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Original Article

miR-26b inhibits total neurite outgrowth, promotes cells apoptosis and downregulates neprilysin in Alzheimer's disease

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Abstract: This study aimed to investigate the effect of miR-26b expression on neurites outgrowth and cells apoptosis in PC12 cellular model of Alzheimer's disease (AD). PC12 cells were stimulated by nerve growth factor and insulted by $A\beta_{1-42}$ to establish PC12 cellular AD model. Methyl thiazolyl tetrazolium (MTT) assay was then used to detect cells viability. Blank mimic, miR-26b mimic, blank inhibitor and miR-26b inhibitor plasmids were transferred into PC12 cellular AD models as NC1-mimic, miR-26b mimic, NC2-inhibitor and miR-26b inhibitor groups respectively. mRNA level, protein level, total neurite outgrowth and cells apoptosis were determined by qPCR, western blot, microscope and Hoechst/PI, respectively. MTT reduction rate was decreased in $A\beta_{1-42}$ insult group compared to control group ($P<0.001$). After plasmids transfection, the total neuritis growth was found to be reduced in miR-26b mimic group compared with NC1-mimic group ($P<0.05$) while was elevated in miR-26b inhibitor group compared with NC2-inhibitor group ($P<0.01$). As to cells apoptosis, the percentage of apoptosis cells was increased in miR-26b mimic group than NC1-mimic group ($P<0.05$), and was decreased in miR-26b inhibitor group than NC2-inhibitor group ($P<0.05$). In addition, neprilysin (NEP) protein and mRNA expressions were decreased in miR-26b mimic group than NC1-mimic group and was increased in miR-26b inhibitor group than NC2-inhibitor group. However, protein or mRNA expression of EIF2S1 and αTTP was not affected by miR-26b. In conclusion, miR-26b inhibits neurite outgrowth, induces cells apoptosis and downregulates NEP expression in PC12 cellular AD model.

Keywords: miR-26b, PC12, Alzheimer's disease, neurite outgrowth, apoptosis

Introduction

Alzheimer's disease (AD) is a progressive and irreversible neurological disorder with marked atrophy of cerebral cortex and loss of cortical or subcortical neurons, which is characterized pathologically by deposition of amyloid beta-protein (Aβ) in brain to form senile plaques (SP), and numerous neurofibrillary tangles (NFT) derived from filaments of microtubule associated highly phosphorylated tau proteins. In addition, neuronal synaptic dysfunction and vertebral nerve cell loss is also a characteristic of AD [1, 2]. Its clinical manifestations include progressive memory impairment, mental decline, altered behavior and personality and even death [3, 4]. However, it is not entirely clear how these typical characteristics are formed in AD.

miRNA, a class of small endogenous non-coding RNAs, regulates gene expression at the transcriptional level or post transcriptional level. They are widely distributed in the central nervous system, also play an important role in neurodevelopment, differentiation and maturation [5]. Several studies disclose that some pivotal AD proteins are served as targets of miRNAs, whose dysregulated expressions are correlated with the development and progress of various neurodegenerative diseases [6-9]. While some previous studies have suggested miR-26b upregulation, observed in AD, perturbs signaling pathways associated with neuronal cell cycle, and thereby causes pleiotropic phenotypes associated with the disease [10]. It indicated that miR-26b may be involved in neuronal aging and in the pathogenesis of neurodegenerative disorders, but their precise etiology

Table 1. Primers information

Gene name	Sequence (5'→3')
MiR-26b	F: *CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACCTATCC*
	R: ACACTCCAGCTGGGTTCAAGTAATTCAGGATA
U6	F: CTCGCTTCGGCAGCACA
	R: AACGCTTCACGAATTTGCGT
GAPDH	F: CCTTCTCTTGTGACAAAGTGGACA
	R: CATTTGATGTTAGCGGGATCTCG
NEP	F: GGAAGAAGTGGTTGTTTATGCTCC
	R: AGCATTTCTGGACTCCTTGTAGTT
αTTP	F: AGAATGTCCAGAACTAAGTGCAGA
	R: CTCCTGTACAATGAGCTCTGATGT
EIF2S1	F: GCTATGGTTACGAAGGCATTGATG
	R: GCTCCATCTGAACATTGAACACTC

in AD development and progression are poorly understood. This study aimed to investigate the role of miR-26b on neurite outgrowth and cells apoptosis in PC12 cellular model of AD.

