MiR-485-3p serves as a biomarker and therapeutic target of Alzheimer's disease via regulating neuronal cell viability and neuroinflammation by targeting AKT3

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Abstract

Background: Numerous microRNAs (miRNAs) have been identified as functional molecules in Alzheimer's disease (AD) pathogenesis. This study aimed to investigate the diagnostic value of microRNA-485-3p (miR-485-3p) in AD patients, evaluate the effect of miR-485-3p on neuronal viability and neuroinflammation, as well as the underlying molecular mechanisms.

Methods: Quantitative Real-Time PCR was used to estimate expression of miR-485-3p and AKT3. A ROC analysis was used to evaluate the diagnostic value of miR-485-3p. The correlation of miR-485-3p with patients' MMSE score and inflammatory response was analyzed. Using Aβ-treated SH-SY5Y and BV2 cells models, the effects of miR-485-3p on neuronal proliferation, apoptosis, and neuroinflammation were explored. A luciferase reporter assay was used to confirm the target gene of miR-485-3p in both SH-SY5Y and BV2 cells.

Results: Serum miR-485-3p expression was significantly upregulated in AD patients and cell models, which had a high diagnostic accuracy and correlated with MMSE score and inflammatory response in AD patients. The knockdown of miR-485-3p in SH-SY5Y and BV2 cells was found to significantly reverse the effect of Aβ treatment on neuronal viability and neuroinflammation. AKT3 was determined as a target of miR-485-3p, which might mediate the biological function of miR-485-3p in AD pathogenesis.

Conclusion: All the data indicated that increased serum miR-485-3p serves as a diagnostic biomarker in AD patients, and knockdown of miR-485-3p exerts a neuroprotective role by improving neuronal viability and weakening neuroinflammation, which may be mediated by AKT3. This study may provide a novel biomarker and therapeutic target for AD therapy.

Keywords
AKT3, Alzheimer's disease, apoptosis, diagnosis, microRNA-485-3p, neuroinflammation, proliferation
1 | INTRODUCTION

Neurodegenerative disease (ND) is a common and growing cause of global mortality and morbidity, especially in the elderly (Erkkinen et al., 2018). The most common neurodegenerative diseases include Alzheimer’s disease (AD), frontotemporal dementia (FTD) and its variants, progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), Parkinson’s disease (PD), dementia with lewy bodies (DLB), multisystem atrophy (MSA), and Huntington’s disease (HD) (Erkkinen et al., 2018). As a common ND, AD is by far the most common cause of dementia, accounting for 80% of all dementia diagnoses (Weller & Budson, 2018). It is highly incapacitated that progresses from mild memory problems to complete loss of mental function, leading to death (Alkasir et al., 2017). AD is mainly characterized by the formation of extracellular neuritic plaques and intraneuronal accumulation of neurofibrillary tangles (Jiang et al., 2015). Neuroinflammation is now well recognized as a prominent feature of the pathology of AD and a potential target for the treatment and prevention of the disease (Cai et al., 2014). Additionally, microglial activation is a major component of neuroinflammation in the central nervous system and provides the first line of defense when injury or disease occurs (Tang & Le, 2016). Therefore, microglia is an important cell for the occurrence of neuroinflammation and the inflammatory response analysis of microglia can reflect the changes of neuroinflammation laterally. As a result, microglia are often used as cellular models to study neuroinflammation.

And Aβ25-35–induced neuronal cells were wildly used to construct AD cell models (Li et al., 2017; Liu et al., 2018). Thus, novel approaches that could ameliorate neurotoxicity and neuroinflammation are needed to improve AD treatment.

MicroRNAs (miRNAs) are small non-coding RNAs, which regulate gene expression (Bernardo et al., 2015). They can regulate gene expression by directly binding to the 3’ untranslated region (3’-UTR) of the target mRNA (Bhaskaran & Mohan, 2014). MiRNAs are associated with physiological processes and pathological results in a variety of diseases, including ND (Correia de Sousa et al., 2019). In AD, some miRNAs members have been identified as candidate diagnostic biomarkers, such as miR-107 and miR-103a-3p (Chang et al., 2017). Studies have found that miR-485-3p was related to ND (Liu, Lang, et al., 2014). A recent study has indicated that miR-485-3p can interfere with the differentiation and proliferation of neuronal stem cells, meanwhile, the activity of neural stem cells plays a vital role in the pathological mechanisms of ND (Gu et al., 2020). And miR-485 has been found to be involved in the pathogenesis of Parkinson’s disease (Goh et al., 2019). Therefore, we speculated that miR-485-3p might has important function in AD. In addition, the regulatory role of miR-485-3p on cell viability and inflammatory response has also been reported in some human diseases (Sole et al., 2019; Zhang et al., 2019). However, the clinical value and biological function of miR-485-3p in AD is still unclear.

