MiR-361-3p inhibits β-amyloid accumulation and attenuates cognitive deficits through targeting BACE1 in Alzheimer's disease

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The role of miR-361-3p in the pathology of Alzheimer's disease is unknown. The target scan was used to screen potential target genes of miR-361-3p, and β-site amyloid precursor protein (APP) cleaving enzyme 1 (BACE1) was emphasized. Results from western blotting and reverse transcription-quantitative polymerase chain reaction (RT-PCR) showed that down-regulated miR-361-3p was correlated with up-regulated BACE1 in Alzheimer's disease patients' brains. Luciferase assay confirmed that miR-361-3p directly targets BACE1. MiR-361-3p overexpression and knockdown experiments were performed and found that miR-361-3p could regulate the expression of BACE1, and the accumulation of APP-β in APPswe transfected SH-SY5Y cell. A Morris water maze test was performed and showed that overexpression of miR-361-3p improved cognitive deficits in APP/PS1 mice. We found miR-361-3p inhibited β-amyloid accumulation by targeting BACE1, which thus weakened cognitive deficits in Alzheimer's disease.

Keywords
Alzheimer's disease; miR-361-3p; BACE1; APP-β; cognitive deficits; APPswe cell

1. Introduction
Alzheimer's disease (AD) becomes the most common neurodegenerative dementia among the world as population grows and life expectancy increases (Ziegler-Graham et al., 2008). Multiple genetic and environmental factors are related to AD, especially sporadic AD (Lahiri et al., 2016). The extracellular accumulation of APP-β and the formation of amyloid plaques are the hallmarks of AD. The Aβ peptide is cleaved from the APP by γ-secretase complex and β-secretase (BACE1) (Selkoe and Hardy, 2016). BACE1 is a crucial enzyme that takes part in the production of β-amyloid and promotes initial cleavage at the β site of APP (Vassar et al., 1999). AD mouse models with amyloid or TAU pathology showed hippocampus-dependent memory deficits (Lo et al., 2013; Webster et al., 2014). It was reported that modest rather than full inhibition of Aβ in AD patients could lead to a reduction of pathology (Carney, 2018; McConlogue et al., 2007).

MicroRNAs (miRNAs) are non-coding RNAs found in multiple organisms, which can decrease levels of target mRNAs largely by binding to the 3′-untranslated regions (UTR) of target mRNAs (Bartel, 2004; Guo et al., 2010; Wu et al., 2006). MiRNAs proved to be potential biomarkers in many diseases in previous studies (Braga et al., 2017; Fkih M’hamed et al., 2017; Liu et al., 2017; Patoulia, 2018; Switlik et al., 2018). Among them, some studies have shown several miRNAs being abnormally expressed and may contribute to neuronal dysfunction (Hebert et al., 2008; Hebert and De Strooper, 2009). For example, the miR124/PTPN1 pathway is thought to be a potential therapeutic target for AD (Wang et al., 2018). MiR-142a-5p and miR-146a-50, etc. were found to be involved in the pathological process in AD (Sierksma et al., 2018). Inhibition of miR-206 increased the level of brain-derived neurotrophic factor (BDNF) improved memory of AD mice (Lee et al., 2012). Particularly, miR-361-3p, an important miRNA in the regulation of non-small cell lung cancer (Chen et al., 2016), pancreatic ductal adenocarcinoma (Hu et al., 2018), cervical cancer (Hu et al., 2018) and cutaneous leishmaniasis lesions (Lago et al., 2018), have been reported to be downregulated in AD (Wang et al., 2011). However, the pathological effect of miR-361-3p in AD and the possible molecular mechanisms are still poorly investigated.

To better understand the role of miR-361-3p in AD, the relationship between miR-361-3p and BACE1 was investigated. Also, we studied the effects of miR-361-3p in APPswe transfected SH-SY5Y cells and APP/PS1 mice. Functional studies were performed to confirm the biological significance of miR-361-3p in AD.

2. Materials and methods
2.1 Ethical statement
All experiments were authorized by the ethics committee of the Zhengzhou Central. All the experiments were performed according to the ethical standards in the 1964 Declaration of Helsinki and its later amendments and were reviewed and approved by the Zhengzhou Central Hospital.
2.2 Patients and tissue samples

Human brain tissues were obtained from Zhengzhou Central Hospital. Nine male and 21 female patients (mean age: 80.8 ± 7.2) were diagnosed with AD and included in the study. The control groups were 11 male and 19 female non-dementia patients with an average age of 79.7 ± 10.3. Autopsied and histopathologically confirmed AD and control frozen frontal cortex samples were obtained from Zhengzhou Central Hospital. Before the operation, the written informed consent was obtained by individual patient to use the excised brain tissue and approved by the Institutional Review Board of the Zhengzhou Central Hospital, which in accordance with the National Institutes of Health guidelines as well.