Materials and methods

Cells culture

Rat pheochromocytoma cells (PC12 cells) were obtained from Shanghai Institutes for Biological Science (Shanghai, China). After cells resuscitation, these cells were cultured in Dulbecco Modified Eagle medium (DMEM) (Gibco, USA) with 10% (v/v) horse serum (Gibco, USA), 5% (v/v) fetal bovine serum (FBS) (Gibco, USA), 100 units/ml penicillin, and 100 μg/ml streptomycin (Corning, Lowell, USA). The PC12 cells were incubated in a humidified atmosphere (95% air and 5% CO_2) at 37°C. The medium was replaced every 2 days.

Preparation of oligomerized $A\beta_{1-42}$

$A\beta_{1-42}$ was purchased from Sigma company (USA) and was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 mM. Prior to the treatment, peptides were pre-incubated at 37°C for 7 days to promote aggregation and then diluted in medium to desired concentrations as described previously [11]. Soluble oligomerized $A\beta_{1-42}$ peptides (equivalent to 1 mM peptides) were added to cells to induce damaging effects.

Neurite differentiation and $A\beta_{1-42}$ insult

For induction of neuronal differentiation, the PC12 cells were treated with 20 ng/mL nerve growth factor (NGF) (Sigma, UA) for 72 hours at 37°C with 95% air and 5% CO_2. The medium was changed every 2 days, and fresh NGF was added each time.

At the day 4, 1 mM of $A\beta_{1-42}$ soluble oligomerized $A\beta_{1-42}$ peptides were added to NGF inducted PC12 cells and these cells were incubated for 24 h to establish cell of AD model.

Methyl thiazolyl tetrazolium (MTT) assay

After PC12 cells were induced by NGF and insulted by $A\beta_{1-42}$, cells viability was assessed by MTT reagent (Sigma, USA). Firstly, 100 mg of MTT was dissolved in 20 mL PBS to make a 5 mg/mL solution. Next, 10 μl of MTT solution was added to each well, which contained 100 μl culture medium, and incubated at 37°C for 4 h. Then the culture medium in the wells was sucked, and 100 μl of DMSO (Dimethyl sulfoxide) was added to each well, the solution was dissolved for 10 min. Finally, the plates were analyzed using a microplate reader (Molecular Devices, USA) at 490 nm. All processing groups were triplicated.

miR-26b transfection and subsequent assays

The PC12 cellular PC12 cellular AD model were divided into four groups and then transfected with blank mimic, miR-26b mimic, blank inhibitor, miR-26b inhibitor plasmids as NC1-mimic group, miR-26b-mimic group, NC2-inhibitor group and miR-26b-inhibitor group. All processing groups were triplicated.

After 24 h plasmids transfection, the expression of miR-26b in each group was detected by qRT-PCR. Then, cells morphology was observed and total neurite outgrowth was quantified by inversion fluorescence microscope. Cells apoptosis was detected by Hoechst/propidium (PI) method, and the expressions of apoptosis-related proteins were determined by western blot. In addition, mRNA and protein expressions of miR-26b predicted target genes were detected by qRT-PCR and western blot respectively.

qRT-PCR assay

miRNAs and mRNA expression were evaluated by qRT-PCR. The total RNA from cells was

Table 2. Antibodies information

Antibody name	Company	Country	Dilution ratio
Primary antibody			
Rabbit polyclonal Anti-Caspase-3 antibody	Abcam	USA	1:2000
Rabbit monoclonal Anti-cleaved Caspase-3 antibody	Abcam	USA	1:2000
Rabbit monoclonal Anti-P38 antibody	Abcam	USA	1:2000
Rabbit polyclonal Anti-P38 (phosphoT180+Y182) antibody	Abcam	USA	1:2000
Rabbit monoclonal Anti-GAPDH antibody	Abcam	USA	1:2000
Rabbit polyclonal Anti-Nephrin antibody	Abcam	USA	1:2000
Rabbit monoclonal Anti-EIF2S1 antibody	Abcam	USA	1:2000
Rabbit polyclonal Anti-TTPA antibody	Abcam	USA	1:2000
Second antibody			
Goat Anti-Rabbit IgG H&L	Abcam	USA	1:2000

