miR-135a-5p inhibitor protects glial cells against apoptosis via targeting SIRT1 in epilepsy

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Abstract. Epilepsy is a common neurological disease that can induce severe physiological brain damage, including nerve cell apoptosis. MicroRNAs (miRs) have been widely investigated in epilepsy therapy. miR-135a-5p expression levels in children with temporal lobe epilepsy were found to be significantly increased. However, whether miR-135a-5p participates in epilepsy-induced cell apoptosis is not completely understood. In the present study, an in vitro model of epilepsy in BV2 microglia cells was induced using 6-µm kainic acid (KA). Reverse-transcription quantitative PCR was performed to analyze miR-135a-5p and sirtuin 1 (SIRT1) mRNA expression levels. Western blotting was performed to measure SIRT1 protein expression levels. BV2 cell proliferation and apoptosis were assessed by performing MTT assays and flow cytometry, respectively. A BCA protein assay kit was used to detect caspase-3 and caspase-9 activities. TargetScan and dual luciferase reporter assays were performed to investigate the interaction between miR-135a-5p and the 3'-untranslated region (UTR) of SIRT1. miR-135a-5p expression was significantly increased in the KA-induced in vitro model of epilepsy in BV2 microglia. miR-135a-5p inhibitor effectively promoted BV2 microglia proliferation and inhibited microglia apoptosis, whereas small interfering RNA targeting SIRT1 significantly repressed BV2 microglia proliferation and induced microglia apoptosis. In addition, the results demonstrated that the 3'-UTR of SIRT1 mRNA was targeted by miR-135a-5p, and SIRT1 knockdown attenuated miR-135a-5p inhibitor-mediated effects on epilepsy. In summary, the results of the present study identified the role of miR-135a-5p inhibitor pretreatment in protecting nerve cells against epilepsy-induced apoptosis and provided a novel strategy for the treatment of neural damage in seizures.

Introduction

Epilepsy is a group of brain disorders characterized by recurrent epileptic seizures, which is related to the abnormal synchronous discharge of brain neurons (1,2). Epilepsy has become one of the most common chronic brain diseases, affecting ~1% of the world population (3). Repeated seizures can cause severe physiological and mental damage to patients (4). Previous studies have demonstrated that the pathogenesis of epilepsy might be related to structural or functional damage in the limbic system (5-7). Cell death and synaptic strength and plasticity are the primary causes of epileptic injuries (8-10). Nerve cell apoptosis is the primary form of epilepsy-induced cell death, and is accompanied by metabolic abnormalities, gene expression abnormalities and activation of cell death-related proteases (11,12).

MicroRNAs (miRNAs/miRs) are endogenous non-coding RNAs that are typically composed of 18-23 nucleotides (13). miRNAs bind with target mRNA, leading to the inhibition of protein translation or degradation of the target mRNA. By regulating target genes, miRNAs are involved in multiple biological processes, including energy metabolism, cell proliferation and apoptosis (14). Previous studies have reported that miRNAs participating in epilepsy cause brain damage; thus, miRNAs have been widely investigated in epilepsy therapy (15-17). For example, miR-132 was reported to affect hippocampal neuron plasticity and contribute to epilepsy induction (18), and miR-141 was reported to protect against neural apoptosis in epilepsy via suppressing p53 (19). It was also demonstrated that miR-135a-5p expression in children with temporal lobe epilepsy (TLE) is significantly increased (20). Moreover, miR-135a-5p was reported to induce the apoptosis of glioma, ovarian cancer and cardiomyocyte cells, whereas miR-135a-5p inhibition significantly prevented apoptosis (21,22). However, whether miR-135a-5p participates in epilepsy-induced nerve cell apoptosis, as well as the molecules that are involved in the process, are not completely understood. Hence, the current study aimed to investigate the function of miR-135a-5p in epilepsy.

Materials and methods

Cell culture and transfection. The BV2 microglia cell line (American Type Culture Collection) was cultured in DMEM.
(Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (HyClone, Cytiva), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) at 5% CO$_2$ and 37°C.