2.3 Animals and treatment

The APPswe/PS1dE9 (APP/PS1) transgenic AD model mice (6-7 months old) used in this study were obtained from the Chinese Academy of Medical Science. All mice were housed in a 12/12 h light/dark cycle with free access to food and water. This type of mice is bred in a C57BL/6 genetic background and over-expresses human amyloid precursor protein. Mice were divided into three groups, wild type (WT), APP/PS1, and APP/PS1 + miR-361-3p groups (n = 15 in each group, 45 mice in total). Before stereotoxic injection, mice were anesthetized by isoflurane. There were no signs of peritonitis observed in our study. Stereotoxic injection of Agomir (100 μM, 1.5 μL) was performed on both side’s hippocampus regions (anterior/posterior = ± 1.9, dorsal/ventral = ± 2.4, medial/lateral = ± 1.9) by a Hamilton micro-syringe at the rate of 0.2 μL/min. Behavioral tests were performed two weeks after the injection. After the hippocampi were dissected and stored at -80 °C for biochemical analyses.

2.4 Cell culture

Human neuroblastoma cell line SH-SY5Y and 293 cell lines were purchased from ATCC (catalog number: CRL-2266). Cells were cultured in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) in a 37 °C incubator with 5% CO₂, containing 10% fetal bovine serum.

2.5 Plasmid and transient transfection

For transfection, cells were cultured in 25 cm² flasks. When the cell density achieved 70% ~ 80%, cells were transfected with 3 μg pAPPSwe, which was obtained from GenePharma (GenePharma, Suzhou, China) using 10 μL of Lipofectamine 2000 Reagent (Invitrogen, Waltham, MA, USA).

2.6 Mimic, inhibitor and agomir

M-361-3p mimics, inhibitors, and agomir were purchased from GenePharma (GenePharma, Suzhou, China). The mimics and inhibitor were transfected with Lipofectamine 2000 (Invitrogen, Waltham, MA, USA), with a concentration of 50 nM/well.

2.7 Luciferase reporter assay

WT or seed-mutated (MUT) BACE1 3’-UTR sequences were chemically synthesized (Sangon, Shanghai, China) according to the seed sequence of miR-361-3p. 293 cells were seeded into 96-well plate and transfected with 100 ng of WT BACE1 3’-UTR or MUT BACE1 3’-UTR, using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA). After 72h, a dual-luciferase reporter assay system was used to detected the luciferase activity according to the manufacturer’s recommendations (Promega, WI, USA). Renilla luciferase activity was used to normalize the luciferase activity.

2.8 RT-qPCR

RNA was extracted from the human tissue, 293, and SH-SY5Y cells by TRIzol (Takara, Dalian, China). The purity and concentration were measured by a ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The reverse transcription of RNA was achieved using the Prime ScriptTM RT Reagent Kit (Takara, Dalian, China). The reactions were carried out with SYBR® Green Master Mix (Takara). The primers were as follows: miR-361-3p, F: 5'-UCCCCACCUGUGAUCCUGAUUU-3', R: 5'-GCAAATCAGAATCACACCTG-3'; human BACE1, F: 5'-AGGGCTTGCACCTGTAGGAC-3', R: 5'-GCTGTGATGATGCGCAGATA-3'; human β-actin, F: 5'-GGACTTCGAGCAAGAGATGG-3', and R: 5'-AGGAGGAAGGCTGGAAGA-3'. Cycling conditions were as follows: Predenaturation at 95 °C for 5 min, denaturation for 10 sec at 95 °C, and annealing for 30 sec at 60 °C, with 40 cycles. The 2−ΔΔCt method was used to analyze the data.

