miR-143-3p inhibition promotes neuronal survival in an Alzheimer’s disease cell model by targeting neuregulin-1

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Abstract

Introduction: Alzheimer’s disease (AD) is still the fifth leading cause of death and most common dementia worldwide. To date, there is no efficient strategy that can slow down the progression of AD owing to delayed diagnosis and limited therapies. MiR-143-3p is up-regulated in serum of AD patients, yet the exact role it plays in AD pathology is still poorly understood. The aim of this study was to investigate the effect of miR-143-3p on neuronal survival.

Material and methods: We induced neuronal differentiation in SH-SY5Y cells using all-trans-retinoic acid (RA), and Aβ1-42 was used to establish the in vitro AD cell model. The expression of tubulin β III and neuregulin-1 (NRG1) was evaluated by immunofluorescence. TUNEL assay was performed to assess cell apoptosis. Cell viability was evaluated using the Cell Counting Kit-8 assay. The binding interaction between miR-143-3p and NRG1 was verified using the luciferase reporter assay.

Results: Typical neuronal-like axons were observed in RA-induced SH-SY5Y cells, followed by increased tubulin β III. A dramatically increased apoptotic rate and reduced cell viability were observed in the AD cell model. Then we silenced the miR-143-3p expression, and Aβ1-42 induced cell apoptosis was alleviated after miR-143-3p inhibition, accompanied by decreased cleaved caspase-3 and cleaved caspase-9 levels. Additionally, NRG1 was confirmed to be a downstream target of miR-143-3p, increased cell viability and suppressed cell apoptosis after miR-143-3p inhibition was abolished by NRG1 knockdown.

Conclusions: Our findings reveal that miR-143-3p inhibition promotes neuronal survival in an in vitro cell model via targeting NRG1, and the miR-143-3p/NRG1 axis is a potential therapeutic target and promising biomarker for AD treatment.

Key words: Alzheimer’s disease, neuronal survival, miR-143-3p, NRG1.

Introduction

Alzheimer’s disease (AD) is a type of neurodegenerative disease mainly characterized by memory and cognitive impairment, accompanied by deteriorated behavior and mood [20]. As the most common form of dementia, AD affected approximately 35.6 million people worldwide in 2010 and the number is anticipated to double every twenty years to 115.4 million by 2050, and caring for AD patients is a substantial Medicare cost and a great burden for
society [26]. Advanced age is the most predominant risk factor for AD as AD cases occur most frequently in patients older than 65 at a risk rate of 10.5%, and incidence rises with age [13]. The major pathogenesis of AD is the deposition of β-amyloid (Aβ) peptide cleaved from the amyloid precursor protein, Tau protein hyperphosphorylation and necrosis of neurons [29]. There are only five drug treatments approved for AD, which can merely control the clinical symptoms [4]. AD has an insidious onset and patients remain asymptomatic at the early stage of the disease, and most patients are diagnosed as the clinical symptoms emerge, which leave very limited treatment options, let alone provide a cure [11]. According to the diagnostic guideline for AD from the National Institute on Aging Alzheimer’s Association, the pathophysiological process of AD starts 10 years or even earlier before diagnosis, which makes early detection and intervention at preclinical phases critical [27]. The Aβ level in cerebrospinal fluid (CSF) appears to decrease 25 years before clinical symptoms. Aβ accumulation and an elevated tau protein level were detected 15 years prior to symptom onset, followed by neurodegeneration and cognitive decline [3]. Consequently, it is feasible to find eligible biomarkers for early diagnosis and progression prediction for AD.

MicroRNAs are a class of noncoding transcripts approximately 22 nucleotides in length. MicroRNAs are involved in numerous physiological processes via complementary binding to target sites to down-regulate gene expression [14]. MiR-143-3p is significantly up-regulated in the serum of AD patients with mild cognitive impairment, indicating that miR-143-3p might be implicated in the progression of AD [8]. Also, miR-143-3p is elevated in both acute ischemic stroke patients and in a mouse stroke model. Inhibition of miR-143-3p expression can alleviate neurological deficits as well as blood-brain barrier damage in mice [2]. MiR-143-3p silencing also prevents nitric oxide-induced neuroblastoma cell apoptosis and protects neurons from ischemic brain injury in rats [34,35]. All these previous findings reveal that miR-143-3p inhibition presents a protective effect on neuronal cells, yet the precise underlying mechanisms remain undiscovered.