BV2 cells (2x10$^4$ cells/well) were seeded into six-well plates. miRNA-negative control (NC) inhibitor (100 nM; 5'-CUCGUAGCGAGGCUAGGUACU-3'), miR-135a-5p inhibitor (100 nM; 5'-AUAGCGAAUAAGGUAACUCU-3'), miR-NC mimic (50 nM; 5'-CUGAACUGCAUGACGCGUA-3'), miR-135a-5p mimic (50 nM; 5'-AGUGUAAUCCUAAUUUUCUGUA-3'), si-NC (50 nM; sense, 5'-CGCAACGCCAGCUAAC-3' and antisense, 5'-AGCGCGCUGUCCGACCCGC-3'), and siRNA-sirtuin 1 (SIRT1; 50 nM; sense, 5'-UGGCUAGAUAGCGUUCUCCC-3' and antisense, 5'-GGGAAACAGCUUUACUGACCA-3') were purchased from Shanghai GenePharma Co., Ltd. Transfections into BV2 cells were performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After incubation at 37°C for 48 h, cells were collected for subsequent experiments. For treatment with KA, BV2 microglia cells were stimulated with 0.6 µm KA (Abcam) for 2 h.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from BV2 cells using TRIZol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNA using the PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol. Subsequently, qPCR was performed using ABI Prism 7500 (Applied Biosystems; Thermo Fisher Scientific, Inc.) and SYBR-Green mix (Roche Diagnostics, Shanghai, China). The thermocycling conditions were as follows: Initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec, with a final extension at 72°C for 10 min. The primer sequences were as follows: U6 forward, 5'-CGCAAGGATGACACGGCAAT-3' and reverse, 5'-GCAGGTTCGCCAGGATTTCC-3'; miR-135a-5p forward, 5'-AGTGUAATCTTATTTCATGCAAGGATTTTCA-3' and reverse, 5'-ATCACGGAAATAGATGACTC-3'; β-actin forward, 5'-ATGTCGGAGCAAGAGCAGCAGC-3' and reverse, 5'-TCCTTCTGACCCATACTGACTTCA-3' and SIRT1 forward, 5'-TCTTCAAGGTGTTTCTCAAGGCAAT-3' and reverse, 5'-ATGTCGGCATGAACTTAA-3'. Relative expression levels of miR-135a-5p and SIRT1 were quantified using the 2^(-∆∆Cq) method (23) and normalized to the internal reference gene U6 and β-actin, respectively.

Dual luciferase reporter assay. The binding site between miR-135a-5p and SIRT1 was predicted using TargetScan (version 7.1; www.targetscan.org/vert_71). BV2 cells (1x10$^4$ cells/well) were seeded into 24-well plates and co-transfected with pGL3-luciferase reporter plasmids (Promega Corporation) containing either wild-type (WT; 0.5 µg) or mutant (Mut; 0.5 µg) SIRT1 3'-untranslated region (UTR) and miR-135a-5p mimic (50 nM) or negative control (NC) mimic (50 nM) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After incubation at 37°C for 48 h, luciferase activities were measured using a Dual Luciferase Assay system (Promega Corporation). Firefly luciferase activities were normalized to Renilla luciferase activities (Promega Corporation).

Caspase-3/9 activity. Cultured BV2 cells were washed and harvested with PBS and lysed using RIPA buffer (Roche Diagnostics (Shanghai) Co., Ltd.) with protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA). Total protein concentration was measured using a BCA protein assay kit (Beyotime Institute of Biotechnology). Proteins (5 µg) were used to measure caspase activities using caspase-3 (cat. no. C116) and caspase-9 (cat. no. C1158) activity assay kits (Beyotime Institute of Biotechnology) according to the manufacturer's protocols.

