

Microarray microRNA profiling of urinary exosomes in a 5XFAD mouse model of Alzheimer's disease

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Abstract

Background: Alzheimer's disease (AD) is an incurable and irreversible neurodegenerative disease, without a clear pathogenesis. Therefore, identification of candidates before amyloid- β plaque (A β) deposition proceeds is of major significance for earlier intervention in AD.

Methods: To explore the potential noninvasive earlier biomarkers of AD in a 5XFAD mouse model, microRNAs (miRNAs) from urinary exosomes in 1-month-old pre-A β accumulation 5XFAD mice models and their littermate controls were profiled by microarray analysis. The differentially expressed miRNAs were further analyzed via droplet digital PCR (ddPCR).

Results: Microarray analysis demonstrated that 48 differentially expressed miRNAs (18 upregulated and 30 downregulated), of which six miRNAs – miR-196b-5p, miR-339-3p, miR-34a-5p, miR-376b-3p, miR-677-5p, and miR-721 – were predicted to display gene targets and important signaling pathways closely associated with AD pathogenesis and verified by ddPCR.

Conclusions: Urinary exosomal miRNAs showing differences in expression prior to A β -plaque deposition were identified. These exosomal miRNAs represent potential noninvasive biomarkers that may be used to prevent AD in clinical applications.

KEYWORDS

5XFAD mouse model, Alzheimer's disease, biomarkers, microarray, miRNA, urinary exosome

1 | INTRODUCTION

Alzheimer's disease (AD) is an age-associated, chronic neurodegenerative disease related to irreversible cognitive deficits and progressive dementia.¹ As the course of AD begins decades before clinical features appear, there is an urgent need to identify early-stage markers, particularly before A β plaque accumulation.² Urine

can be collected noninvasively in abundance and is also a significant potential resource for the discovery of novel biomarkers for AD^{2,3} because the kidneys collect most waste matter as final metabolites that have been excreted into the cerebrospinal fluid and blood.⁴ However, relative to those in other body fluids, protein concentrations in normal urine are very low (less than 100 mg/L or 150 mg/d).⁵ Proteins that are highly abundant in urine, including albumin, might

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mask the search of proteins present at lower concentrations that have underlying physiological or pathological significance.⁵

Exosomes, first described in the 1980s, are extracellular nano-sized vesicles 40–160 nm in diameter and can be extracted from various body fluids.⁶ They are amenable to transcriptomic and proteomic analyses, and potentially valuable in disease diagnosis and monitoring.^{7,8} We previously identified 316 proteins, containing 44 brain-associated cell markers, from the urinary exosomes of 1-month-old 5XFAD mouse models and littermate controls. The 5XFAD mouse model predominately generates A β ₄₂, which accumulates in plaques from 2 months of age. Notably, eighteen proteins were found only in the 5XFAD group. Importantly, clusterin and annexin 2 expression was significantly decreased in the Alzheimer's disease model. AOA_H, LY86, and clusterin were the first brain-associated cell biomarkers seen to be differentially expressed in urinary exosomes from AD models.⁹ The urinary exosomes of 5XFAD model mice have a rounded erythrocyte-like shape or present as flattened spheres, as characterized by transmission electron microscopy.⁹

MicroRNAs (miRNAs) are noncoding RNA molecules involved in normal physiological and pathological processes. They are involved in cell signaling, and are also potential diagnostic biomarkers and therapeutic targets for a variety of diseases, including neurodegenerative diseases.^{10,11} Although miRNAs degrade rapidly in post-mortem brain tissue, they are stable in body fluids such as cerebrospinal fluid (CSF) and serum.¹² The evaluation of miRNAs can be informative in health and disease: several studies have reported miRNA dysregulation in AD,¹³ while some studies have collected noninvasive urinary exosomes to investigate AD.⁷

In this study, the 5XFAD mouse model of AD was used to examine differential protein expression in urinary exosomes. miRNA microarray analysis was employed to identify potential early-stage biomarkers for AD, prior to the development of A β ₄₂ deposition in the brains of mouse model (Figure 1A). These findings may help develop methods for the prevention of AD.

2 | METHODS

2.1 | Ethics statement

The experimental procedures complied with the Chinese Regulations for Laboratory Animals, and were examined and authorized by the Institutional Animal Care and Use Committee of the Institute of Laboratory Animal Science, Peking Union Medical College (approval ID: QC19005).

2.2 | Animals

The 5XFAD mouse model of AD expresses five human mutations, specifically in PS1 and APP (B6SJL-Tg[APP**K670N***M671L***I716V***V717I*, PSEN1**M146L***L286V*] 6799Vas/J), that are controlled by the neuron-specific Thy1 promoter. The 5XFAD mice, purchased from

The Jackson Laboratory (600 main street, Bar Harbor, Maine, USA 04 609), were crossed with wild-type SJL mice. Their offspring were hemizygous for the PS1 and APP transgenes.¹⁴ PCR was used to determine the transgenic status of the offspring (Transgene forward primer: AGG ACT GAC CAC TCG ACC AG; transgene reverse primer: CGG GGG TCT AGT TCT GCA T). Transgene-positive female offspring were used, and their transgene-negative female littermates were used as the control group. The mice were provided with a standard diet and water ad libitum, and were fed at 22°C in an environmentally controlled room, with a 12-hours light and dark cycle. Urine samples from 4-week-old models and littermate mice were collected using metabolic cages. To avoid contamination, the mice were given free access to water without food during urine collection.