Neuregulin-1 (NRG1) is a member of the neuregulin family containing an epidermal growth factor-like domain. NRG1 plays a crucial role in the development of the brain, and it is also expressed in adult brain [31]. NRG1 was also reported to be involved in neurodevelopmental processes including neuronal migration and differentiation as well as synapse formation [5]. Recent studies showed that NRG1 is significantly down-regulated in the hippocampus of AD patients, and NRG1 alleviated cognitive impairment and neuropathology in an AD mouse model [18,32]. Furthermore, NRG1 was a putative downstream target of miR-143-3p by bioinformatics prediction in TargetScan. Therefore, we speculate that miR-143-3p is involved in the development of AD through regulating NRG1.

In the present study, human neuroblastoma SH-SYSY cells were treated with Aβ1-42 to imitate the neuronal injury in AD. We investigated the effect of miR-143-3p on viability and apoptosis in SH-SYSY cells. We also assessed the interaction between miR-143-3p and NRG1.

**Material and methods**

**Cell culture, differentiation, transfection, and Aβ1-42 treatment**

SH-SYSY cells were obtained from Procell (China) and cultured with MEM/F12 culture medium (Procell, China) containing 10% fetal bovine serum (FBS) (Biological Industries, Israel) in an incubator at 37°C. Cell differentiation was induced by 10 μM all-trans-retinoic acid (RA) (Aladdin, China) in MEM/F12 culture medium with 1% FBS for 7 days, and culture medium was replaced every 3 days. Morphological changes in SH-SYSY cells after RA treatment were observed under a microscope (200× magnification), and differentiated cells were used for all subsequent investigations. SH-SYSY cells were transfected with microRNAs, microRNA inhibitors or siRNAs using Lipofectamin3000 (Invitrogen, USA) according to the manufacturers’ protocol. To establish the *in vitro* AD cell model, Aβ1-42 (GenScript, USA) was dissolved in pre-cooled hexafluoroisopropanol for 10 minutes, and hexafluoroisopropanol was allowed to volatilize to form an Aβ1-42 protein membrane, followed by dissolving in DMSO. SH-SYSY cells were transfected and treated with 1 μM Aβ1-42 for 24 hours to perform the subsequent experiments.

**Immunofluorescence and TdT-mediated dUTP nick-end labeling (TUNEL) assay**

For immunofluorescence assay, SH-SYSY cell slides were fixed with 4% paraformaldehyde for 15 minutes and permeated with 0.1% tritonX-100
for 30 minutes at room temperature. Cell slides were then washed with PBS three times and blocked with goat serum for 15 minutes, followed by incubation with mouse NRG1 antibody (1 : 50, Proteintech, China) or rabbit tubulin β III antibody (1 : 50, BOSTED, China) overnight at 4°C. Cell slides were washed with PBS buffer three times and incubated with cy3-labeled goat anti-mouse IgG (1 : 200, Proteintech, China) or cy3-labeled goat anti-rabbit IgG (1 : 200, Proteintech, China) for 60 minutes at room temperature. For TUNEL assay, cell slides were permeated with 0.1% tritonX-100 and cell apoptosis was detected using a commercial In Situ Cell Death Detection Kit (Roche, Switzerland). After washing with PBS buffer, cell slides were stained with DAPI (Beyotime, China) and sealed with anti-fluorescence quenching reagent (Solarbio, China). Typical images were captured under a microscope (400× magnification).

Cell Counting Kit-8 assay

SH-SYSY cells were seeded onto a 96-well plate (3 × 10^3 cells/well) and cultured with 100 μl of culture medium containing 10 μl of CCK-8 (Sigma, USA) for one hour. Optical density values at 450 nm were recorded and analyzed.

Quantitative real-time PCR

SH-SYSY cells were lysed and total RNAs were extracted using a commercial RNA isolation kit (Biotek, China). Total RNAs were reverse transcribed into cDNA and quantified using SYBR Green (Bioteke, China). β-actin and small nuclear RNA U6 were used as an internal control. Stem-loop RT primers and real-time PCR primers used in this study are listed below:

- Hsa-miR-143-3p specific stem-loop primer: 5’-GTTGGCTCTGTTGACCGGTCCAGGTATATTGCCAC- CAGACCAAAGAGCTA-3’;
- Hsa-U6 specific stem-loop primer: 5’-GTTGGCTCTGTTGACCGGTCCAGGTATATTGCCAC- CAGACCAAAGAGCTA-3’;
- Hsa-miR-143-3p-F: 5’-GAGAGATGAAGCACTGTAGC-3’; Hsa-miR-143-3p-R: 5’-GCAGGGTCCGAGGTATTC-3’;
- Hsa-U6-F: 5’-GCTTCGGCAGCACATATACT-3’; Hsa-U6-R: 5’-GTGCAGGGTCCGAGGTATTC-3’;
- NRG1-F: 5’-CGGAGGAGCTGGTGAATC-3’;
- NRG1-R: 5’-GCTGGTGGAGCTGGTGAATC-3’;
- β-actin-F: 5’-CACTGTGGCCCATCTAATGCC-3’; β-actin-R: 5’-TAATATGCACGACGGATTTCC-3’.