extracted using TRIZol Reagents (Invitrogen, USA), according to the manufacturer's instructions. The quantitation of RNA samples was performed by OD 260. 1 μg of total RNA from each sample were used for cDNA synthesis with transcription kit (TOYOBO, Japan). And then cDNA products were subjected to qPCR with SYBR Green kit (KAPA, USA). The PCR amplification was performed as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 5 s, 61°C for 30 s. U6 or GAPDH was used as reference gene. All experiments were triplicated. The qRT-PCR results were calculated by the $2^{-\Delta\Delta Ct}$ method. The primers information was presented in **Table 1**.

Total neurite outgrowth measurement

After cells morphology was observed by inverted microscope (Nikon, Japan), Imaging Software Presage (Advanced imaging Concepts, Inc., USA) was used to quantify the total neurite outgrowth per cell which was defined as total length of neurite outgrowth of cells divided by number of cells included.

Hoechst/PI assay

Hoechst 33342 (λ_{ex}350 nm, λ_{em}461 nm) and PI (λ_{ex}535 nm, λ_{em}617 nm) were added to the culture medium at final concentrations of 8 and 1.5 M, respectively, and cultured at 37°C for 30 min. Images were collected by inversion fluorescence microscope (Nikon, Japan), the damaged cells and total cells were calculated under ×200 magnification, and the percentage of damaged cell was calculated. All experiments were triplicated.

Western blot assay

Total proteins were extracted from cells of each group with 1 ml ice-cold RIPA buffer (Thermo Fisher Scientific, USA), then total protein concentration in each sample was measured using the bicinchoninic acid (BCA) kit (Pierce Biotechnology, Rockford, IL, USA) and compared with the standard curve. Next, 20 μg total protein samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed, and separated proteins were electrophoretically transferred to polyvinylidene fluoride membranes (PVDF) membrane (Millipore, Bedford, USA). After blocked with 5% skim milk for 2 h, membranes were incubated with the corresponding primary antibody overnight at 4°C. Then, membranes were incubated with the appropriate HRP-conjugated secondary antibody for 1 h at room temperature. The bands were visualized using an enhanced chemiluminescence (ECL) kit (Millipore, Bedford, USA) followed by exposure to X-ray film. All experiments were triplicated. Specific antibody was used to detect the corresponding protein. Antibodies used for Western blot are listed in **Table 2**.

miR-26b target genes assay

The miR-26b target gene was predicted by these following analysis: (1) Target mRNAs of miR-26b were firstly predicted by miRwalk 2.0 with 6 or above positive results by 12 methods (miRwalk, Microt4, miRanda, mirbridge, miRDB, miRMap, miRNAMap, pictar2, PITA, RNA22, RNAhybrid, Targetsacn) (zmf.umm.uni-Heidelberg.de/apps/zmf/mirwalk2) [12]. (2) Genes correlated with AD pathology or risk were ana-

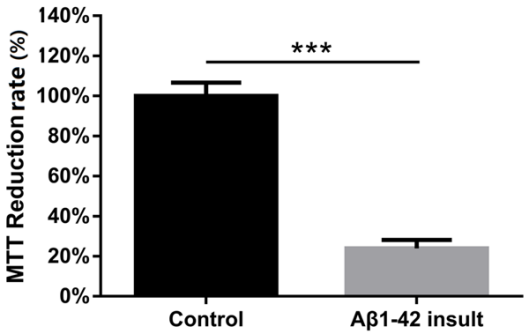

Figure 1. Effect of $A\beta_{1-42}$ insult on PC12 cellular AD model. Comparison between two groups was determined by t test. *P*<0.05 was considered significant. **P*<0.05, ***P*<0.01, ****P*<0.001.