2.3 | Exosome purification

2.3.1 | Preparation of urine samples

All urinary exosome samples were extracted from 30 mL of urine using the Exosome Isolation Q3 kit (Wayen Biotechnologies, EI03-03001, Shanghai, China). The urine samples (6 samples, *n* = 15 mice/sample) were initially stored on ice. The frozen samples were completely thawed in a 37°C water bath, then placed on ice. Each sample was then centrifuged at 3000× *g* at 4°C for 15 minutes. The supernatants were switched to new tubes and placed on ice until exosome isolation.

2.4 | Exosome isolation

Reagent A (of the Exosome Isolation Q3 kit) was warmed to room temperature before use. The manufacturer's instructions recommend using a 20 mL starting volume of urine. The process of exosome isolation was performed as described in a previous study.⁹ The supernatants, containing the isolated exosomes, were then transferred into new tubes. The exosomes were then immediately used in the analytical protocols or were aliquoted and deposited at –80°C for later use.

2.5 | MicroRNA microarray

Total RNA was isolated using the miRNeasy Micro Kit (Qiagen, GmbH, Germany). Each RNA sample was then used to generate biotinylated cRNA targets for the GeneChip[®] miRNA 4.0 Array. Briefly, each labeled sample was added to a hybridization cocktail, incubated, and injected into miRNA arrays according to the manufacturer's instructions. After 16 hours of hybridization at 48°C, the arrays were washed and then stained in Fluidics Station 450, and then scanned by a GeneChip scanner. Microarray experiments were performed following the protocol of Affymetrix Inc.