Western blot

SH-SYSY cells were lysed with RIPA buffer (Beyotime, China) and fractionated on SDS polyacrylamide gels. Then total proteins were transferred onto PVDF membranes (Thermo Fisher, China) and blocked with 5% nonfat milk. PVDF membranes were incubated with primary antibodies overnight at 4°C, rinsed with PBS buffer three times and incubated with secondary antibodies for 40 minutes at room temperature. Primary antibodies used were NRG1 antibody (1 : 500, Abclonal, China), pro-caspase-3/cleaved caspase-3 antibody (1 : 500, CST, USA), pro-caspase-9/cleaved caspase-9 antibody (1 : 500, CST, USA) and β-actin antibody (1 : 2000, Proteintech, China). Secondary antibodies used were HRP-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG (1 : 10000, Proteintech, China). Target protein bands were visualized using ECL reagent (Beyotime, China) and analyzed using Gel-Pro-Analyzer.

Luciferase reporter assay

A luciferase reporter assay was performed to verify the interaction between miR-143-3p and NRG1. Wildtype and mutant type of NRG1 3’-UTR containing a binding site, termed as NRG1-WT and NRG1-MUT, were amplified and cloned into a pmirGLO plasmid (Promega, USA). HEK-293T cells were seeded onto a 12-well plate and co-transfected with pmirGLO plasmids and NC/miR-143-3p mimics. Luciferase activity was assessed using a commercial Firefly Luciferase Assay Kit (Promega, China) following the manufacturer’s instructions.

Statistical analysis

All data were analyzed using the GraphPad Prism 7 software. The results were displayed as means ±SD. Student’s unpaired t-test was used for determining the differences between two groups and for comparisons of more than two groups one-way ANOVA followed by Tukey’s multiple comparison test was used. A statistically significant difference in this study was adopted as p < 0.05.

Results

Retinoic acid treatment successfully induced SH-SYSY cell differentiation

SH-SYSY cells were treated with RA for 7 days and typical neuronal-like axons were observed under
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A microscope (Fig. 1A). Immunofluorescence assay showed that the expression of tubulin β III was dramatically increased after RA treatment (Fig. 1B). These results indicated that SH-SY5Y cells were successfully differentiated into neuronal-like cells after RA treatment and these cells were used in all subsequent detections.

**In vitro Alzheimer's disease cell model was successfully established**

SH-SY5Y cells were treated with Aβ1-42 and obvious cell apoptosis was detected after treatment (Fig. 2A, B). Aβ1-42 also significantly reduced cell viability in SH-SY5Y cells (Fig. 2C), indicating that an in vitro AD cell model was established. Also, the expression level of miR-143-3p was remarkably increased in the AD cell model while the protein level of NRG1 was significantly reduced (Fig. 2D-F).