Western blotting. Cell harvest and lysis were performed as aforementioned. Protein concentration was determined using a BCA assay (Beyotime Institute of Biotechnology). Protein samples were mixed with SDS loading buffer. Proteins (20 µg per lane) were separated via SDS-PAGE (8%) and electrotransferred into PVDF membranes. Following blocking with 5% dry milk in 0.1% TBS-Tween-20 (TBS-T) at room temperature for 2 h, the membranes were incubated overnight at 4°C with anti-SIRT1 (cat. no. 9475; 1:1,000; Cell Signaling Technology, Inc.) and anti-GAPDH (cat. no. 2118; 1:1,000; Cell Signaling Technology, Inc.) antibodies in TBS-T. Following washing with 0.1% TBS-T and incubation with goat anti-rabbit IgG HRP-conjugated secondary antibody (cat. no. 7074; 1:2,000; Cell Signaling Technology, Inc.) for 1 h at 37°C, the membranes were thoroughly washed again. Protein bands were visualized using ECL Prime Western blot analysis detection reagent (EMD Millipore). GAPDH was used as the loading control. Densitometry was analyzed using ImageJ (version 1.50; National Institutes of Health).

Flow cytometric apoptosis assay. Cell apoptosis was assessed using the Annexin V/Dead Cell Apoptosis kit (Thermo Fisher Scientific, Inc.). Cultured BV2 cells (1x10$^4$ from each group) were harvested and washed with PBS. Following centrifugation at 2,000 x g for 3 min at room temperature, cells were suspended in 100 µl PBS, mixed with 5 µl Annexin V-FITC and 5 µl PI and incubated for 15 min in the dark at room temperature. Apoptotic cells at early (FITC+/PI-) and advanced stages (FITC+/PI+) were considered as apoptotic cells. The apoptotic cell percentage were measured by FACSCalibur flow cytometry (BD Biosciences) and data were analyzed by FlowJo (version 7.6.3; FlowJo LLC).

MTT assay. BV2 cells (1x10$^4$ cells/well) were cultured at 37°C in 96-well plates for 0, 24, 48 or 72 h. Cells were incubated with 20 µl MTT solution at 37°C for 3 h. Subsequently, the supernatant was removed, and 150 µl DMSO (Sigma-Aldrich; Merck KGaA) was added to dissolve the formazan product. The optical density was measured using a microplate reader at a wavelength of 570 nm. Statistical analysis. Each experiment was conducted three times. Data are analyzed by SPSS (version 19; IBM Corp.). Data are presented as the mean ± SEM. Comparisons between two groups were analyzed using a Student's t-test. Comparisons among multiple groups were analyzed using one-way ANOVA followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.
Results

**Increased miR-135a-5p expression in KA-induced in vitro epilepsy models.** To assess whether miR-135a-5p was involved in epilepsy, changes of miR-135a-5p expression in BV2 microglia were investigated. KA-treated BV2 microglia have been reported to effectively induce abnormal protein expression, cell apoptosis and other typical post-epileptic reactions (24,25). The results demonstrated that miR-135a-5p expression levels were significantly increased in KA-treated cells compared with controls (Fig. 1).

**miR-135a-5p inhibitor blocks KA-induced apoptosis.** To further investigate whether miR-135a-5p participated in KA-induced post-epileptic responses, BV2 cells were transfected with miR-135a-5p inhibitor prior to KA treatment. Subsequently, the effects of miR-135a-5p inhibitor pre-administration on KA-induced cells were assessed. miR-135a-5p inhibitor significantly decreased miR-135a-5p expression compared with the NC group (Fig. 2A). In addition, at 48 and 72 h post-KA treatment, the MTT assay results demonstrated that miR-135a-5p inhibitor significantly increased BV2 cell numbers compared with the NC group (Fig. 2B).

To assess whether miR-135a-5p is involved in epilepsy-induced apoptosis, flow cytometry was performed to detect the rate of apoptosis in KA-treated BV2 cells. The rate of apoptosis in miR-135a-5p inhibitor-transfected cells was significantly decreased compared with the NC group (Fig. 3A and B). Subsequently, caspase-3 and caspase-9 activities, which are closely related to apoptosis, were measured (26,27). The results demonstrated that caspase-3 and caspase-9 activities were significantly suppressed in the miR-135a-5p inhibitor group compared with the NC group (Fig. 3C and D). The results indicated that miR-135a-5p participated in the pathological response of KA-induced epilepsy models by promoting apoptosis.