To improve the diagnosis and treatment of AD, this study sought to assess the serum expression of miR-485-3p in AD patients, evaluate the diagnostic value of miR-485-3p, and explore the effect of miR-485-3p on neuronal viability and neuroinflammation. Moreover, we attempt to confirm the relationship between miR-485-3p and AKT3 to further understand the underlying molecular mechanisms involving in the biological function of miR-485-3p.

2 | MATERIALS AND METHODS

2.1 | Patients and sample collection

This study was performed with the approval by the Ethics Committee of Shengli Oilfield Central Hospital. A total of 89 AD patients were recruited from Shengli Oilfield Central Hospital between 2013 and 2017. The diagnosis of AD patients was performed with the diagnostic criteria of National Institute of Neurological and Communication Disorders and Stroke/Alzheimer’s disease and Related Disorder Association (NINCDS-ADRDA) (McKhann et al., 1984). Additionally, 62 healthy volunteers were enrolled from the individuals, who received physical examination in Shengli Oilfield Central Hospital. There was no statistically difference in age, gender and education duration between the AD patients and health controls. Venous blood was collected from participants and centrifuged for the extraction of serum, which was further stored at −80°C for use. To evaluate the cognitive function of AD patients, the Mini-Mental State Examination (MMSE) score was measured and recorded with following definition: 21 ≤ MMSE score ≤26 represents mild dementia; 15 ≤ MMSE ≤20 represents moderate dementia; MMSE score <15 represents severe dementia (Kahle-Wrobleski et al., 2017). Written informed consent was obtained from each participant.

2.2 | Cell culture and treatment

A human neuroblastoma cell line SH-SY5Y and a human microglia cell line BV2 were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco’s modified Eagle’s medium (DMEM: Invitrogen, Thermo Fisher Scientific, CA, USA) in a humidified incubator with 5% CO2 at 37°C. The SH-SY5Y cells treated with Aβ25-35 (Sigma-Aldrich, Saint Louis, MO, USA) for 24 h was used to construct the neuronal cell injury model, and the BV2 cells treated with Aβ25-35 (Sigma-Aldrich, Saint Louis, MO, USA) was used to construct the neuronal inflammatory cell model.
2.3 | Cell transfection

To regulate the expression of miR-485-3p in SH-SY5Y and BV2 cells, miR-485-3p mimic, miR-485-3p inhibitor and the corresponding negative controls (mimic NC and inhibitor NC) were transfected into the cells using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Twenty-four hours after the transfection, the SH-SY5Y and BV2 cells transfected with miR-485-3p inhibitor and inhibitor NC were treated with Aβ25-35 and the subsequent cell experiments were performed.

2.4 | RNA extraction and quantitative real-time PCR

Total RNA was extracted from the serum and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized from RNA using a PrimeScript RT reagent Kit (Takara, Japan) according to the manufacturer's instruction. The relative expression levels of miR-485-3p and mRNA expression of AKT3 were measured using quantitative real-time PCR (qRT-PCR), which was carried out using a SYBR Green I Master Mix kit (Invitrogen, Thermo Fisher Scientific, Inc.) and the 7500 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Inc.). The final expression values were calculated by the 2−ΔΔCt method, and U6, and GAPDH, were respectively, used as internal controls for miR-485-3p and AKT3.

2.5 | Enzyme-linked immunosorbent assay

The inflammatory response in AD patients and the neuroinflammation of BV2 cell lines were evaluated by examining the levels of pro-inflammatory cytokines. The Enzyme-linked immunosorbent assay kit (BioSource, San Diego, CA, USA) was used to examine the concentrations of IL-1β, IL-6, and TNF-α in the serum samples and cell supernatant according to the protocols of manufacturer.

2.6 | MTT assay

The cell proliferation of SH-SY5Y cells was evaluated using an MTT assay. The cells with a density of 3 × 10^6 cell/well were seeded into 96-well plates and incubated at 37°C for 24 h. At the time point of 48 h, 0.5 mg/mL MTT (Sigma-Aldrich, MO, USA) was added into the wells with a further 4 h of incubation. Then, the MTT solution was removed and 200 μL dimethyl sulfoxide (DMSO) was added into the wells. The cell proliferation was examined by reading the absorbance at 490 nm using a microplate reader (Bio-Rad, model 550, Philadelphia, PA, USA).