**MiR-143-3p inhibition promoted viability and suppressed apoptosis in SH-SY5Y cells**

SH-SY5Y cells were transfected with specific miR-143-3p inhibitor and treated with Aβ1-42. Aβ1-42 treatment significantly induced apoptosis in SH-SY5Y cells, which was partially reversed by miR-143-3p inhibitor (Fig. 3A, B). Aβ1-42 treatment inhibited cell viability in SH-SY5Y cells while further miR-143-3p inhibition abolished the effect (Fig. 3C).
Fig. 2. *In vitro* AD model was successfully established. SH-SY5Y cells were treated with 10 μM Aβ1-42 for 24 hours. A, B) Cell apoptosis was evaluated with TUNEL assay and apoptosis rate was analyzed. C) Cell viability was assessed using commercial CCK-8. D) The miR-143-3p level was measured by quantitative real-time PCR. E, F) The protein level of NRG1 was determined by western blot. AD – Alzheimer’s disease; TUNEL – TdT-mediated dUTP nick-end labeling; Aβ1-42 – amyloid-β peptide 1-42; CCK-8 – Cell Counting Kit-8; NRG1 – neuregulin 1; **p < 0.01; ***p < 0.001.
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Fig. 3. Effects of miR-143-3p inhibition on viability and apoptosis in SH-SYSY cells. SH-SYSY cells were transfected with miR-143-3p inhibitor or non-specific control inhibitor for 24 hours and treated with Aβ1-42 for 24 hours. A, B) TUNEL assay was performed to estimate cell apoptosis. C) Cell viability was assessed by CCK-8 assay. D) Protein levels of pro-caspase-3, cleaved caspase-3, pro-caspase-9 and cleaved caspase-9 were determined by western blot. **p < 0.01; ***p < 0.001.
**Fig. 4.** NRG1 expression was up-regulated after miR-143-3p inhibition in AD cell model. SH-SY5Y cells were transfected with miR-143-3p inhibitor or non-specific control inhibitor for 24 hours and treated with Aβ1-42 for 24 hours. **A, B** Expression levels of miR-143-3p and NRG1 were measured using quantitative real-time PCR. **C** The protein level of NRG1 was determined by western blot. **D** Localization and expression of NRG1 were detected by immunofluorescence assay. **p < 0.01; **p < 0.001.
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Also, the protein levels of cleaved caspase-3 and cleaved caspase-9 were elevated in SH-SY5Y cells after Aβ1-42 treatment while further miR-143-3p inhibition reversed the effect (Fig. 3D). These results indicated that miR-143-3p inhibition promoted viability and suppressed apoptosis in the AD cell model.

**NRG1 expression was up-regulated after miR-143-3p inhibition in AD cell model**

The expression of miR-143-3p was significantly elevated in the AD cell model, which was partially abrogated after miR-143-3p inhibition (Fig. 4A). NRG1 expression was reduced after Aβ1-42 treatment, which was reversed by further miR-143-3p inhibition at both mRNA and protein levels (Fig. 4B, C). A similar trend was observed in the immunofluorescence assay, and NRG1 was mainly localized in the cytoplasm (Fig. 4D).

**NRG1 was a downstream target of miR-143-3p**

The binding site of miR-143-3p on NRG1 is shown in Figure 5A. MiR-143-3p mimics significantly suppressed the luciferase activity in NRG1-WT yet had no obvious effect on NRG1-MUT compared to NC mimics (Fig. 5B). Also, miR-143-3p mimics inhibited NRG1 expression while miR-143-3p inhibitor

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**Fig. 5.** NRG1 was a downstream target of miR-143-3p. A, B) Wildtype and mutant type of NRG1 3′-UTR containing binding site of miR-143-3p were co-transfected with NC/miR-143-3p mimics into HEK-293 cells. Binding site was shown, and interaction between miR-143-3p and NRG1 was verified using luciferase reporter assay. C, D) SH-SYSY cells were transfected with NC/miR-143-3p mimics or NC/miR-143-3p inhibitor. Expression level of NRG1 was determined using quantitative real-time PCR and western blot, *p < 0.05; **p < 0.01; ***p < 0.001.
Fig. 6. MiR-143-3p regulated apoptosis and viability in SH-SYSY cells via targeting NRG1. SH-SYSY cells were co-transfected with NRG1 siRNA and miR-143-3p inhibitor for 24 hours, and treated with 10 μM Aβ1-42 for 24 hours. A) SH-SYSY cells were transfected with NRG1 siRNAs and NRG1 protein level was determined by western blot. B, C) Cell apoptosis was evaluated using immunofluorescence assay and apoptosis rate was calculated. D) CCK-8 assay was performed to evaluate cell viability, *p < 0.05; **p < 0.01; ***p < 0.001.
MiR-143-3p regulated apoptosis and viability in SH-SY5Y cells via targeting NRG1

We silenced NRG1 expression using three specific siRNAs, among which siNRG1-2 showed the highest efficiency and was used for subsequent investigations (Fig. 6A). MiR-143-3p inhibitor alleviated Aβ1-42-induced cell apoptosis, which was further abrogated after siNRG1 transfection (Fig. 6B, C). Also, miR-143-3p inhibitor promoted cell viability in Aβ1-42-treated SH-SY5Y cells, and the effect was abolished by further NRG1 silencing (Fig. 6D). These results indicated that miR-143-3p inhibition suppressed apoptosis and promoted viability in SH-SY5Y cells via targeting NRG1.