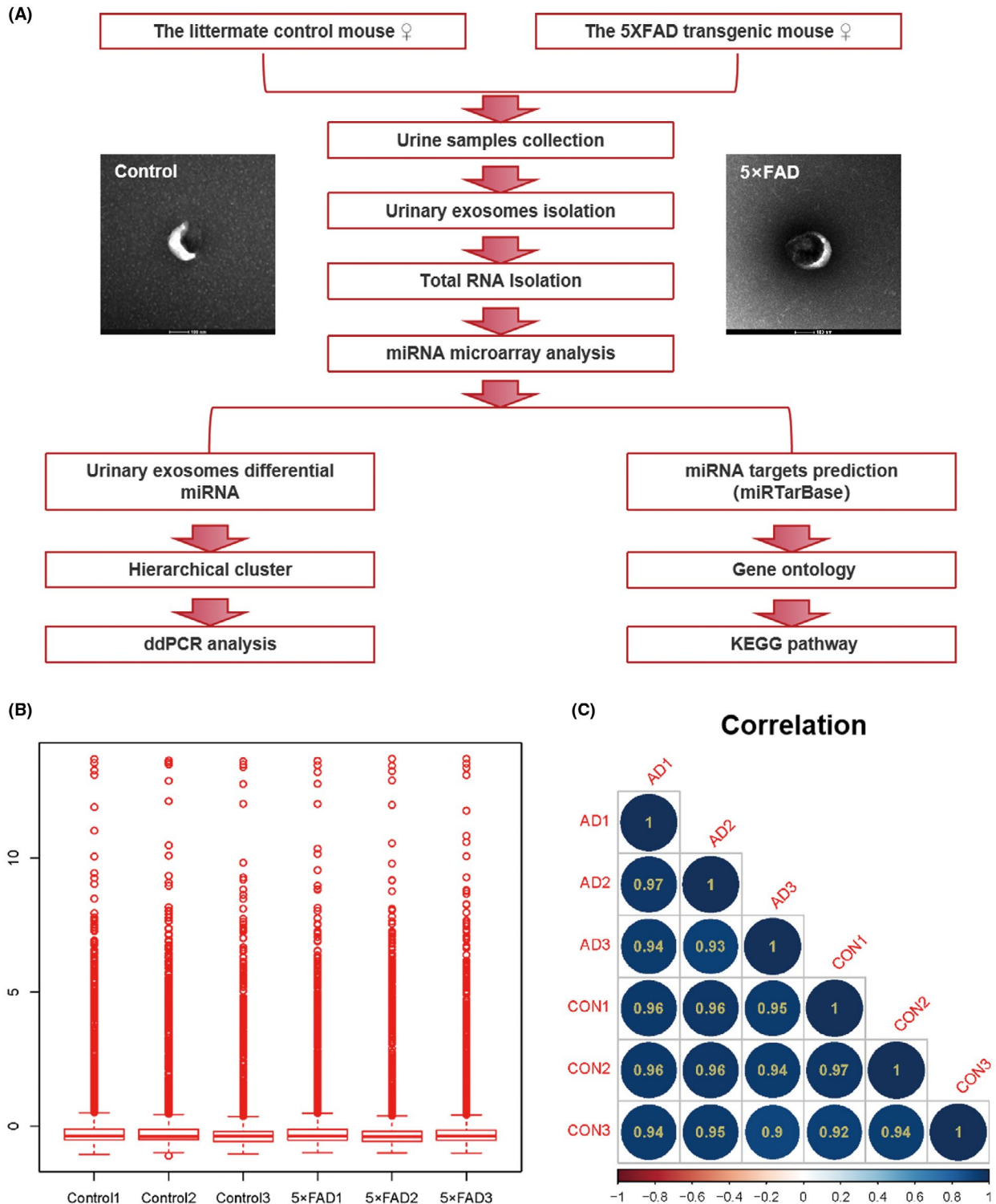


FIGURE 1 Workflow of the miRNA microarray analysis of urinary exosomes in 5XFAD mice models of Alzheimer's disease and their littermate controls. A, Urinary exosome sampling and isolation, and miRNA microarray evaluation. B, Boxplot showing the sample distribution of the microarray data. C, Correlation plot of the urinary exosome samples in the 5XFAD models and littermate controls

2.6 | Microarray data analysis

The raw data were normalized using TAC 4.0.1, using a robust multiarray average including background correction, quantile normalization,

and summarization. This global normalization method was used because there are no reliable endogenous controls for exosomal miRNAs, and currently no agreed procedure for normalization in urinary exosomal miRNA detection. We used U6 snRNA, which has previously been

used to calculate the relative level of urinary miRNA expression for qPCR detection,¹⁵ to evaluate the quality of the target miRNA samples and to confirm the threshold for each sample. Differential expression of miRNAs was measured as fold change (FC), with a threshold for up-regulation and downregulation of FC ≥ 1.2 . The target genes of the intersecting differentially expressed miRNAs were predicted using three databases including Targetscan, microRNAorg, and PITA. Heatmaps for the target genes were generated using the R package "pheatmap".

Gene ontology (GO) pathway enrichment analysis of the target genes associated with the differentially expressed miRNAs was performed, to explore the associated molecular functions (MF), cellular components (CC), and biological processes (BP) (the top 15 categories are shown). GO was performed using the R package "clusterProfiler", with Fisher's exact test. GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed to confirm the roles of the target genes. Significantly enriched ($P < .05$) GO categories were selected.

Differentially expressed miRNAs, and their putative target genes, were determined by screening the miRTarBase database (of experimentally validated miRNA target genes), followed by KEGG database screening, to identify pathways contributing to neurodegenerative diseases and those related to the nervous system.

2.7 | Droplet digital polymerase chain reaction (ddPCR)

Absolute quantification and high-precision identification of nucleic acid target sequences is possible via ddPCR. ddPCR has greater sensitivity, reproducibility, and accuracy than qPCR.¹⁶ ddPCR measures the end point of the reaction, classifying each droplet as positive or negative, thereby enabling examination at the low concentrations of miRNA found in urinary exosomes. ddPCR was performed according to the manufacturer's protocol (Bio-Rad, Hercules, CA, USA). The PCR reaction mixture, containing cDNA, Super Mix, and nuclease-free water, is described in Table 1. For every miRNA, the TaqMan microRNA assay utilizes primers and probes that are specific to its mature form (Table 2). The ddPCR reaction system is presented in Table 3. The reaction plates were loaded using a QX200 automatic droplet generator (Bio-Rad). Following PCR amplification, the plate holding the droplets was loaded into a QX200 Droplet Reader, which evaluates each droplet individually using a two-color examination system.¹⁶

3 | RESULTS

3.1 | miRNA expression differed significantly between the 5XFAD and control mice

Urinary exosomes were extracted from each 30 mL sample of urine (six samples, $n = 15$ mice/sample), and analyzed using the

TABLE 1 The PCR reaction mixture

Ingredient	Volume
First strand synthesis of cDNA	
5x miScript HiFlex Buffer	4 μ L
10x miScript Nucleics Mix	2 μ L
miScript Reverse Transcriptase Mix	2 μ L
Template RNA + H ₂ O	12 μ L
Total	20 μ L
Evagreen ddPCR	
2x ddPCR Supermix Evagreen	10 μ L
Primer F(10 μ M)	0.4 μ L
Primer R(10 μ M)	0.2 μ L
DNA + ddH ₂ O	9.4 μ L
Total	20 μ L

TABLE 2 The primers utilized in TaqMan microRNA assay

Primer Name	Sequence (5'-3')
U6	TTCGTGAAGCGTTCCATATTTT
mmu-miR-196b-5p	TAGGTAGTTTCTGTTGTTGGG
mmu-miR-339-3p	CGCCTCGGCGACAGA
mmu-miR-34a-5p	GGCAGTGTCTTAGCTGGTTGT
mmu-miR-376b-3p	ATCATAGAGGAACATCCACTTAAAAA
mmu-miR-677-5p	TTCAGTGATGATTAGCTTCAAAAA
mmu-miR-721	AGTGCAATTAAGGGGGAA

