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Suppression of lncRNA-ATB prevents amyloid-β-induced neurotoxicity in PC12 cells via regulating miR-200/ZNF217 axis

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ABSTRACT

Objective: Alzheimer's disease (AD) is a neurodegenerative disease characterized by progressive cognitive decline with loss of memory. The objective of this study was to investigate the role and regulatory mechanism of lncRNA-ATB in regulating amyloid-β-induced neurotoxicity in neuronal PC12 cells.

Material and methods: The expression levels of lncRNA-ATB in cerebrospinal fluid (CSF) and serum of patients with Alzheimer's disease (AD) were determined. In addition, PC12 cells were incubated with 20 μM $A\beta_{25-35}$ to induce cell injury. The lncRNA-ATB expression in $A\beta_{25-35}$-treated PC12 cells was also determined. Moreover, the effects of lncRNA-ATB suppression on $A\beta_{25-35}$-induced PC12 cell injury were investigated by assessing cell viability, apoptosis, cytotoxicity, and oxidative stress (intracellular Ca^{2+} and ROS concentrations and JC-1 mitochondrial membrane potential). Moreover, the regulatory relationships between lncRNA-ATB and miR-200 were explored, as well as the targets of miR-200 were identified.

Results: The results showed that lncRNA-ATB was increased expressed in the CSF and serum of patients with AD. $A\beta_{25-35}$-induced injury in PC12 cells and increased the expression of lncRNA-ATB. Suppression of lncRNA-ATB alleviated $A\beta_{25-35}$-induced PC12 cell injury. Further studies showed that miR-200 was negatively regulated by lncRNA-ATB. Suppression of lncRNA-ATB alleviated $A\beta_{25-35}$ injury by regulation of miR-200. Moreover, miR-200 negatively regulated ZNF217 expression and ZNF217 was a target of miR-200.

Conclusions: Our findings indicate that lncRNA-ATB is highly expressed in AD patients. Suppression of lncRNA-ATB may protect PC12 cells against $A\beta_{25-35}$-induced neurotoxicity via regulating miR-200/ZNF217 axis. LncRNA-ATB/miR-200/ZNF217 axis may provide a new insight for preventing AD.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterized by progressive cognitive decline with loss of memory, accounting for 50–70% of all cases of dementia [1–3]. It is a polygenic/complex disorder that can be caused by epigenomic, genomic, and environmental factors [4]. It is estimated that there are approximately 35 million people suffering from AD around the world in 2015 [5]. Most of the clinical symptoms of AD occur at a very later stage, thus, identification of disease markers for early detection of AD is essential to slow down its progression [6].

Long noncoding RNAs (lncRNAs) are longer than 200 nucleotides. Accumulating studies have confirmed that lncRNAs play a crucial role in a wide range of biological functions [7,8], as well as diverse pathological diseases [9,10]. The lncRNAs in the central nerve system are widely involved in the regulation of neuronal differentiation, synaptic plasticity and behaviors [11]. Moreover, aberrant expressions of lncRNAs are shown to underlie the disease pathophysiology of AD pathophysiology and therefore may serve as promising biomarkers and potential therapeutic targets [12–14]. For instance, suppression of lncRNA BDNF-AS can exert protective functions against $A\beta_{25-35}$-induced neurotoxicity in PC12 cells through inhibiting cell apoptosis and oxidative stress [15]; lncRNA EBF3-AS can promote neuron apoptosis in AD [16]; lncRNA BACE1 is found to increase in the plasma of AD patients and has a high specificity for AD, indicating that plasma lncRNA BACE1 may be a promising biomarker for the diagnosis of AD [14]. Therefore, identification of crucial lncRNAs will not only deepen understanding of the pathogenesis of AD but also provide new ideas for the diagnosis and prevention of this disease. However, the crucial lncRNAs involved in AD are largely unknown.

The lncRNA activated by transforming growth factor-β (lncRNA-ATB) has been identified to be involved in many pathological processes. For instance, lncRNA-ATB promotes the progression of colon cancer and is correlated with poor prognosis [17]. LncRNA-ATB is also found

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to function as an oncogene in colorectal cancer and may serve as an indicator of poor prognosis [18]. In pancreatic cancer, however, low expression of lncRNA-ATB is found to be associated with clinical progression and unfavorable prognosis [19]. In addition to cancer field, lncRNA-ATB is also found to be involved in HCV-related hepatic fibrosis [20], keloids [21], and coal workers' pneumoconiosis [22]. However, there were limited reports regarding the role of lncRNA-ATB in neurodegenerative diseases including AD.

In the present study, the expression levels of lncRNA-ATB in cerebrospinal fluid (CSF) and serum of patients with AD were determined. In addition, PC12 cells were incubated with 20 μM $A\beta_{25-35}$ to induce cell injury, and the effects of lncRNA-ATB suppression on $A\beta_{25-35}$-induced PC12 cell injury were investigated by assessing cell viability, apoptosis, cytotoxicity, and oxidative stress (intracellular Ca2+ and ROS concentrations and JC-1 mitochondrial membrane potential). Moreover, the regulatory relationships between lncRNA-ATB and miR-200 were explored, as well as the targets of miR-200 were identified. Our data will provide a theoretical basis for better elucidation of the role and regulatory mechanism of lncRNA-ATB in AD.