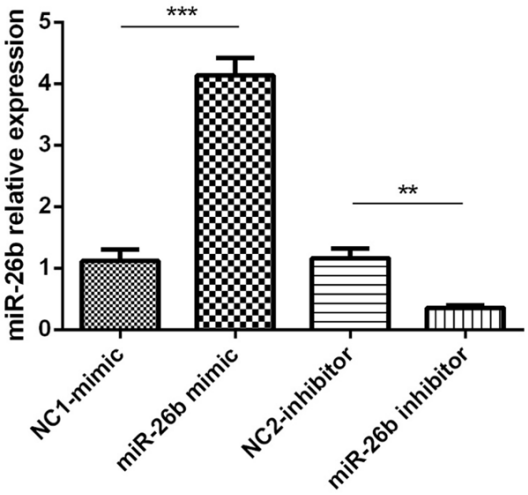

Figure 2. Effect of plasmids transfection on miR-26b expression in PC12 cellular AD model. Comparison between two groups was determined by t test. *P*<0.05 was considered significant. **P*<0.05, ***P*<0.01, ****P*<0.001.

lyzed by DisGene (www.disgenet.org) [13]. (3) Potential targeted genes were then analyzed by combining of miR-26b target genes and AD related genes. Thus, top three miR-26b gene (neprilysin (NEP), α-tocopherol transfer protein (αTTP) and in eukaryotic translation initiation factor 2 subunit 1 (EIF2S1) were selected to be determined by qRT-PCR and western blot.

Statistics

Statistical analysis was performed using SPSS software 22.0 (IBM, USA) and GrapgPad prism V5.01 (IBM, USA). Data was presented as mean ± standard. Comparison between two groups was determined by t test. *P*<0.05 was considered significant.

Results

Cells viability after $A\beta_{1-42}$ insult

In order to detect the establishment of AD model, we used MTT assay to determine the reduction rate of NGF inducted PC12 cells after $A\beta_{1-42}$ insult. We found that MTT reduction rate was decreased in $A\beta_{1-42}$ insult group compared to control group (*P*<0.001, **Figure 1**).

miR-26b expression after plasmids transfection

At 24 h following plasmids transfection, the result of qRT-PCR showed that, compared to NC1-mimic group, miR-26b-mimics induced an upregulation of miR-26b level in miR-26b-mimic group (*P*<0.001, **Figure 2**), and compared with NC2-inhibitor group, the miR-26b expression in miR-26b-inhibitor group was markedly decreased (*P*<0.01, **Figure 2**).

Effect of miR-26b on total neurite outgrowth

The effect of miR-26b on total neurite outgrowth was presented in **Figure 3A**, after transfection for 24 h, The mean length of total neurite outgrowth was markedly shorter in miR-26b-mimic group compared with the NC1-mimic group (*P*<0.05, **Figure 3B**) and was longer in miR-26b-inhibitor group compared with NC2-inhibitor group (*P*<0.01, **Figure 3B**).

miR-26b promoted cells apoptosis

Cells apoptosis were presented in **Figure 4A**, 24 h following plasmids transfection, cells apoptosis percentage in miR-26b-mimic group was obviously elevated than NC1-mimic group (*P*<0.05, **Figure 4B**), besides, cells apoptosis percentage was decreased in miR-26b-inhibitor group compared with NC2-inhibitor group (*P*<0.05, **Figure 4B**).

Meanwhile, compared with NC1-mimic group, the cleaved caspase-3 (C-Caspase 3) levels was elevated and the Phosphorylated P38 (*P*-P38) was reduced in miR-26b-mimic group (*P*>0.05, **Figure 4C**). While, the C-Caspase 3 levels was diminished and the P-P38 was enhanced in miR-26b-mimic group compared with NC2-inhibitor group (*P*>0.05, **Figure 4C**). Furthermore, the levels of total caspase 3 and P38 protein in miR-26b-mimic group and miR-26b-inhibitor group were not different from those in the control groups (All *P*>0.05, **Figure 4C**). This indicates that miR-26b promoted cells apoptosis in PC12 cellular AD model.