2.9 Western blotting

Human tissues, SH-SY5Y cells, or mice hippocampi were lysed in RIPA buffer (Biotime biotechnology, Shanghai, China). 5× Laemmli SDS sample buffer was added into the supernatant of centrifuged lysate. Samples were boiled at 95 °C for 5 min. The boiled samples were loaded onto 10% SDS-PAGE, and transferred onto nitrocellulose filter membranes (Pall Corp., USA). Membranes were blocked in 5% nonfat milk for 2 hours at room temperature, and after that, incubated with the primary antibodies (BACE1, APP-β, cleaved caspase 3, Bcl-2, Bax, Proteintech Group, USA) with 1:1000 at 4 °C overnight. After washing, HRP-conjugated secondary antibodies (Proteintech Group, USA) were used for a 2 hours incubation at room temperature. After washing, the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA, USA) and a Kodak medical X-ray film (Denville Scientific Inc, USA) were used to detect the signals of the protein–antibody complexes. The protein was quantified by the Multi Gauge software V3.0 from Fuji Film (Santa Clara, CA, USA).

2.10 Apoptosis analysis

SH-SY5Y cells were seeded into 6-well plates one day before transfection. The cells were harvested 3 days after the treatment. After washing with phosphate-buffered saline, cell apoptosis was assessed by an Annexin V-APC Apoptosis Detection Kit (BD Biosciences, San Jose, CA, United States). Cells were collected, centrifuged, and re-suspended in 500 μL of binding buffer to the density of 10⁶ cells/mL. After that, 100 μL suspended buffer was incubated with 5 μL 7-AAD and 5 μL Annexin V-FITC avoiding light for 15 min at room temperature. Flow cytometry was performed for the apoptosis analysis.

2.11 Morris water maze test

The Morris water maze test was performed two weeks after the injection. The water tank was filled with water (25 ± 1 °C), and a hidden platform and the tank had four quadrants. The mice were trained for five consecutive days, four times each day. Every
Figure 1. Down-regulated miR-361-3p was correlated with high-regulated BACE1 in AD patients’ brains. A. The protein level of BACE1 in AD patients’ brains (n = 30) and normal (n = 30). The gray value of the western blots was quantified. The data were normalized to β-actin; B, C. The mRNA levels of miR-361-3p and BACE1 in AD and normal were determined by RT-qPCR. β-Actin was used as an internal control. D. The correlation between miR-361-3p and BACE1 level was analyzed. The error bar indicates the SEM. *** P < 0.001.

mouse was released into the water by facing the wall in one of four quadrants. Each mouse was allowed 60 seconds at most to find the hidden platform. If the mouse failed to reach the platform, it would directed to the platform and stayed there for 15 seconds. After training, exploratory tests were performed. In the test, the platform was removed; each mouse was allowed 60 seconds for free swimming. The recorded data were analyzed by computerized software (Wilmette, IL, USA).

2.12 Statistical analysis

GraphPad Prism 7 was used in this study. All data were presented as the mean ± SEM. Each experiment was repeated for three times. Two-way ANOVA and Student’s t-test were used for analysis. P < 0.05 indicates a statistically significant difference.

3. Results

3.1 Down-regulated miR-361-3p was correlated with up-regulated BACE1 in AD patients’ brain

The protein level of BACE1 and APP-β in the human AD frontal cortex tissues were found significantly increased as compared to the control (Fig. 1A). The mRNA expression of miR-361-3p was decreased (Fig. 1B) and BACE1 was increased (Fig. 1C) in the human AD frontal cortex tissues as compared to the control. The results showed negative correlation between the expression level of miR-361-3p and BACE1 (Fig. 1D).

3.2 BACE1 was a target of miR-361-3p

The target scan was used to screen potential target genes of miR-361-3p, and BACE1 was emphasized. To further study whether miR-361-3p directly target BACE1, the 3’-UTR sequence of BACE1 (WT) and mutated BACE1 3’-UTR sequences (MUT) (Fig. 2). pGL3-control plasmid or pRL-TK plasmid were co-transfected with miR-361-3p mimics or NC in 293 cells, respectively. Luciferase activity was measured 48h after the transfection. The result showed that miR-361-3p mimics markedly reduced the activity of the WT plasmid, but not MUT plasmid. Thus, it is concluded that miR-361-3p directly target BACE1 (Fig. 2).

