <0.05 (denoted as $*p<0.05$) was considered statistically significant.

III. Results

3.1 Identification of Serum Exosomes

Exosomes were isolated from serum using ultracentrifugation, and their morphology and marker proteins were further identified by TEM and Western blot. TEM results showed that the isolated vesicles exhibited typical cup-shaped or spherical membrane structures of exosomes. Western blot analysis further confirmed that these vesicles expressed the exosome marker protein CD63, while the endoplasmic reticulum marker Calnexin was negative. Together, these results indicate that in this study, high-purity exosomes were successfully isolated from serum using ultracentrifugation.

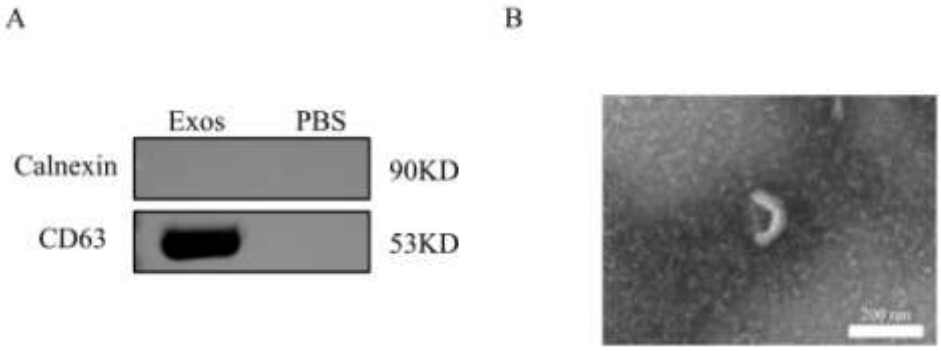

Figure 1: Isolation and characterization of serum exosomes. (A) Expression of the exosome marker protein CD63 detected by western blot, with Calnexin as a negative control protein. (B) Morphological structure of exosomes observed by TEM (scale bar: 200 nm).

3.2 Serum Exosomal Small RNA Sequencing

To elucidate the expression profile characteristics of small RNAs in plasma exosomes of Alzheimer's disease (AD) patients and healthy controls, this study performed small RNA sequencing on the isolated exosome samples (Figure 2A).

By systematically classifying and annotating the small RNA sequences obtained from sequencing, we further analyzed their composition and distribution characteristics. As shown in Figure 1B, the small RNA transcriptome composition of all samples showed high consistency. Among them, piRNA accounted for the highest proportion, approximately 70%–80% of the total sequencing reads; miRNA was the second largest category, accounting for about 10%–15%. The remaining non-coding RNAs, such as tRNA, snRNA, snoRNA, and rRNA, only accounted for a very small portion. This distribution pattern is consistent with the typical characteristics of exosomal small RNAs, indicating that the sample processing and sequencing procedures were reliable and the data quality was high, providing a solid data basis for subsequent screening of AD-related differentially expressed miRNAs.

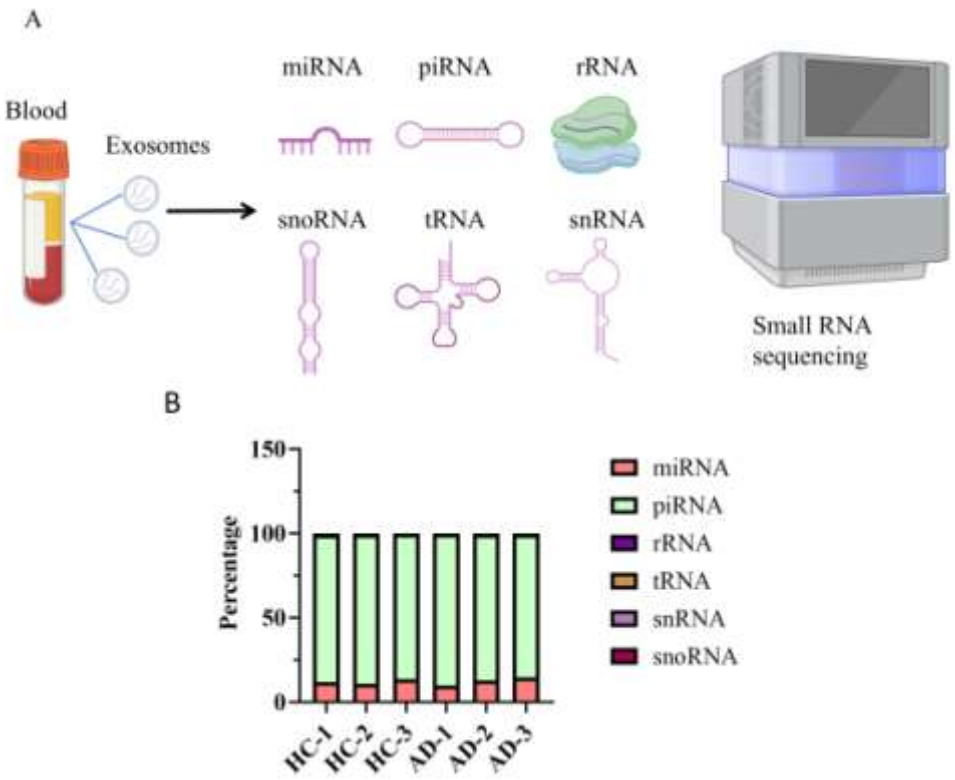

Figure 2 Serum exosomal small RNA sequencing. (A) Analysis of plasma-derived exosomal small RNA sequencing in the AD group (n=3) and the control group (n=3). (B) Percentage composition of various small RNA biotypes in the small RNA sequencing.