**miR-135a-5p regulates SIRT1 expression.** To detect how miR-135a-5p participates in cell apoptosis, the potential target genes of miR-135-5p were identified using TargetScan (www.targetscan.org/vert_71). The results indicated that the 3'-UTR of SIRT1 mRNA might be targeted by miR-135a-5p (Fig. 4A). To assess whether miR-135a-5p regulated SIRT1 expression, a mutant SIRT1 3'-UTR sequence, which was unable to bind to miR-135a-5p, was designed (Fig. 4A). Subsequently, BV2 cells were co-transfected with miR-135a-5p or NC mimic and WT or Mut SIRT1 3'-UTR-containing luciferase reporter plasmids. In miR-135a-5p mimic-transfected BV2 cells, a significant increase in miR-135a-5p expression was detected (Fig. 4B). The dual luciferase assay results demonstrated that miR-135a-5p mimic suppressed the luciferase reporter activities of WT SIRT1 3'-UTR-transfected BV2 cells, but not Mut SIRT1 3'-UTR-transfected BV2 cells, which indicated that miR-135a-5p targeted the 3'-UTR of SIRT1 (Fig. 4C).

To further detect the effects of miR-135a-5p on SIRT1 expression, BV2 cells were transfected with miR-135a-5p inhibitor. The results indicated that SIRT1 mRNA and protein expression levels were significantly enhanced compared with NC groups (Fig. 5A-C).

**siRNA-SIRT1 enhances KA-induced apoptosis.** SIRT1 participates in a variety of important biological processes, including cell apoptosis and survival (28,29). To assess whether SIRT1 participated in KA-induced post-epileptic responses, siRNA-SIRT1 was designed and transfected intoBV2 cells prior to KA treatment to investigate the effects of siRNA-SIRT1 pre-administration on KA-induced epilepsy. SIRT1 mRNA and protein expression levels were significantly decreased in SIRT1 siRNA-transfected BV2 cells compared with NC groups (Fig. 6A-C). In addition, at 48 and 72 h after KA treatment, the MTT assay results demonstrated that siRNA-SIRT1 significantly decreased BV2 cell numbers compared with the siRNA-NC group (Fig. 6D).

To confirm that SIRT1 was involved in epilepsy-induced apoptosis, flow cytometry was performed to detect the rate of apoptosis in KA-treated BV2 cells. The rate of apoptosis of siRNA-SIRT1-transfected cells was significantly higher compared with the siRNA-NC group (Fig. 7A and B). Moreover, caspase-3 and caspase-9 activities were increased in the siRNA-SIRT1 group compared with the siRNA-NC group (Fig. 7C and D). The results suggested that SIRT1 might be involved in the pathological response of KA-induced epilepsy model by inhibiting apoptosis.

**SIRT1 participates in the regulation of miR-135a-5p on epilepsy.** To study the relationship between SIRT1 and the biological phenotype induced by miR-135a-5p inhibitor, KA-treated BV2 cells were co-transfected with miR-135a-5p inhibitor and SIRT1 siRNA. Subsequently, flow cytometry was performed (Fig. 8A and B). miR-135a-5p inhibitor-induced reductions in the rate of apoptosis were attenuated by SIRT1 knockdown. In addition, miR-135a-5p inhibitor-mediated reductions in caspase-3 and caspase-9 activities were also reversed by co-transfection with SIRT1 siRNA (Fig. 8C and D). As presented in Fig. 8E, miR-135a-5p inhibitor-induced increases in cell number were inhibited by co-transfection with siRNA-SIRT1. The aforementioned results suggested that the effects of the miR-135a-5p inhibitor on the KA-induced epilepsy model were impaired by SIRT1 knockdown.
Discussion

miRNAs are widely involved in neural damage following seizures (30), but the molecular mechanisms are not completely understood. In the present study, miR-135a-5p expression was significantly upregulated in a KA-induced in vitro epilepsy model, and miR-135a-5p inhibitor effectively increased BV2 cell proliferation and inhibited cell apoptosis. Moreover, the results indicated that SIRT1 expression was regulated by miR-135a-5p, and SIRT1 knockdown impaired the effects of the miR-135a-5p inhibitor on KA-treated BV2 cells.