Discussion

Despite great efforts made in investigating the etiology of AD, AD remains the fifth leading cause of death around the world [36]. Accumulated evidence has shown that Aβ plays a role in AD pathogenesis [6,21]. Although the function of Aβ in AD remains controversial, Aβ is still the most well-known causative factor for the disease [33]. In recent years, a variety of cell models have been developed for a better understanding of AD pathogenesis [12,28]. Amyloid β accumulation is one of the hallmarks of AD, among which Aβ1-42 is the most toxic form and widely used in AD in vitro model induction [25]. Loo et al. [22] established an AD cellular model by adding Aβ to the cell culture medium, and they found that apoptosis may play a role in the neuronal loss associated with AD. Hu et al. [17] suggested that trans-caryophyllene may be able to exert a protective effect against Aβ-induced neuroinflammation in AD. Human neuroblastoma cell line SH-SY5Y is widely used in research on neurodegenerative diseases including Parkinson’s disease and AD [1]. In the present study, we induced neuronal differentiation of SH-SY5Y using 10 μM RA, a vital signaling molecule during neuronal differentiation [23]. Typical neuronal-like morphological features were observed, concomitant with an elevated level of tubulin β-III, one of the neuron-specific markers [9]. We induced SH-SY5Y with Aβ42 in our study; reduced cell viability and increased neuronal apoptosis were detected, indicating that we successfully built an in vitro cell model to mimic AD pathologic condition.

Treatment of AD patients is always delayed owing to lack of efficient early diagnosis, which prevents early intervention [19]. Several microRNAs are emerging as critical regulators for AD pathogenesis including tau protein hyperphosphorylation, neuroinflammation and neuronal apoptosis [10]. Circular RNA DLGAP4 ameliorates neurological deficits and promotes cerebrovascular integrity by sponging miR-143-3p in an ischemic stroke mouse model [2]. MiR-143-3p inhibition also alleviates oxygen-glucose deprivation by targeting the glycolysis enzyme hexokinase 2 and further ameliorates ischemia injury [35]. Furthermore, excessive production of nitric oxide is implicated in various neurodegenerative diseases, including AD, by inducing neuronal apoptosis through regulating miR-143-3p [34]. These previous works suggest that miR-143-3p inhibition exerts a neuroprotective effect in neurodegenerative disorders. In the present study, miR-143-3p was up-regulated in an AD cell model, which was highly consistent with the previous study in serum of AD patients [8]. Moreover, miR-143-3p inhibition improved cell viability and reduced cell apoptosis, accompanied by elevated cleaved caspase-3 and caspase-9. Aβ42 induces neuronal apoptosis via activating pro-apoptotic proteins, inducing cytochrome c release and further activating caspases, followed by the mitochondrial mechanism of cell apoptosis [15]. Thus our results indicated that miR-143-3p inhibition alleviated neuronal apoptosis in the AD cell model through the mitochondrial pathway.

NRG1 level was decreased in both AD patients and an in vivo mouse model. NRG1 is imperative for neural and cardiac development. NRG1 functions via specifically binding to the ErbB family of receptor tyrosine kinases, especially ErbB4, which also acts as a susceptibility gene for AD pathology [30]. The activation of NRG1 is based on the β-site of amyloid precursor protein-cleaving enzyme (BACE1), a crucial enzyme involved in the pathogenesis of AD [24]. BACE1-dependent cleavage of NRG1 is critical for its functions in synaptic plasticity and normal psychiatric behaviors [16]. NRG1 modulates the cell surface expression of amyloid precursor protein (APP), affects the production of Aβ and further hinders the progress of AD [18]. NRG1 can also attenuate cognitive deficits and neuropathology to play a neuronal-
protective role in an AD animal model, accompanied by a reduced Aβ level [32]. Furthermore, soluble NRG1 was reported to be a potential biomarker for AD diagnosis as there is a significant difference of soluble NRG1 levels between mild AD patients and control subjects [7]. The negatively correlated expression of NRG1 and miR-143-3p inspired us to investigate their interaction in the AD cell model. Furthermore, we proved that NRG1 is a downstream target of miR-143-3p. Aβ1-42 treatment inhibited NRG1 expression, while further miR-143-3p inhibition abolished the effect at both mRNA and protein levels. Also, miR-143-3p inhibition suppressed cell apoptosis and improved cell viability, which was further reversed after NRG1 knockdown by specific siRNAs. Our current results revealed that miR-143-3p inhibition has a neuroprotective effect in an AD cell model and alleviates neuronal apoptosis via targeting NRG1. The limitation of our study is the lack of in vivo experiments to confirm the effect of miR-143-3p on AD.

In conclusion, we successfully established an in vitro AD cell model. Our findings demonstrated that miR-143-3p regulates neuronal survival through the intrinsic apoptosis pathway by targeting NRG1. The miR-143-3p/NRG1 axis may serve as a potential diagnostic and therapeutic target for AD treatment.

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Disclosure

The authors report no conflict of interest.

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