INTRODUCTION

Alzheimer’s disease (AD) is an age-associated, chronic neurodegenerative disease related to irreversible cognitive deficits and progressive dementia. As the course of AD begins decades before clinical features appear, there is an urgent need to identify early-stage markers, particularly before Aβ plaque accumulation. Urine can be collected noninvasively in abundance and is also a significant potential resource for the discovery of novel biomarkers for AD because the kidneys collect most waste matter as final metabolites that have been excreted into the cerebrospinal fluid and blood. 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). Proteins that are highly abundant in urine, including albumin, might
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 nanosized 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.\(^7\) 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\(\beta_{42}\), 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. AOAH, LY86, and clusterin were the first brain-associated cell biomarkers 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\(\beta_{42}\) deposition in the brains of mouse model (Figure 1A). These findings may help develop methods for the prevention of AD.

## 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\(^{K670N*M671L*I716V*V717I, PSEN1*M146*L286V}\) 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 04609), 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, EIQ3-03001, 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 3000x 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\(^6\) 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.
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,\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 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
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/Cycs/Akt1/Irak1/Cflar/Bcl2), neurotrophin signaling pathway (7; 0.04), prion diseases (3; 0.02) (Elk1/Lac11/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) (Pparg1c/Cycs/Grm1/Pparg/Potr2l/Sin3a), amyotrophic lateral sclerosis (ALS) (2; 0.01) (Cycs/Bcl2), phagosome (5; 0.03).
oxidative phosphorylation, Alzheimer’s disease (1; 0.01) (Cycs) and Parkinson’s disease (1; 0.01) (Cycs) (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).

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 Apvia induction of TGF-β1-induced antiapoptotic factor self-aggregation in AD.17 Neurogenic locus notch homolog protein

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.
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.
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. 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.

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, 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. 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, relative to its expression in healthy controls.

Lysosomal neuraminidase-1 (NEU1) constitutes a multienzyme complex with β-galactosidase and CTSA. 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. Importantly, for the same model, we previously found that urinary exosomal NEU1 is differentially expressed in 1-month-old mice models and controls. 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 fluid.
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, 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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