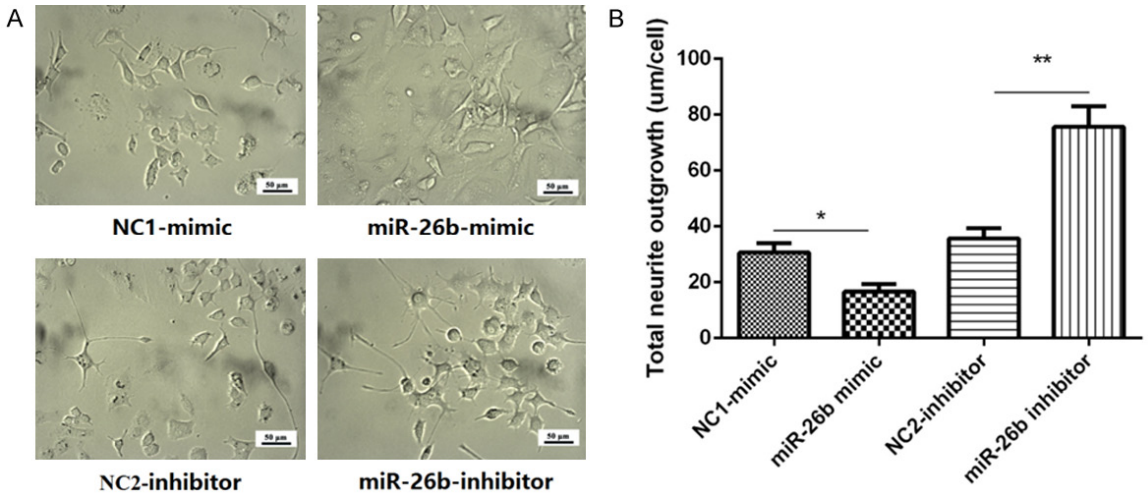

Figure 3. Effect of plasmids transfection on total neurite outgrowth in PC12 cellular AD model. A. The morphology of PC12 cellular AD model after transfected with miR-26b mimic or inhibitor. B. The mean length of neurite outgrowth in PC12 cellular AD model. Comparison between two groups was determined by t test. $P<0.05$ was considered significant. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

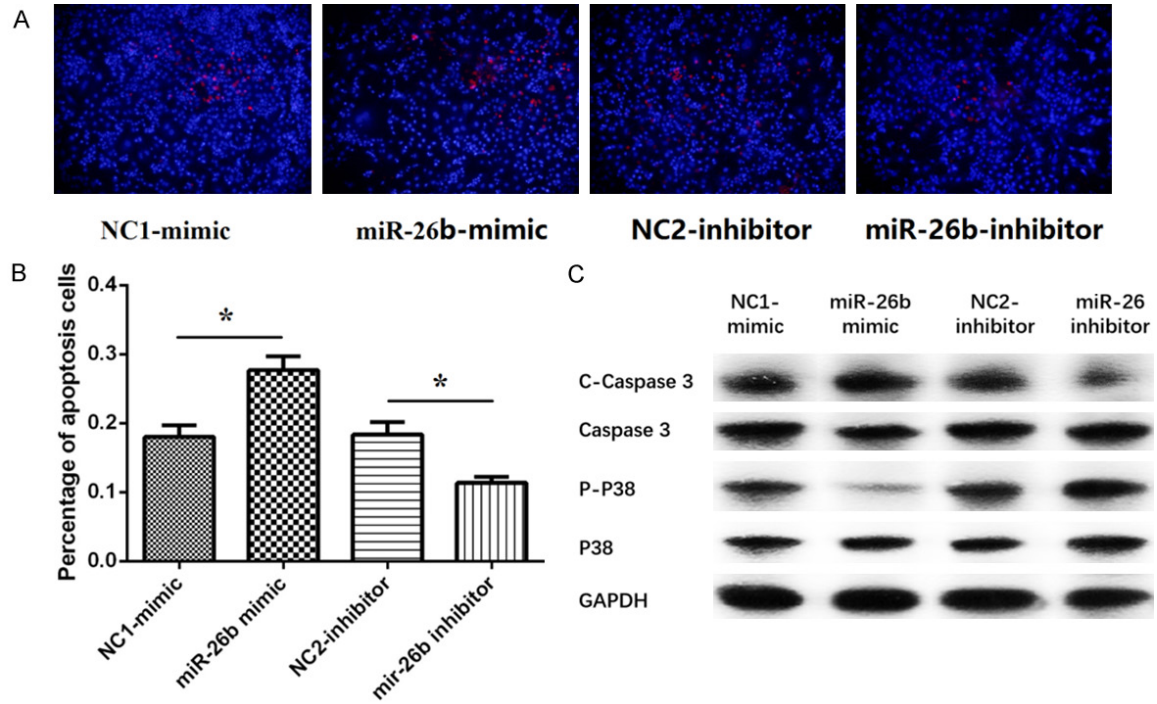

Figure 4. Effect of plasmids transfection on apoptosis in PC12 cellular AD model. A. The cells apoptosis of PC12 cellular AD model under fluorescence microscope. B. The cells apoptosis percentage of PC12 cellular AD model in miR-26b-mimic group and miR-26b-inhibitor group. C. The expression of C-Caspases 3, Caspases 3, P-P38 and P38 activity in miR-26b-mimic group and miR-26b-inhibitor group. Comparison between two groups was determined by t test. $P<0.05$ was considered significant. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Expression of candidate target genes