3.3 Analysis of differential miRNAs in serum-derived exosomes

After quality control and annotation of serum exosomal small RNA sequencing data, a total of 559 known miRNAs were identified in samples from AD patients and healthy controls. Using |log2 fold change|> 1 and p <0.05 as the screening threshold, 19 differentially expressed miRNAs (DEMs) were identified. These results were visualized using a volcano plot (Figure 3A), where the x-axis represents $\log_2$ FC (AD vs. HC) and the y-axis represents $-\log_{10}$ (p-value). Gray dots represent miRNAs with no significant difference, while significantly upregulated and downregulated miRNAs are marked in red and blue, respectively. Among the 19 DEMs, 14 were significantly upregulated, and 5 were significantly downregulated in AD patient serum exosomes. Hierarchical clustering heatmap analysis (Figure 3B) showed that the expression profiles of these 19 DEMs clearly distinguished AD patients from healthy controls, indicating disease group-specific expression patterns.

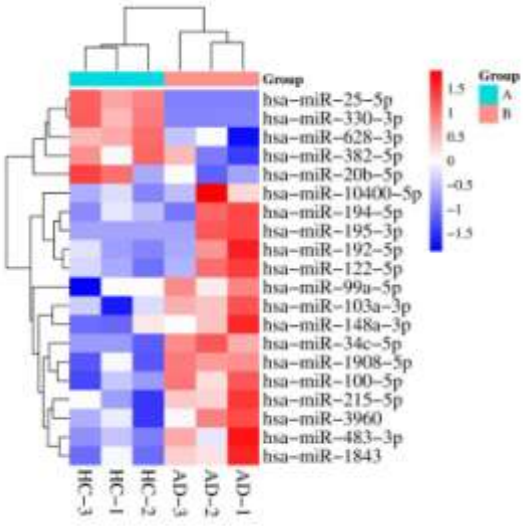

Figure 3 Differentially expressed miRNA clustering heatmap. Red represents upregulated expression, and blue represents downregulated expression.

3.4 Validation of Differential miR-103a-3p in Serum Exosomes of AD Patients

To verify the reliability of the small RNA sequencing results and further clarify the expression level of miR-103a-3p in serum exosomes of AD patients, the relative expression of miR-103a-3p in serum exosomes of AD patients and healthy controls was detected using U6 small nuclear RNA (U6 snRNA) as an internal reference. The qRT-PCR results showed that, compared with the healthy control group, the expression level of miR-103a-3p in serum exosomes of AD patients was significantly upregulated (Figure 3A). This result is consistent with the expression trend observed in our previous small RNA sequencing study and further supports the potential ofmiR-103a-3p as a circulating biomarker for AD.

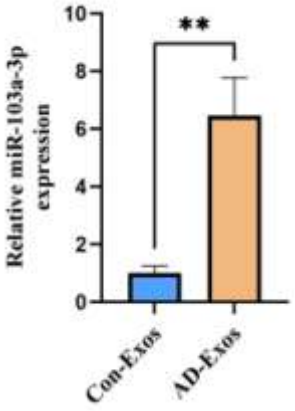

Figure 3 Expression of miR-103a-3p in serum exosomes of AD patients

3.4 **Prediction of miR-103a-3p Target Genes and Functional Enrichment Analysis**

To elucidate the potential functions of the differentially expressed miR-103a-3p identified in this study in the pathogenesis of AD, their target genes were first predicted using the TargetScan database, yielding a total

of 2,262 potential targets. Subsequently, Gene Ontology (GO) functional annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed to systematically investigate the biological processes and pathways associated with these target genes.

GO analysis revealed (Figure 4A) that in the Biological Process (BP) category, the target genes were significantly enriched in processes such as RNA polymerase II-mediated transcriptional regulation, nervous system development, and protein phosphorylation. Regarding Cellular Component (CC), the target genes were predominantly localized to structures including the glutamatergic synapse, dendrites, axons, and neuronal cell bodies. In the Molecular Function (MF) category, enrichment was observed in functions such as protein binding, DNA-binding transcription factor activity, and protein kinase activity. KEGG pathway enrichment analysis further demonstrated (Figure 4B) significant enrichment of target genes in multiple signaling pathways closely associated with AD pathology, including the Wnt signaling pathway, pathways in cancer, neurodegenerative disease pathways, and nucleocytoplasmic transport.

