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miR-106b inhibits tau phosphorylation at Tyr18 by targeting Fyn in a model of Alzheimer's disease

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ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by β-amyloid deposits and neurofibrillary tangles consisting of hyperphosphorylated tau protein. Increasing evidence has revealed that microRNAs (miRNAs) are implicated in the pathogenesis of AD. However, the effect of miRNAs on abnormal tau phosphorylation remains largely unclear so far. In this study, we investigated the role of miR-106b in tau phosphorylation and identified a new molecular mechanism of the hyperphosphorylation of tau. The results of qRT-PCR showed that the expression level of miR-106b was decreased, but Fyn was increased in the temporal cortex of AD patients. Overexpression of miR-106b inhibited $A\beta_{1-42}$-induced tau phosphorylation at Tyr18 in SH-SY5Y cells stably expressing tau (SH-SY5Y/tau), whereas no changes were observed in tau phosphorylation at Ser396/404. Dual-luciferase reporter gene assay validated that Fyn was a direct target gene of miR-106b. In addition, western blot analysis revealed that Fyn protein expression was suppressed when SH-SY5Y cells were transfected with miR-106b mimics. Endogenous Fyn expression was knockdown by transfection with a small interfering RNA specific for Fyn (si-Fyn). The phosphorylation level of tau at Tyr 18 was decreased in the si-Fyn group compared with the negative control group, but the inhibitory effect of si-Fyn on tau phosphorylation was attenuated when miR-106b expression was inhibited. Taken together, these data suggest that miR-106b inhibits $A\beta_{1-42}$-induced tau phosphorylation at Tyr18 by targeting Fyn. Our findings extend the knowledge about the regulation of tau phosphorylation and the regulatory mechanism of Fyn gene expression.

1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder in the old people and characterized by neuronal cell apoptosis associated with a progressive decline in learning, memory and cognitive functions, causing dementia [1]. The neuropathological hallmarks of AD are extracellular deposits of amyloid β (Aβ) protein and the accumulation of intracellular neurofibrillary tangles which are made up of hyperphosphorylated microtubule associated protein (tau) [2]. At present, there is no strategy for preventing or curing AD except for a few medications that can improve cognitive and memory skills. So, it is extremely urgent to further elucidate the pathogenesis of AD and to develop new pharmacological strategies.

Fyn, a non-receptor type tyrosine kinase, exerts a variety of biological actions in T-cell development and activation, brain development, neuroinflammation, synaptic function and plasticity [3]. Aβ oligomer interacted with postsynaptic prion protein to activate Fyn kinase, reducing the function of NMDA receptors as well as the synaptic function of neurons [4]. Upregulation of Fyn expression promotes human amyloid precursor protein (hAPP)/Aβ-dependent neuronal and behavioral deficits in Fyn/$hAPP_{low}$ mice, while downregulation of Fyn expression rescued these deficits. Increased expression of Fyn does not affect amyloid deposition in Fyn/$hAPP_{low}$ mice [5]. The tau tyr18 has been shown to be phosphorylated in SH-SY5Y cells overexpressing Fyn [6]. In 1993, Shirazi and Wood found that the Fyn immunoreactivity in a proportion of neurons of AD brain was remarkably higher than that in neurons of normal brains, and that tau protein was aberrantly phosphorylated in these AD neurons [7]. Fyn protein is mainly expressed in the dendrite of neurons, where it has been proved to interact with tau

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to stabilize receptor complexes in the postsynaptic density. Evidence has showed that Fyn protein is co-localized with tau in neurons with tau tangles in AD, implying that the tau-Fyn interaction plays a pathogenic role in the disease progression of AD [8].

microRNAs (miRNAs), a class of small noncoding RNA molecules (18–25 nucleotides), downregulate protein expression of more than 60% of protein-coding genes by directly binding to target messenger RNAs (mRNAs) [9]. In recent years, a large amount of miRNAs have been revealed to influence cell proliferation, differentiation, and death in the pathogenesis and progression of various human diseases, including AD [10]. Several miRNAs have been shown to be aberrantly expressed in both AD patients and transgenic mouse models of AD [11,12]. miR-26b, upregulated in postmortem brains of early-stage AD patients, results in DNA replication, aberrant cell cycle entry and tau phosphorylation, which induce apoptotic cell death of rat primary postmitotic neurons [13]. miR-125b, one of the most abundant miRNAs in the brain, is elevated in frontal cortex of AD patients compared to age-matched healthy controls. Overexpression of miR-125b in the brain of C57BL/6 wild-type mice results in tau hyperphosphorylation and impairs learning and memory capabilities of these mice [14]. Hébert et al. have reported that miR-106b expression is down-regulated in sporadic AD patients, suggesting that miR-106b may be implicated in the pathogenesis of AD [15]. We speculate that ectopic expression of miR-106b may be related to the hyperphosphorylation of tau protein and play an important role in the progression of AD.

In this study, we examined the expressions of miR-106b and Fyn in brains of AD patients, and investigated the effect of miR-106b on $A\beta_{1\text{-}42}$-induced tau phosphorylation at Tyr18 and the exact underling mechanisms.