Compared with NC1-mimic group, the mRNA and protein expression of NEP gene was reduced in miR-26b-mimic group ($P<0.05$, **Figure 5A**, **5D**), and their expressions were increased in miR-26b-inhibitor group ($P<0.01$, **Figure 5A**, **5D**). However, no difference of the mRNA and protein expression of EIF2S1 and αTTP was found (all $P>0.05$, **Figure 5B-D**).

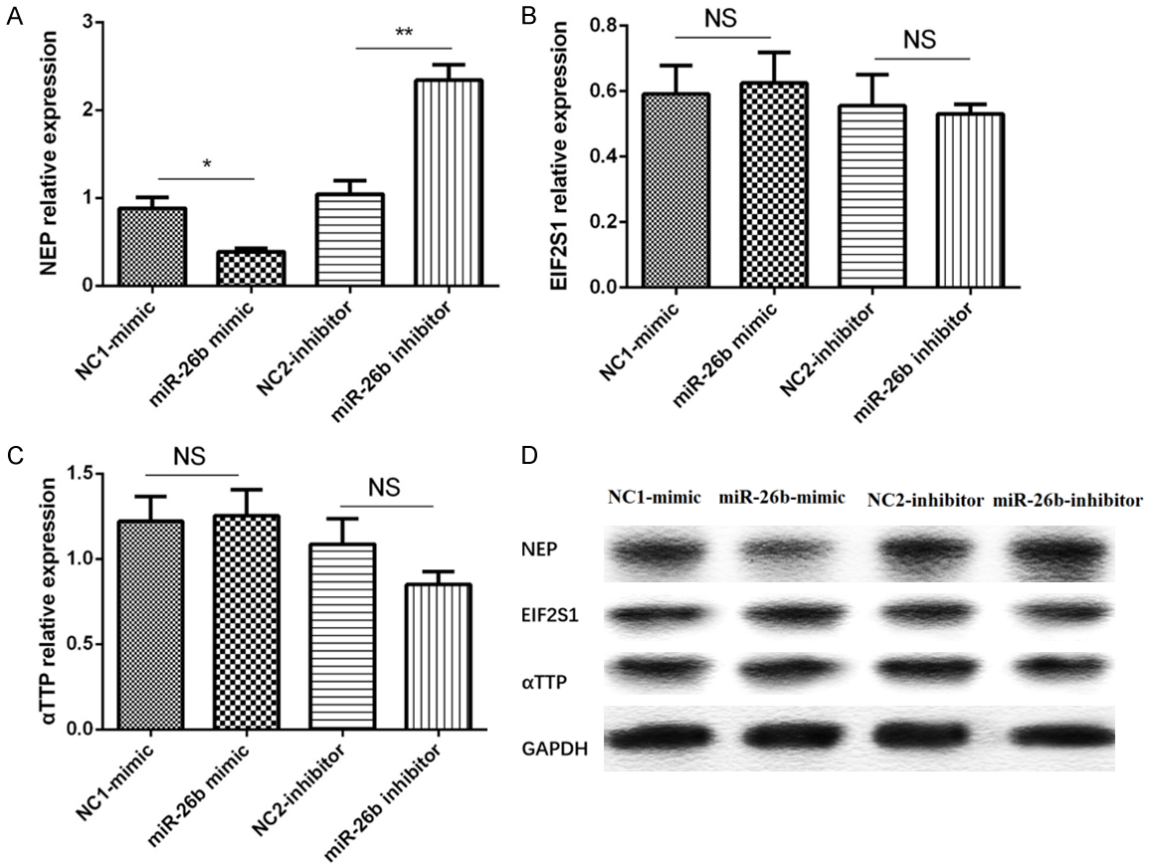

Figure 5. Effect of miR-26b levels on NEP, EIF2S1, αTTP genes. A. The expression of NEP mRNA in different groups. B. The expression of EIF2S1 mRNA in different groups. C. The expression of αTTP mRNA in different groups. D. The expression of NEP, EIF2S1, αTTP protein in different groups. Comparison between two groups was determined by t test. $P<0.05$ was considered significant. $*P<0.05$, $**P<0.01$, $***P<0.001$.