3.3 MiR-361-3p regulates the expression of BACE1, and the accumulation of APP-β in APPswe transfected SH-SY5Y cell

SH-SY5Y/APPswe cells were successfully constructed. The results showed decreased expression of miR-361-3p (Fig. 3A), but the expression of BACE1 and APP-β were notably increased in SH-SY5Y/APPswe cell, confirmed by RT-PCR and Western Blotting (Fig. 3A,B). Cell apoptosis was significantly increased in SH-SY5Y/APPswe cell compared with SH-SY5Y cells (Fig. 3C). Compared with SH-SY5Y cells, the expression levels of apoptosis-related proteins caspase 3 and bax in SH-SY5Y/APPswe cells were significantly increased, while the expression of Bcl-2 was decreased (Fig. 3D). miR-361-3p mimics and inhibitor can successfully increase and inhibit its expression, which confirmed by RT-PCR (Fig. 3E). After SH-SY5Y/APPswe cells were transfected with miR-361-3p mimics, the expression of BACE1 and APP-β were decreased significantly. On the contrary, after SH-SY5Y/APPswe cell were transfected with miR-361-3p inhibitor, the expression of BACE1 and APP-β were increased significantly (Fig. 3F,G). These results indicated that miR-361-3p regulated the expression of BACE1 and the accumulation of APP-β in APPswe transfected SH-SY5Y cells.

3.4 MiR-361-3p suppressed the apoptosis of SH-SY5Y/APPswe cell

After SH-SY5Y/ APPswe cells were transfected with miR-361-3p mimics, the cell apoptosis was notably decreased. MiR-361-3p inhibitor transfection showed the opposite result (Fig. 4A). The apoptosis related-proteins cleaved caspase 3 and Bax were markedly decreased, and Bcl-2 was increased in SH-SY5Y/APPswe cell. MiR-361-3p inhibitor transfection showed the opposite results (Fig. 4B).
Figure 2. BACE1 was a target of miR-361-3p. A Construction schematic of WT or mutant BACE1 3’-UTR vectors. B. Relative luciferase activity was analyzed in 293 cells. The error bars indicate SEM. \( **P < 0.001 \).

Figure 3. MiR-361-3p regulated the expression of BACE1 and the accumulation of APP-\( \beta \) in SH-SY5Y cells. A. The relative miR-361-3p, BACE1, and APP-\( \beta \) mRNA expression were determined by RT-PCR; B. The protein levels of BACE1 and APP-\( \beta \) were detected by western blot; C. The apoptotic rate of SH-SY5Y and SH-SY5Y/APPswe cells. D. The protein levels of cleaved caspase3, Bcl-2, and Bax; E. The expression of miR-361-3p in SH-SY5Y/APPswe cells transfected with mimics and inhibitor was confirmed by RT-PCR; F. The relative miR-361-3p, BACE1, and APP-\( \beta \) mRNA expression were determined by RT-PCR; G. The protein levels of BACE1 and APP-\( \beta \) were detected by western blot. The error bar indicates the SEM. \( **P < 0.01, ***P < 0.001 \).

3.5 Injection of miR-361-3p improved cognitive deficits in APP/PS1 mice

The Morris water maze test was performed to assess the effect of miR-361-3p on mice spatial learning and memory. As shown in Fig. 5A, APP/PS1 mice showed increased latency and decreased the frequency of crossing platform locations compared to WT mice. Injection of miR-361-3p in APP/PS1 mice decreased the latency and increased the frequency of crossing platform location as compared to that in APP/PS1 mice. The body weight showed no significant difference between groups of mice. These results showed impaired spatial learning and memory in APP/PS1 mice, while injections of miR-361-3p restored these impairments.

APP/PS1 mice were sacrificed, and their hippocampi were dissected. The expression of miR-361-3p was decreased in APP/PS1 mice. MiR-361-3p was successfully overexpressed in APP/PS1 mice, which was confirmed by RT-PCR (Fig. 5B). The protein level of BACE1 and APP-\( \beta \) was markedly increased in APP/PS1 mice, and this was reversed by injection of miR-361-3p into the mice’s hippocampi (Fig. 5C). These results indicated that miR-361-3p exerted effects on spatial learning and memory improvement in APP/PS1 mice and BACE1 was involved in this pathological process.
Figure 4. MiR-361-3p suppressed apoptosis of SH-SY5Y/APPswe cell. A. Flow cytometric analysis of the apoptotic rate in SH-SY5Y cells; B. The protein levels of cleaved caspase3, Bcl-2, and Bax were determined by western blot, gray values of the western blot were quantified. The data were normalized to β-actin. The error bar indicates the SEM. **P < 0.01, ***P < 0.001.