2.7 | Cell apoptosis analysis

Cell apoptosis was detected by flow cytometry (Cytomics FC 500 MPL, Bechman Coulter). Briefly, after designed treatment, cells were harvested and stained with propidium iodide (PI) and annexin V staining according to the manufacturer's instructions. Then stained cells were analyzed by flow cytometry.

2.8 | Luciferase reporter assay

According to the bioinformatical prediction with miRanda (http://www.microrna.org/microrna/home.do), a complementary sequence of miR-485-3p was searched at the 3′-UTR of AKT3. To confirm the interaction between miR-485-3p and AKT3, a luciferase reporter assay was used. The wild-type (WT) or mutant type (MT) 3′-UTR of AKT3 was cloned into pGL3-luciferase basic vector (Promega, Madison, WI, USA). The combined vectors were co-transfected with miR-485-3p mimic, miR-485-3p inhibitor or the NCs into SH-SY5Y and BV2 cells by Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) according to the protocols of manufacturers. After 24 h transfection, the relative luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega).

2.9 | Statistical analysis

All the statistical analyses were carried out by SPSS 21.0 software (SPSS, Inc., Chicago, USA) and GraphPad Prism 7.0 software (Inc., Chicago, USA). All data were presented as mean ± standard deviation (SD). Differences between groups were analyzed using Student’s t test or one-way ANOVA followed by Tukey’s test. The correlation between indicators was assessed using Pearson correlation coefficient. A receiver operating characteristic (ROC) curve was plotted based on serum miR-485-3p expression levels to assess the diagnostic value of miR-485-3p in AD patients. A p < 0.05 indicated statistically significant.

3 | RESULTS

3.1 | Upregulated expression of miR-485-3p in AD patients and cell models

From the qRT-PCR results, the serum expression of miR-485-3p was increased in AD patients compared with the health controls (p < 0.001, Figure 1a). Consistently, the serum expression of miR-485-3p was also increased in Aβ-treated
SH-SY5Y and BV2 cells compared with the normal controls (all $p < 0.001$, Figure 1b,c).

3.2 Relationship of miR-485-3p with dementia severity and diagnostic value of miR-485-3p in AD patients

In this study, MMSE score were detected to reflect the dementia severity in AD patients, and the diagnostic value of miR-485-3p was evaluated by plotting a ROC curve. From the Figure 2a, we found that the serum miR-485-3p levels was negatively correlated with MMSE score of AD patients ($r = -0.817$, $p < 0.001$), and the number of patients in the mild, moderate and severe groups was 7, 59, and 23, respectively. The ROC curve shown in Figure 2b indicated that miR-485-3p had high diagnostic accuracy with an AUC of 0.933. At an optimal cutoff value of 1.435, the sensitivity was 84.3% and the specificity was 96.8%. Thus, the serum miR-485-3p expression, which was negatively correlated with the dementia severity, had a high diagnostic accuracy for the differentiation between AD patients and health controls.

3.3 Correlation of miR-485-3p with inflammatory response in AD patients

To evaluate the correlation between miR-485-3p expression and inflammatory response in AD patients, the serum inflammatory cytokines levels were detected. As shown in Table 1, the miR-485-3p levels was positively correlated with IL-1β ($r = 0.732$, $p < 0.001$), IL-6 ($r = 0.655$, $p < 0.001$), and TNF-α ($r = 0.690$, $p < 0.001$) in AD patients. These indicated that the miR-485-3p was positively correlated with the inflammatory response.

3.4 Effect of miR-385-3p on neuronal proliferation and apoptosis in SH-SY5Y cells

By cell transfection, the upregulated expression of miR-485-3p by Aβ treated was successfully downregulated by the miR-485-3p inhibitor ($p < 0.001$, Figure 3a). The neuronal proliferation analyses indicated that the decreased neuronal proliferation induced by Aβ in SH-SY5Y cells was upregulated by the knockdown of miR-485-3p ($p < 0.01$, Figure 3b). Furthermore, the increased cell apoptosis rate induced by Aβ...
in SH-SY5Y cells was inhibited by the knockdown of miR-485-3p \((p < 0.05, \text{Figure 3c})\).

### 3.5 | Effect of miR-485-3p on neuroinflammation in BV2 cells

By cell transfection, the upregulated expression of miR-485-3p by Aβ treated was successfully downregulated by the miR-485-3p inhibitor \((p < 0.001, \text{Figure 4a})\). In addition, the concentrations of inflammatory cytokines in cell supernatant were measured and showed that the increased levels of IL-1β (Figure 4b), IL-6 (Figure 4c) and TNF-α (Figure 4d) induced by Aβ in BV2 cells were all decreased by the knockdown of miR-485-3p \((all \ p < 0.001)\). Therefore, the knockdown of miR-485-3p could downregulate neuroinflammation.