2. Materials and methods

2.1. Patient samples

Eighteen probable AD patients (7 males and 9 females, 70.3 ± 8.8 years) and 16 healthy individuals (8 males and 10 females, 68.3 ± 9.6 years) were enrolled in this study. According to the criteria of the National Institute of Neurological and Communicative Disorders and Stroke as well as Alzheimer's Disease and Related Disorders Association (NINCDS/ADRDA), a diagnosis of probable AD was confirmed based on clinical evaluation, neuropsychological testing, and magnetic resonance imaging [23]. Assessment for neurological disorders was also preform to confirm that there was no neurological disorder of healthy individuals. The CSF samples of AD patients and healthy individuals were collected by lumbar puncture in polypropylene tubes, centrifuged, and stored at −80 °C. Notably, only CSF samples with < 4 leukocytes/μl and < 200 erythrocytes/μl were selected. In addition, the serum was harvested from both patients and healthy controls. This study was approved by the ethic committee of our hospital and patients were informed for using their data and samples in further scientific purposes.

2.2. Cell culture

The neuronal PC12 cells (Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai, China) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 5% fetal bovine serum, 10% horse serum and 1% Pen-Strep (Thermo Fisher Scientifc, Inc., Waltham, MA, USA), and maintained in a 37 °C humidified atmosphere with 5% CO_2 for 2–3 d. To further induce neuronal differentiation, PC12 cells were exposed to NGF (50 ng/ml) for 2 days at 37 °C. Differentiated PC12 cells were confirmed by examination with a microscope [24,25], which were used in all subsequent experiments.

2.3. Aβ preparation and treatment

$A\beta_{25-35}$ aggregation was prepared as described previously [26]. In order to induce aggregation, lyophilized $A\beta_{25-35}$ was added into RPMI 1640 medium, followed by incubation at 37 °C with constant oscillation for 3 days. The aggregated $A\beta_{25-35}$ was then diluted to 100 μg/ml (100 μM) and stored at −20 °C. To induce cell injury, PC12 cells were incubated with 20 μM $A\beta_{25-35}$.

2.4. Transient transfection

Short hairpin RNA (shRNA) targeting lncRNA-ATB (sh-ATB), small interfering RNAs targeting ZNF217 (si-ZNF217) and their negative controls (sh-NC or si-NC), miR-200 mimic, mimic NC, miR-200 inhibitor, and inhibitor NC were purchased from GenePharma (Shanghai, People's Republic of China). For cell transfection, PC12 cells (2×10^5) were seeded in 6-well plates and then transfected with sh-ATB, sh-NC, si-ZNF217, si-NC, miR-200 mimic, mimic NC, miR-200 inhibitor, and inhibitor NC using Lipofectamine 2000 Reagent (Life Technologies, CA, USA) according to the manufacturer's instructions.

2.5. MTT assay

Cell viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyl tetrazolium bromide (MTT) cell proliferation assay kit (Sigma-Aldrich, St. Louis, MO, USA). Briefly, PC12 cells (5000 cells per well) were seeded in a 96-well plate. After different treatments, PC12 cells were incubated with 20 μL 0.5 mg/mL MTT for 4 h in a 37 °C humidified incubator with 5% CO_2. Next, the supernatant was removed after centifugation and 100 μL dimethyl sulfoxide (DMSO) was added to dissolve the MTT formazan crystals. The absorbance of each sample at 490 nm was measured on a microplate reader (Becton Dickinson, Mountain View, CA, USA).

2.6. Flow cytometry

Cell apoptosis assay was conducted using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double staining apoptosis detection kit (BD Biosciences Pharmingen, San Jose, CA, USA). PC12 cells were seeded in a 96-well plate. After different treatments, cells were digested with 0.05% trypsin (Sigma-Aldrich), washed twice with pre-cold PBS and then resuspended in 1× binding buffer. Afterwards, cells were stained with Annexin V-FITC and PI for 20 min in the dark at room temperature, followed by identification of the apoptotic cells by a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). The obtained data were analyzed using the Cell Quest software (Becton Dickinson).

2.7. LDH assay

The cytotoxicity was evaluated by measuring the LDH leakage into the culture medium which was detected using Cytoscan-LDH cytotoxicity assay kit (G-Biosciences Inc., St Louis, MO, USA). Briefly, PC12 cells (5000 cells per well) were seeded in a 96-well plate. After different treatments, 50 μL supernatant of each wells were transferred to another fresh 96-well plate and then 50 μL reconstituted Substrate Mix was added to incubate cells at 37 °C in the dark for 30 min. Subsequently, the reaction was terminated by adding 50 μL Stop Solution. The absorbance at 490 nm was recorded by Microplate Reader.

2.8. Measurement of intracellular concentrations of calcium (Ca^{2+}) and reactive oxygen species (ROS)

The intracellular concentrations of Ca^{2+} and ROS in PC12 cells were measured using the fluorescence Ca^{2+} indicator Fluo-3 AM and a ROS kit, respectively. For detecting Ca^{2+} levels, PC12 cells were collected, stained with 5 μg/ml of Fura-3 AM for 60 min, and then incubated for 30 min at 37 °C. Fluorescence-intensity was then detected by a flow cytometer and the Ca^{2+} levels in cells were expressed as NRFU (U/cell). For detecting intracellular ROS level, PC12 cells were collected and then incubated with 10 μM of the cell permeable 2′,7′-dichlorofluorescin diacetate (DCFH-DA) for 20 min at 37 °C. Subsequently, the fluorescence was detected using a flow cytometer, and the data were presented as an average of the intensity of the recorded fluorescence (NRFU) (U/cell).

2.9. Measurement of mitochondrial membrane potential (ΔΨm)

ΔΨm of PC12 cells was determined using JC-1 mitochondrial