Discussion

In this study, we found that (1) miR-26b inhibited the total neurite outgrowth and promoted cells apoptosis in PC12 cellular AD model. (2) miR-26b reduced the NEP expression but not αTTP or EIF2S1 expression in PC12 cellular AD model.

miRNAs are involved in post-transcriptional regulation of gene expression in multicellular organisms by affecting both the stability and translation of mRNAs [14]. A deal of miRNAs has been proposed to be involved in pathogenesis of AD and other neurological disorders [10, 15-17]. A research shows that overexpression of miR-125b in primary neurons is associated with tau hyperphosphorylation and cognitive deficits [18]. In AD mouse models, the miR-15 family regulates the level of tau phosphorylation by targeting Erk1 kinase [19]. Moreover, miR-98 increases Aβ formation and tau phosphorylation by inhibiting the translation of IGF-1 in HEK293 cells, which leads to the occurrence of AD [20]. These indicate miRNAs play crucial functions in AD development and progression.

miR-26b, a member of the miR-26 family, is located on human chromosome 2q35, which is abundantly expressed in nerve [21-23]. Several studies illustrate that miR-26b expression is dysregulated in AD samples. A research exhibits that miR-26b expression is upregulated in AD human postmortem brains compared with healthy brains [18]. Another investigation indicates that miR-26b is increased in blood of 48 AD patients compared with 22 normal control [17]. Furthermore, an animal experiment shows that miR-26b level is elevated in cholesterol fed rabbit model of AD compared to the control samples [24]. These suggest that miR-26b expression correlates with the risk of AD.

An *in vitro* experiment also confirms that miR-26b activates cell cycle entry (CCE), tau-phosphorylation, and promotes cells apoptosis in rat primary post mitotic neurons, which further indicates miR-26b might be involved in AD pathogenesis [10]. Consistent with prior studies, we observed that miR-26b mimic reduced total neurite outgrowth and promoted cells apoptosis in PC12 cellular AD model, while the result of miR-26b inhibitor group was opposite. This indicated miR-26b was involved in the pathology of AD by regulating neurite outgrowth and cells apoptosis. And the possible explanations might be as follows: (1) miR-26b induces CCE and promotes cells apoptosis through activating the expression of cyclin E1 (CCNE1), DNA polymerase excitability factor (PCNA) and ki67 protein [10]. (2) miR-26b stimulates NFT formation by increasing Rb1 mRNA to increase cyclin-dependent kinase 5 (Cdk5) activity level and to induce tau phosphorylation, thus inhibits the neurite outgrowth [10].

NEP, also known as membrane metallo-endopeptidase (MME), is located on human chromosome 3q25.2, whose coding protein is a type II transmembrane glycoprotein [25]. NEP is found in a variety of normal tissues, particularly on presynaptic membranes of neuron [26]. A research observes that in brain tissue of AD patients, NEP mRNA expression is remarkably decreased compared with normal control [27]. Recently, an animal experiment exhibits that upregulation of NEP expression reduces the concentration of Aβ in the mouse brain [26]. In addition, another *in vivo* and *in vitro* experiment reveals that a decreased NEP level in AD mice model and primary neurons results in an increased Aβ level and vice versa [28-30]. These imply that NEP plays an important role in delaying disease progression in AD. However, the interaction between miR-26b and NEP has not been studied so far. In our study, we observed that miR-26b inversely regulated expression of NEP but not EIF2S1 or αTTP in cellular AD model. These indicate that miR-26b might be involved in delaying AD progress by regulating NEP but not EIF2S1 or αTTP.

In conclusion, miR-26b inhibits neurite outgrowth, induces cells apoptosis and downregulates NEP expression in PC12 cellular AD model.

Disclosure of conflict of interest

None.

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