### 3.6 | MiR-485-3p directly regulates AKT3 expression in SH-SY5Y and BV2 cells

A luciferase reporter assay was performed to confirm the interaction of miR-485-3p with AKT3. The putative binding site of miR-485-3p at the 3′-UTR of AKT3 was showed in Figure 5a. In SH-SY5Y cells, by cell transfection, the relative expression of miR-485-3p was upregulated by the miR-485-3p mimic, while was downregulated by the miR-485-3p inhibitor \((all \ p < 0.001, \text{Figure 5b})\). As represented in Figure 5c, the relative luciferase activity was significantly inhibited by the overexpression of miR-485-3p, whereas was promoted by the knockdown of miR-485-3p \((all \ p < 0.001)\). In contrast, no changes were observed in luciferase activity in the MUT group \((all \ p > 0.05)\). From the Figure 5d, we found that the relative mRNA expression of AKT3 was inhibited by the overexpression of miR-485-3p, while was promoted by the knockdown of miR-485-3p \((all \ p < 0.05)\).

Consistently, in BV2 cells, the miR-485-3p mimic upregulated the miR-485-3p expression, whereas the miR-485-3p inhibitor downregulated the miR-485-3p expression \((all \ p < 0.001, \text{Figure 5e})\). As shown in Figure 5f, the relative luciferase activity was significantly suppressed by the overexpression of miR-485-3p, whereas was promoted by the knockdown of miR-485-3p \((all \ p < 0.05)\). However, there was no change in the MUT group \((all \ p > 0.05)\). From the Figure 5g, the miR-485-3p overexpression inhibited, while the miR-485-3p knockdown promoted the mRNA expression of AKT3 \((all \ p < 0.01)\).

### 4 | DISCUSSION

In recent decades, the important role of miRNAs in a variety of human diseases has been discovered (Rupaimoole & Slack, 2017). Some miRNAs, which are closely related to the pathogenesis of disease, also play a key role in AD. For example, Liu et al. found that miR-384 regulated both Amyloid Precursor Protein and β-Secretase expression, suggesting the role of miR-384 in AD progression (Liu et al., 2014). A study conducted by Jiang et al. investigated the role of miR-137 in the development of AD, which showed that miR-137 and expression of the CACNA gene inhibited the Tau hyperphosphorylation in AD (Jiang et al., 2018). Liu et al. confirmed that miR-106b inhibited Tau phosphorylation at yr18 by targeting Fyn in a model of

| Cytokines | \( R \) value | \( P \) value |
|-----------|-------------|-------------|
| IL-1β     | 0.732       | <0.001      |
| IL-6      | 0.655       | <0.001      |
| TNF-α     | 0.690       | <0.001      |

**TABLE 1** Correlation between serum miR-485-3p levels and inflammatory cytokines in AD patients

**FIGURE 3** Effect of miR-485-3p on neuronal proliferation and apoptosis of SH-SY5Y cells. (a) The miR-485-3p expression was increased by Aβ treatment, and the increased miR-485-3p expression induced by Aβ was downregulated by the miR-485-3p inhibitor. (b) The decreased neuronal cell proliferation induced by Aβ was promoted by the knockdown of miR-485-3p. (c) The increased neuronal cell apoptosis induced by Aβ \((**p < 0.01, *p < 0.05 vs. Controls;***p < 0.001, **p < 0.01, *p < 0.05 vs. Aβ)\)

**FIGURE 5** Effect of miR-485-3p on neuronal proliferation and apoptosis of SH-SY5Y cells. (a) The miR-485-3p expression was increased by Aβ treatment, and the increased miR-485-3p expression induced by Aβ was downregulated by the miR-485-3p inhibitor. (b) The decreased neuronal cell proliferation induced by Aβ was promoted by the knockdown of miR-485-3p. (c) The increased neuronal cell apoptosis induced by Aβ \((**p < 0.01, *p < 0.05 vs. Controls;***p < 0.001, **p < 0.01, *p < 0.05 vs. Aβ)\)
AD, which provided evidence for the inhibition of AD by miR-106b (Liu et al., 2016). These studies highlighted the vital effect of miRNAs in AD. In this study, we observed that the expression levels of miR-485-3p in AD patients and AD models were upregulated compared with the health controls. The abnormal expression of miR-485-3p in AD suggested that miR-485-3p may be involved in the development of AD.