Figure 5. Overexpress miR-361-3p improved cognition in APP/PS1 mice. A. The escape latency to the hidden platform during training, frequency of crossing platform location and body weight were analyzed; B. The mRNA level of miR-361-3p; C. The protein levels of BACE1 and APP-β were detected by western blot. The error bar indicates the SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

4. Discussion

Our results have shown that miRNAs are involved in the AD pathological process. For example, the miR-124/PTPN1 pathway was reported its importance in synaptic deficits in AD, and it is a promising new AD therapeutic target (Wanget al., 2018). MiR-128 knockout reduced Aβ production and inflammatory responses by targeting PPARγ and weakened AD-like performances in AD mice (Wang et al., 2018). Besides that, miR-9, miR-124a, miR-125b, were dysregulated and contributed to neuronal dysfunction in AD (Lukiw, 2007). It is a prognostic biomarker in cutaneous leishmaniasis caused by L. braziliensis (Lagoetal., 2018). Its expression was associated with better survival outcomes in cervical cancer (Hu et al., 2018). It also functions as an oncomiR in pancreatic ductal adenocarcinoma (Hu et al., 2018) and acts as a novel tumor suppressor for non-small cell lung cancer (Chen et al., 2016).

The expression and biological functions of miR-361-3p in AD were investigated, which was found markedly decreased in the AD brain. A previous study (Zhang and Pan, 2009) suggested that miR-361 was significantly up-regulated in the brain but down-regulated in the liver after hexogen exposure, which could cause neurotoxicity.

The protein and mRNA levels of BACE1 and APP-β in the human AD frontal cortex brain tissues were found markedly increased as compared with that in the control brain in our study.
It is reported that soluble oligomers of Aβ affect synaptic functions and induce neurotoxicity, and thus contribute to the disease process (Haass and Selkoe, 2007; Mucke and Selkoe, 2012; Selkoe and Hardy, 2016). Amyloid accumulation is thought to be an early factor in AD (Jack et al., 2010). BACE1 knockdown could decrease Aβ accumulation, which is expected to contribute to synaptic dysfunction and other downstream effects (Ferreira et al., 2015; Jin et al., 2011; Oz et al., 2013). Potential benefits resulting from pharmacologic inhibition of BACE1 were summarized and reported (Koelsch, 2017). In our study, we found a negative correlation between down-regulated miR-361-3p and up-regulated BACE1. Also, luciferase reporter assay verified that miR-361-3p directly targets and negatively regulates BACE1.

We found miR-361-3p regulated the expression of BACE1 and the accumulation of APP-β in APPswt transfected SH-SY5Y cells. MiR-361-3p overexpression markedly decreased the expression of BACE1 inhibited APP-β accumulation, and reduced the apoptosis of SH-SY5Y/APPswt cell. Taken together, we concluded that down-regulated miR-361-3p contributed to the pathological process by increased BACE1 expression, thus promoted the accumulation of Aβ and induced nerve cell apoptosis in vitro. Since degeneration mostly occurs in the hippocampal and cortex brain tissues, which leading to learning and memory deficits (Nofray and Prowenzale, 2002; Selkoe, 2002), we thus performed the in vivo experiment. These results revealed that overexpression of miR-361-3p in AD mice effectively reverses the deficits in spatial learning and memory of AD mice, and the expression of BACE1 and APP-β was significantly decreased. The inhibition of BACE1 is recently thought to provide a treatment strategy for AD (Vassar et al., 2014). Thus, this regulation mechanism is worthwhile to investigate. It seems that miR-361-3p may be an attractive therapeutic candidate for AD. It was reported that reactive oxygen species (ROS) could regulate the expression of miR-9, miR-128, and miR-125b in cultured neurons, which indicates that miRNAs may promote ROS’s pathogenic in AD (Lukiw, 2007). Salta et al. (2016) showed 1,4,5-trisphosphate 3-kinase B (ITPKB) regulates the activity of BACE1 and the phosphorylation level of tau protein by loss function of miR-132. Therefore, these studies open a door for further investigation of these aforementioned issues.

In conclusion, our study showed that miR-361-3p contributes to the pathological process of AD through promoting the accumulation of Aβ, inducing nerve cell apoptosis, and improving spatial learning and memory via targeting BACE1. MiR-361-3p may be considered as a potential therapeutic target for AD.

Abbreviations
AD: Alzheimer's disease; APP: Amyloid precursor protein; RT-PCR: reverse transcription-quantitative polymerase chain reaction.

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Conflict of interest
The authors declare no conflict of interest.

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