TABLE 3 The reaction system of ddPCR

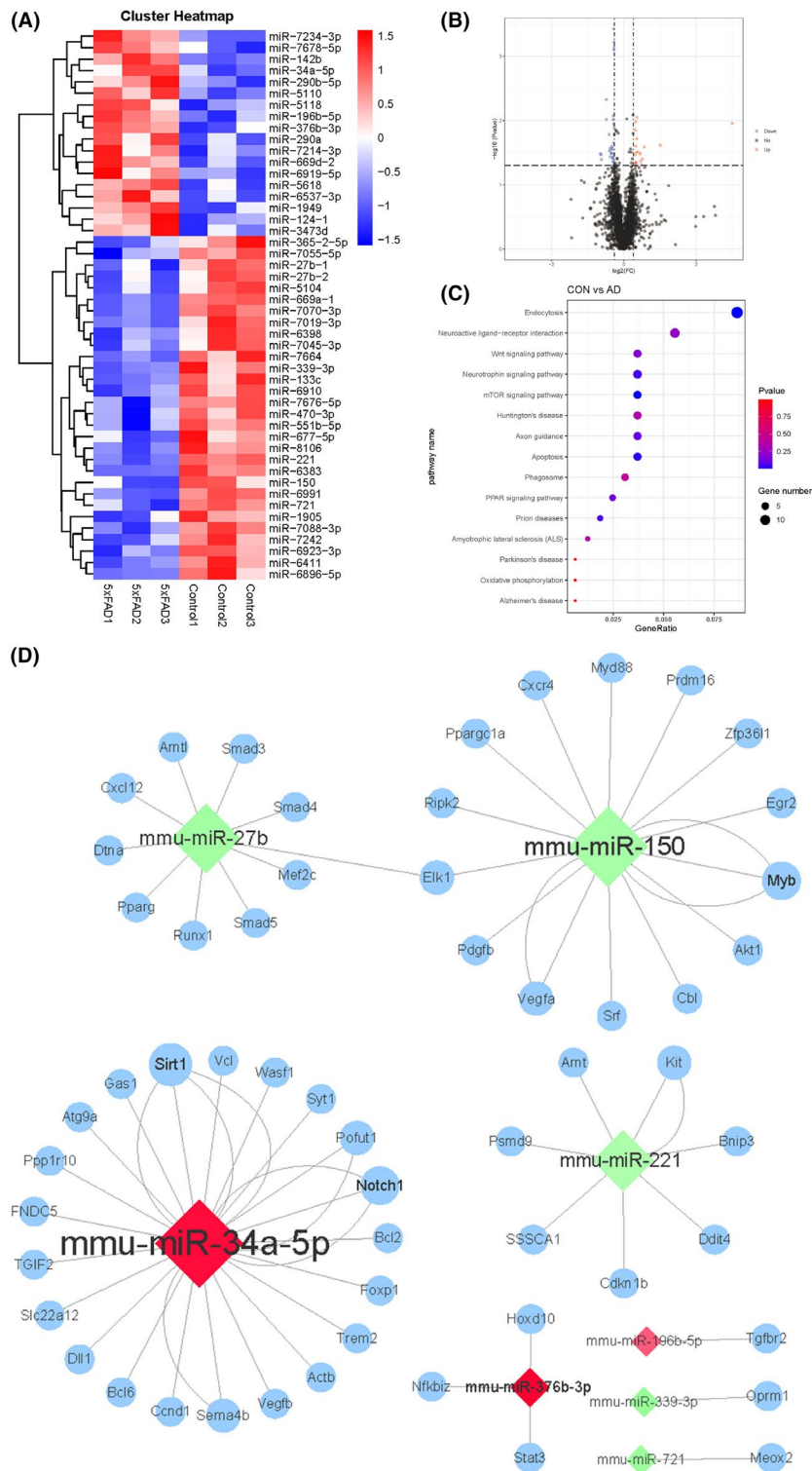
Temperature ($^{\circ}$ C)	Time	Ramp rate	Cycle(s)
95	5 min	2 $^{\circ}$ C/s	1
95	30 s		40
60	1 min		40
4	5 min		
98	10 min		1
4	∞		1

microarray assay. The miRNA levels were evenly distributed in all samples (Figure 1B). Figure 1C illustrates Pearson's correlation of the samples. There were 48 differentially expressed miRNAs, with 18 upregulated and 30 downregulated (FC ≥ 1.2 , $P < .05$), as shown in the heatmap and scatter plot (Figure 2A,B). miR-34a-5p was upregulated by 1.37-fold ($P = .03$).

3.2 | Function and pathway analysis

KEGG pathway analysis (Figure 2C) revealed that the differentially expressed microRNA-related target genes were associated with functions and signaling pathways involved in learning and memory

FIGURE 2 Comparison of the microarray analysis by hierarchical clustering, scatter plot, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, and regulatory network analysis. A, Heatmap reveals the classification of 5XFAD mice (x-axis) according to differentially expressed miRNA (y-axis) and unbiased clustering analysis. Every row represents an miRNA, and every column represents a urinary exosome sample. B, Scatter plot comparison of the fold changes identified by the microarray detection and analysis and their corresponding P values. The vertical dashed lines correspond to 1.2-fold upregulation (red points) and downregulation (blue points), separately, and the horizontal line represents $P = .05$. C, Dot plot of the KEGG pathway analysis of the enriched pathways associated with the differentially expressed proteins. x-axis: fold enrichment of each pathway; y-axis: pathway name; color: statistical significance (P value); dot size reflects the number of genes distributed to each KEGG pathway. D, miRNA-target interaction network. Putative targets were confirmed by exploring the miRtarBase database of experimentally validated miRNA target genes, followed by screening of the KEGG database for pathways participating in neurodegenerative diseases and the nervous system. Red and pink diamonds: upregulated pathways; green diamonds: downregulated pathways. The line linking the miRNA and its target gene represents the published reference; the number of lines indicates the extent of evidence



and related to neurodegenerative diseases. These included the following pathways (followed by count and GeneRatio in parentheses): Endocytosis (14; 0.09), mTOR signaling pathway (6; 0.04), apoptosis (6; 0.04) (*Myd88/Cyca/Akt1/Irak1/Cflar/Bcl2*), neurotrophin signaling pathway (7; 0.04), prion diseases (3; 0.02