In summary, bioinformatics analysis suggests that the serum exosomal differentially expressed miRNAs identified in this study may be extensively involved in biological processes such as neuronal transcriptional regulation, synaptic function, cell adhesion, and key signal transduction pathways by modulating the aforementioned target genes. These findings provide a crucial foundation for the subsequent identification of key regulatory factors associated with AD.

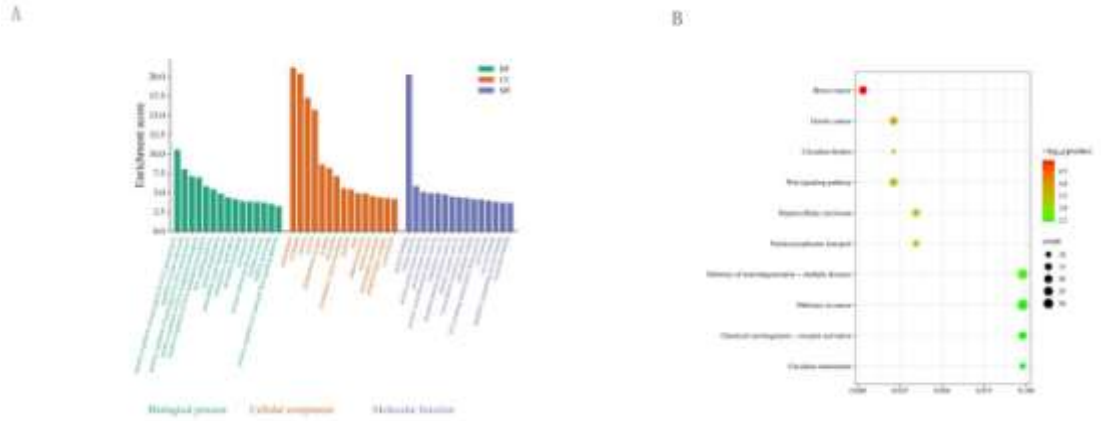

Fig. 4 Functional enrichment analysis of differentially expressed miR103a-3p target genes. (A) GO feature enrichment analysis. The abscissa is the GO entry, and the ordinate is the enrichment significance ($-\log_{10}$ P value). (B) KEGG pathway enrichment analysis. The top 10 pathways with the most significant enrichment were shown, with the color gradient indicating the size of the P value and the abscissa representing the proportion of enriched genes.

IV. Discussion

In this study, the expression profile characteristics of small RNAs in circulating exosomes and their potential regulatory role in AD were revealed by sequencing small RNAs in serum exosomes of AD patients and healthy controls. The core findings suggest that there is a set of specifically differentially expressed miRNAs in the plasma exosomes of AD patients, and their predictive target genes are significantly enriched in pathways closely related to AD pathology, such as neuronal development, synaptic signaling, cytosis, and autophagy[26, 27]. These results suggest that peripheral blood exosomal miRNAs may not only be used as biomarkers to reflect the pathological progression of AD, but may also actively participate in the regulation of diseases through cell-to-cell communication.

Further bioinformatics analysis provides direction for the functional interpretation of these differential miRNAs. GO analysis showed that target genes were significantly enriched in biological processes such as nervous system development and protein phosphorylation, and were highly localized in neuronal synapses and cell bodies. This result supports the hypothesis that peripherally derived exosomal miRNAs may indirectly regulate intraneuronal transcription and synaptic function through the blood-brain barrier or peripheral neuronal-glial interaction. KEGG pathway enrichment analysis further linked differential miRNAs to the classical pathological mechanism of AD: enrichment of the Wnt pathway suggests that it may be involved in neuronal survival and metabolic regulation[28, 29] ; while the association between the neurodegenerative pathway and the hezhi transport pathway points to its potential impact on Aβ and Tau protein metabolism[30, 31].

It is worth noting that the conclusions of this study also have certain limitations. First, based on the sample size of the discovery cohort, the differential miRNAs screened need to be validated in a larger independent cohort to confirm their reliability as a peripheral marker of AD. Secondly, whether these miRNAs affect

AD-related pathways through exosome-mediated delivery pathways still needs to be confirmed by further functional experiments. For example, key miRNAs can be overexpressed or knocked down in AD models to clarify their regulatory effects on pathological deposition, synaptic loss, and cognitive impairment in vivo.
In summary, this study reveals the expression characteristics of serum exosomal miRNAs in AD patients and their potential biological networks from the perspective of peripheral systems, providing new ideas for exploring the molecular mechanism of AD. Future research can further focus on the functional validation and mechanism profiling of key differential miRNAs and evaluate their translational potential as disease diagnostic markers or intervention targets.

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Compliance with ethical standards
Conflict of interest
Authors declare that they have no potential conflicts of interests.

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