2. Materials and methods

2.1. Patients and brain samples

Brain tissues of 5 AD patients and 5 age-matched health controls were acquired from Huaihe Hospital of Henan University. The temporal cortex were dissected from brain tissues and quickly frozen in liquid nitrogen for further analysis. The aim and protocol of this study were informed to all subjects and caregivers (usually the next of kin) and all participants gave written informed consents. The study procedure was approved by the institutional review board of Huaihe Hospital of Henan University.

2.2. Cell culture and transfection

SH-SY5Y cells (American Type Culture Collection, Rockville, MD, USA) stably expressing human tau protein (SH-SY5Y/tau) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Tianhang Biological Technology, Hangzhou, China) at 37 °C in a humid incubator with 5% CO_2. Transient or stable transfection of SH-SY5Y cells were carried out using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The plasmid which carries tau gene was constructed by amplifying the CDS of tau using polymerase chain reaction (PCR), and then the PCR fragment was subcloned into pcDNA3.1 (Invitrogen). $A\beta_{1\text{-}42}$ and the scrambled control peptide were purchased from rPeptide (Athens, GA, USA).

RNA isolation and quantitative real-time PCR (qRT-PCR).

To quantify the expression of Fyn mRNA, total RNA was isolated from tissues and cells using Trizol reagent (Invitrogen). Quality and concentration of RNA were evaluated by NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA). cDNA was amplified using SuperScript III RT (Invitrogen) as per the protocol provided by the manufacturer. qPCR was conducted using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) on an ABI-7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The primers used for qPCR were as follows: Fyn sense: 5′-GACTCTTAAACCAGGCAC-3′, and anti-sense: 5′-CAGGTTTTCACCAGGTTG-3′; β-actin sense: 5′-GTCTTCCCCTCCATCGTG-3′, and anti-sense: 5′-AGGGTGAGGATGCCTCTCTT-3′. β-actin was used as a loading control for Fyn. For the miR-106b expression analysis, isolation of miRNAs was performed with an miRNeasy Kit (Qiagen, Valencia, CA, USA). cDNA was synthesized by a TaqMan miRNA Reverse Transcriptase Kit (Applied Biosystem). The miRNA expression was detected by using a TaqMan miRNA assay kit (Applied Biosystem) according to the manufacture's protocol. The primers used for qPCR were as follows: miR-106b sense: UAAAGUGCUGACAGUGCAGAU, and anti-sense: CUGCACUGUCAGCACUUUGUU; small nuclear RNA (snRNA) U6 sense: 5′-CTCGCTTCGGCAGCACA-3′, and antisense: 5′-AACGCTTCACGAATTTGCGT-3′. snRNA U6 was used as an internal control for miR-106b. Data were analyzed using the 2-ΔΔCT method.

2.3. Western blot

Cells were harvested 36 h post-transfection and lysed in ice-cold RIPA lysis buffer (Sigma-Aldrich, St. Louis, MO, USA). Cell lysates were separated by SDS-polyacrylamide gel (SDS-PAGE) electrophoresis and then transferred to a polyvinylidene fluoride (PVDF) membrane (Merck Millipore, Billerica, MA, USA). After blocking with 5% non-fat dry milk in tris buffered saline containing 0.1% Tween-20 (TBST) for 45 min, membranes were probed with PHF-1 antibody against tau phosphorylated at Ser396/404 (Abcam, Cambridge, MA, USA), PY18 antibody against tau phosphorylated at Tyr18 (MediMabs, Montréal, Québec, Canada), anti-Fyn antibody (Santa Cruz Biotechnology, CA, USA), tau-5 antibody against total tau (Abcam) or anti-β-actin antibody (Santa Cruz) overnight at 4 °C. Membranes were rinsed three times (10 min each) in TBST and then incubated with appropriate secondary antibodies conjugated with horseradish peroxidase (HRP; Santa Cruz) for 1 h at room temperature. After three washes (15 min each) in TBST, the indicated protein signals were visualized by enhanced chemiluminescence (Thermo Scientific) according to the manufacturer's instructions. Blot quantification was performed using Image J software.

2.4. Dual-luciferase reporter assay

miR-106b mimics and scramble mimics (negative control; miR-NC) were purchased from a commercial company (GeneChem Co., Ltd, Shanghai, China). Luciferase reporter plasmids carrying wild-type (WT) or mutant (Mut) Fyn 3′-untranslated region (3′-UTR) were obtained from GeneChem Co., Ltd. One day before transfection, SH-SY5Y cells (3×10^4 cells per well) were planted into a 24-well plate and grown in complete DMEM for 24 h at 37 °C. SH-SY5Y cells of 80% confluence were co-transfected with 50 nM miR-106b mimics or miR-NC along with 0.4 μg of firefly luciferase reporter vector or pRL-TK Renilla luciferase vector (Promega, Madison, WI, USA). Luciferase activity was carried out 36 h after transfection using a Dual-Luciferase Reporter Assay System (Promega) as per the manufacturer's instruction. Measurements of luminescence were conducted on a Glomax multi luminometer (Promega). The experiments were repeated independently three times and each assay was done in triplicate. Renilla luciferase was used for normalization.

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