(*Elk1/Lamc1/Notch1*), axon guidance (6; 0.04), PPAR signaling pathway (4; 0.02), Wnt signaling pathway (6; 0.04), neuroactive ligand-receptor interaction (9; 0.06), Huntington's disease (6; 0.04) (*Pparg1a/Cyca/Grm1/Pparg/Polr21/Sin3a*), amyotrophic lateral sclerosis (ALS) (2; 0.01) (*Cyca/Bcl2*), phagosome (5; 0.03),

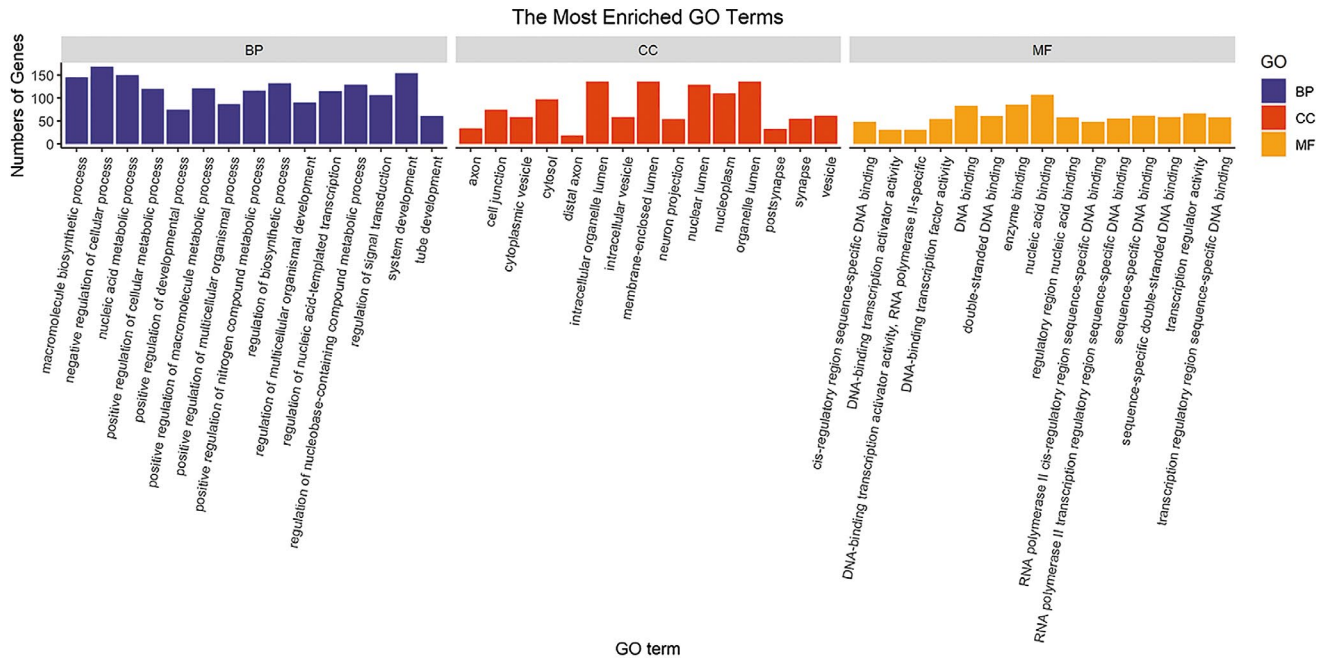


FIGURE 3 Gene ontology (GO) analysis of urinary exosome miRNA microarray data for a 5XFAD mice model of AD. Histogram of the differentially expressed proteins in the biological processes, cellular components, and molecular functions categories; the top 15 proteins are displayed. Comparison of the numbers of genes identified for the various GO terms between the control and 5XFAD mice model. The top 15 terms ($P < .05$) are shown

oxidative phosphorylation, Alzheimer's disease (1; 0.01) (*Cyca*) and Parkinson's disease (1; 0.01) (*Cyca*) (Figure 2C).

The differentially expressed miRNAs that we identified included mmu-miR-27b, mmu-miR-150, mmu-miR-34a-5p, mmu-miR-221, mmu-miR-336b-3p, mmu-miR-196b-5p, mmu-miR-339-3p, and mmu-miR-721 (Figure 2D). GO functional enrichment analyses suggest that the differentially expressed miRNAs were mainly associated with negative regulation of cellular processes related to neuron projection and (post)synapse, and with specific DNA binding that might participate in the physiological function and the progression of Alzheimer's disease (Figure 3).