MiRNAs are attractive molecules that have been identified as diagnostic tools for various human diseases, including AD (Bekris & Leverenz, 2015; Swarbrick et al., 2019). MiR-455-3p (Kumar et al., 2017) and miR-501-3p (Hara et al., 2017) have been found to be biomarkers for the diagnosis of AD. The diagnostic value of miR-485-3p has been demonstrated in other diseases, such as gastric cardia adenocarcinoma (Wang, Zhang, et al., 2018) and non-invasive glioblastoma (Ebrahimkhani et al., 2018). Due to the increased serum miR-485-3p level in AD, ROC analysis was used in this study to evaluate its diagnostic performance in AD. The analysis results showed that serum miR-485-3p had high diagnostic accuracy in AD patients. Additionally, the serum miR-485-3p level was negatively correlated with MMSE score of AD patients, suggesting that miR-485-3p was correlated with the severity of AD.

To simulate neuronal cell injury and neuroinflammation in AD, Aβ25-35–induced neuronal cells were widely used to construct AD cell models (Liu et al., 2018; Wang, Sun, et al., 2018). In the pathogenesis of AD, impaired neuronal viability and uncontrolled inflammatory response lead to neurotoxicity. Therefore, it is necessary to improve neuronal viability and decrease neuroinflammation. For example, a study by Liu et al. showed that downregulation of miR-155 alleviated cognitive impairment and involvement of neuroinflammation in AD (Liu et al., 2019). Another study by Zhang et al. provided evidence for the neuroprotective effect of miR-200a-3p by promoting neuronal viability (Zhang et al., 2017). In addition, the regulatory effects of miR-485-3p on cell proliferation and inflammatory response have been studied in diseases such as AD (Gu et al., 2020) and cutaneous lupus erythematosus (Sole et al., 2019). In AD patients, we found that miR-485-3p was positively correlated with serum inflammatory cytokine levels. Further cell experiments showed that miR-485-3p expression is upregulated in Aβ-stimulated BV2 cells, and knockdown of miR-485-3p levels can reverse the promoting effect of Aβ on neuroinflammation of BV2 cells. Furthermore, knockdown of miR-485-3p promoted the decreased cell
proliferation induced by Aβ, whereas reduced the increased cell apoptosis induced by Aβ. Therefore, we believe that miR-485-3p may play a neurotoxic role in AD by reducing neuronal viability and improving neuroinflammation, and may be a therapy target.

In this study, bioinformatics analysis was performed to find the 3'-UTR presumed binding site of miR-485-3p in AKT3, and subsequent luciferase activity results showed that AKT3 was a direct target of miR-485-3p. The effect of AKT3 on cell viability has been widely studied in breast cancer tumor cells, which could promote cell proliferation and inhibit cell apoptosis (Hu et al., 2018). In addition, the regulatory role of AKT3 in regulating inflammatory response has also been reported in the literature (DuBois et al., 2019). Therefore, we speculate that AKT3 may also be involved in regulating neuronal cell proliferation, apoptosis and neuroinflammation, and mediating the neurotoxic effect of miR-485-3p in AD. Further experiments proved that in two cell lines, AKT3 was inhibited by the overexpression of miR-485-3p and promoted by the knockdown of miR-485-3p. This indicates that miR-485-3p may directly promote the AD process through negative regulation of AKT3.

5 | CONCLUSION

In conclusion, this study found that the serum miR-485-3p expression was upregulated in AD patients and Aβ-induced cell lines. In addition, miR-485-3p was correlated with MMSE score, suggesting that miR-485-3p was related to the severity of AD. Moreover, the increased miR-485-3p expression may be a candidate diagnostic biomarker in AD patients. Furthermore, AKT3 serves as a direct target of miR-485-3p, and knockdown of miR-485-3p in AD promoted neuronal cell proliferation, and inhibited neuronal cell apoptosis and neuroinflammation, which may be mediated AKT3. This
study may provide evidence for a novel diagnostic biomarker and a potential therapeutic target for AD therapy.

CONFLICTS OF INTEREST
The authors have declared no conflict of interest.

AUTHORS’ CONTRIBUTIONS
L. Yu, H. Li, and M. Li designed the study, analyzed and interpreted the data regarding, wrote and revised the manuscript. W Liu and L. Zhang collected clinical samples and analyzed and interpreted the clinical and cell experimental data. Q. Tian and H. Li performed the examination of cells. All authors reviewed the manuscript and approved the final version.

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