3.3 | Predicted differentially expressed miRNA targets

The six differentially expressed miRNAs (mmu-miR-196b-5p, mmu-miR-339-3p, mmu-miR-34a-5p, mmu-miR-376b-3p, mmu-miR-677-5p, and mmu-miR-721) associated with Alzheimer's disease pathogenesis were then selected for correlation and validation of miRNA expression in urinary exosomes, via ddPCR (Figure 4A). The ratio between the target gene Ct value and U6 snRNA reference gene Ct value was calculated for all samples (Figure 4B). Based on ddPCR validation, the changes in expression of miR-196b-5p (1.96-fold upregulation), miR-339-3p (0.84-fold downregulation),

miR-34a-5p (3.64-fold upregulation), and miR-376b-3p (4.53-fold upregulation) were consistent with the microarray results (Table 4). The Venn diagram intersections indicate that three differentially expressed targeted protein and corresponding genes, beta-actin (*Actb*), cathepsin A (*Ctsa*), and major urinary protein 19 (*Mup19*)⁹ were common to the model and control groups (Figure 4C).

A single miRNA can downregulate a large number of target mRNAs, and the suit of miRNA target genes co-mediated by a distinct miRNA generally constitutes a biologically comprehensive network of functionally related to molecules. Using bioinformatics pathway analysis tools, we therefore created a global molecular network of experimentally validated targets for distinct miRNAs. To elucidate the interactions between the miRNA gene targets and their related signaling pathways, we summarized the network for the six targeted miRNAs, their validated target genes, and the important related signaling pathways (Figure 5). The six candidate miRNAs display functional roles in production of proteins directly associated with neurodegenerative diseases including AD pathology, as well as other important proteins known to take part in AD, such as protein related to mitochondrial oxidative chain (*Cytochrome c*), apoptosis regulatory protein (*Bcl-2*), autophagy and metabolism associated protein-NAD-dependent protein deacetylase sirtuin-1 (*Sirt1*). Transforming growth factor-beta (*Tgfb*) targeted by miR-196b-5p results in the generation of A β via induction of TGF- β 1-induced antiapoptotic factor self-aggregation in AD.¹⁷ Neurogenic locus notch homolog protein

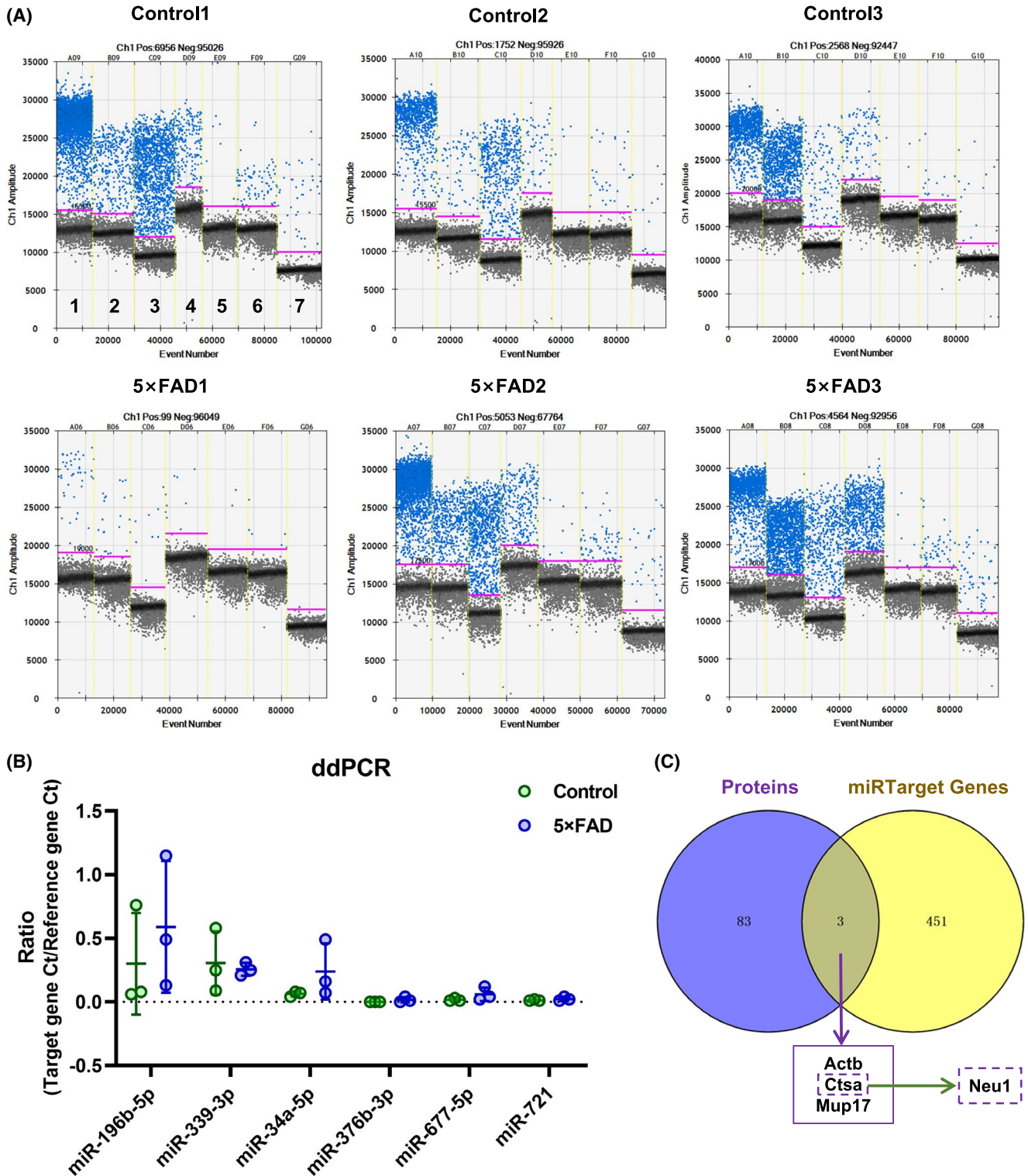


FIGURE 4 Differentially expressed candidate miRNA in the urinary exosome miRNA microarray analysis of a 5XFAD mice model of AD. A, Scatterplot of the droplet digital PCR data. Grey dots are negative droplets. Blue and green dots are positive droplets (above the pink horizontal threshold). x-axis: number of droplets; y-axis: signal amplitude. The miRNA quality thresholds for each sample are shown in the first lane of each panel: Lane 1, U6; Lane 2, mmu-miR-196b-5p; Lane 3, mmu-miR-339-3p; Lane 4, mmu-miR-34a-5p; Lane 5, mmu-miR-376b-3p; Lane 6, mmu-miR-677-5p; and Lane 7, mmu-miR-721. B, Quantitative analysis of the target miRNA ratio after normalization of the ddPCR absolute values. C, Venn diagram of the number of significantly altered miRNAs (with FC ≥ 1.2 and $P < .05$) for the targeted genes; the plot represents the intersections of the significantly altered urinary exosome proteins, comparing month-old 5XFAD mice and their control littermates. Purple: differentially expressed miRNAs; yellow: shared differentially expressed urinary exosome proteins



TABLE 4 Summary of the data analyzed by microarray and ddPCR

miRNA	ProbeName	Accession	Chromosome	Strand	Start	Stop	Fold change (microarray)	P value	Fold change (ddPCR)
mmu-miR-196b-5p	20 501 773	MIMAT0001081	chr6	-	52 230 129	52 230 150	1.25	.01	1.96
mmu-miR-339-3P	20 500 985	MIMAT0004649	chr5	-	139 369 673	139 369 695	0.79	.01	0.84
mmu-miR-34a-5p	20 500 916	MIMAT0000542	chr4	+	150 068 473	150 068 494	1.37	.03	3.64
mmu-miR-376b-3p	20 501 791	MIMAT0001092	chr12	+	109 723 508	109 723 528	1.38	.04	4.53
mmu-miR-677-5p	20 504 674	MIMAT0003451	chr10	+	128 085 291	128 085 312	0.82	.04	3.10
mmu-miR-721	20 504 754	MIMAT0003515	chr5	-	136 375 777	136 375 797	0.79	.03	1.96

1(*Notch1*) targeted by miR-34a-5p may be related to the risk of AD as the expression of *Notch1* mRNA was significantly increased in human brain microvascular endothelial cells from AD patients compared to normal subjects.¹⁸ This network allowed us to display an integrated profile of possible functional microRNA-mRNA-genetic interactions and signaling pathways.

4 | DISCUSSION

Diagnostics for AD has developed from use of assessments focused only on neuropsychological symptoms to the use of combined assays involving molecular signatures. Research on therapy has focused on minimizing invasiveness and increasing availability. Reliable, noninvasive methods for diagnosing early-stage AD is crucial for increasing the efficiency of available therapeutic treatments.¹⁹

To address this need, we screened for urinary exosomal miRNAs in a well-characterized mouse model of AD, in the early stage, and identified 48 differentially expressed miRNAs (18 upregulated and 30 downregulated). Importantly, miR-34a-5p, which contributes to the pathological development of AD and is supported as a preclinical biomarker of AD,^{20,21} was upregulated in our model.

We identified various signaling pathways associated with the differentially expressed miRNAs and their target genes. It is notable that we detected these particular putative targets, and their associated signaling pathways, using 1-month-old model mice. This suggests that dysregulation of the related signaling pathways occurs in the early stage of AD.

Patients with AD are known to have upregulated miR-27b in the cerebellum, hippocampus, and medial frontal gyrus.^{22,23} A recent study, based on platelets from patients with Alzheimer's disease, suggests an miRNA-specific imbalance in the miR-150 precursor. Moreover, our findings regarding the expression of miR-150 are consistent with previous studies showing that miR-150 was downregulated in plasma-derived exosomes from patients with Alzheimer's disease,²²⁻²⁴ relative to its expression in healthy controls.

Lysosomal neuraminidase-1 (NEU1) constitutes a multienzyme complex with β -galactosidase and CTSA.²⁵ A site on NEU1 takes part in binding to CTSA; in the absence of CTSA, NEU1 self-associates into chain-like oligomers, similar to the situation that occurs in the lysosomal storage disease known as galactosialidosis.²⁵ Importantly, for the same model, we previously found that urinary exosomal NEU1 is differentially expressed in 1-month-old mice models and controls.⁹ These differences in urinary exosomal protein expression may clarify the potential interactions that occur during pathogenesis in Alzheimer's disease.

The six best candidate biomarkers identified here were among the top biomarkers identified during our pilot screening of 15 miRNAs. These candidate biomarkers were mapped to AD-related pathology via *in silico* screening against two independent databases of predictive targets (TargetScan) and validated targets (MirTarBase).

These results suggest that urinary exosomal miRNAs are promising candidates to supplement or replace invasive cerebrospinal

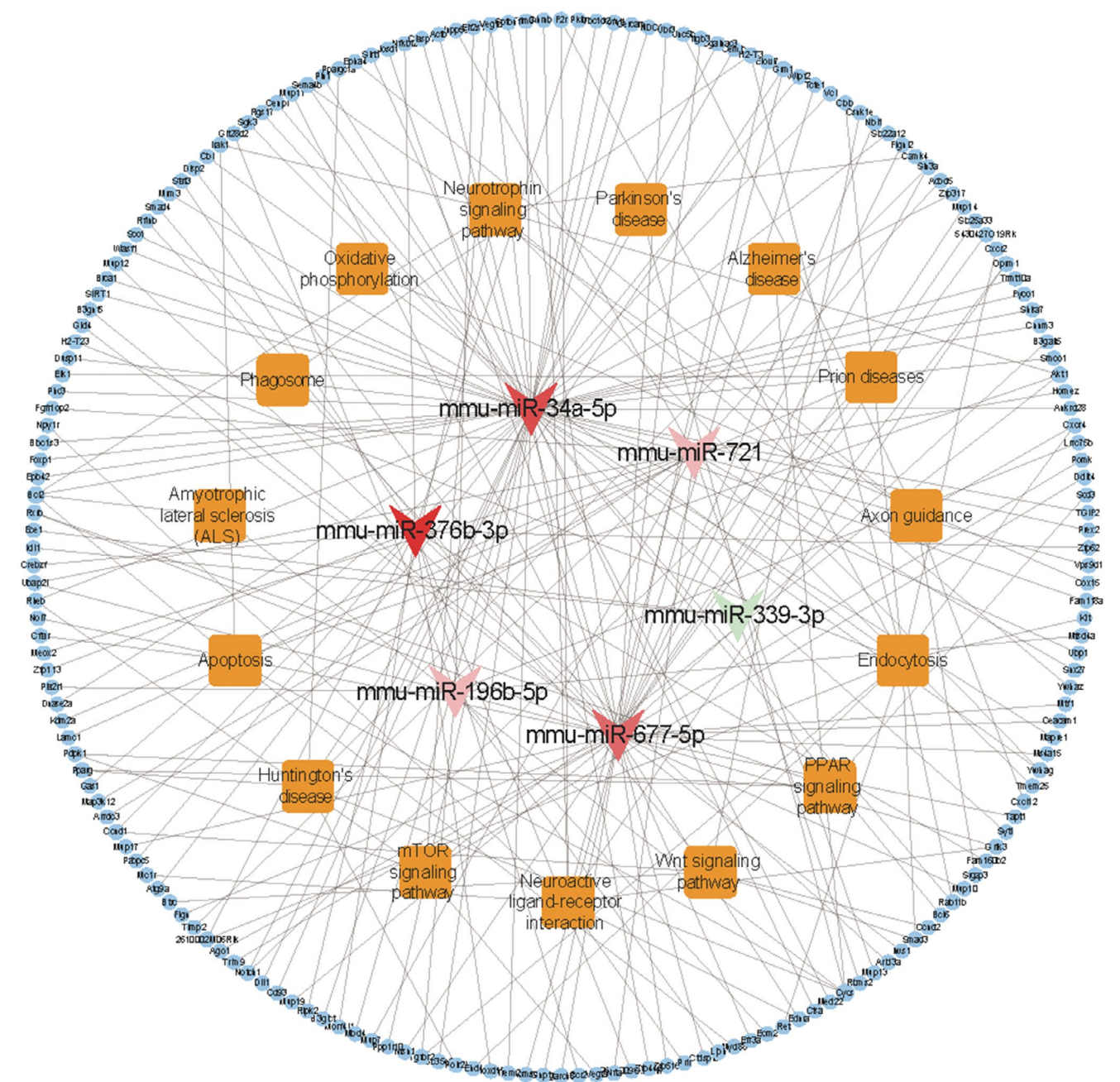


FIGURE 5 Combined molecular analysis of the miRNA-target interaction network, for the urinary exosome miRNA microarray analysis of a 5XFAD mice model of AD. Network visualization of functional annotations of experimentally validated target genes and their miRNAs. Node size reflects the degree of connectivity. Orange nodes: functional annotations obtained via KEGG functional enrichment analysis. Red: upregulated; green: downregulated

fluid-derived markers for identifying early AD (patent pending: PCT/IB2016/052440). These markers should be verified using larger cohorts.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

CQ and ZQS designed the project. ZQS, YJQ, YFX, LZ, LZ, LZ, YLH, WJZ, PY, YZ and XLL performed most of the experiments. CQ and ZQS wrote the manuscript. All authors analyzed the data.

DATA AVAILABILITY STATEMENT

All data supporting the conclusions of this manuscript will be made available by the corresponding authors, without undue reservation, to any qualified researcher.

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