Numerous miRNAs have been reported to be abnormally expressed in patients with epilepsy and epilepsy models, and certain miRNAs have been confirmed to be involved in neural damage following seizures (21,22,31). In the present study, miR-135a-5p expression levels in a KA-induced epilepsy model were markedly increased. Similarly, Wu et al (20) reported an abnormal increase in miR-135a-5p expression levels in children with TLE, which was also observed in a rat model. Alsharafi and Xiao (32) also confirmed that miR-135a-5p was upregulated in adult patients with TLE and animal models. The aforementioned results indicated that increased miR-135a-5p expression...
was a co-occurrence and characteristic phenotype in different epilepsy models, which suggested that miR-135a-5p might serve as a potential biomarker of epilepsy. In addition, previous studies demonstrated that miR-135a-5p inhibition effectively alleviated epilepsy-induced nerve injury (20,33). In the present study, the number of BV2 cells was significantly increased and the rate of apoptosis was notably decreased by miR-135a-5p inhibitor transfection, which suggested that miR-135a-5p might serve as a potential therapeutic target for epilepsy. However, the mRNA targets of miR-135a-5p require further investigation.

Using TargetScan, SIRT1 was predicted as a potential mRNA target for miR-135a-5p. Additionally, the dual luciferase reporter assay results further suggested that SIRT1 was targeted by miR-135a-5p. SIRT1 expression levels were

Figure 4. miR-135a-5p targets SIRT1. (A) SIRT1 is predicted to be a target of miR-135a-5p via TargetScan analysis. (B) Reverse transcription-quantitative PCR showed that compared with the miR-NC mimic group, miR-135a-5p mimic significantly increased the levels of miR-135a-5p in BV2 microglia cells, indicating the successful transfection of miR-135a-5p mimic in BV2 microglia cells. (C) Dual luciferase assay showed that miR-135a-5p mimic decreased the luciferase activity in BV2 microglia cells transfected with WT SIRT1 3'-UTR but not mutant SIRT1 3'-UTR, indicating that miR-135a-5p targeted the 3'-UTR of SIRT1.

Figure 5. miR-135a-5p inhibits SIRT1 expression in an in vitro model of epilepsy. In a kainic acid-induced in vitro model of epilepsy in microglia cells, reverse transcription-quantitative PCR and western blotting indicated that SIRT1 (A) mRNA and (B and C) protein levels were increased by miR-135a-5p inhibitor, indicating that miR-135a-5p inhibited SIRT1 expression by targeting its 3'-untranslated region.

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Using TargetScan, SIRT1 was predicted as a potential mRNA target for miR-135a-5p. Additionally, the dual luciferase reporter assay results further suggested that SIRT1 was targeted by miR-135a-5p. SIRT1 expression levels were
Figure 6. siRNA-SIRT1 reduces nerve cell proliferation in an in vitro model of epilepsy. In a KA-induced in vitro model of epilepsy in microglia cells, reverse transcription-quantitative PCR and western blotting showed that compared with the siRNA-NC group, siRNA-SIRT1 significantly decreased the (A) mRNA and (B and C) protein levels of SIRT1, indicating its successful transfection. (D) At 48 and 72 h after KA treatment, MTT assay showed that compared with the siRNA-NC group, siRNA-SIRT1 significantly reduced BV2 cell number. *P<0.05 and **P<0.01 vs. siRNA-NC. SIRT1, sirtuin 1; siRNA, small interfering RNA; NC, negative control; KA, kainic acid.

Figure 7. siRNA-SIRT1 enhances nerve cell apoptosis in an in vitro model of epilepsy. (A and B) In a kainic acid-induced in vitro model of epilepsy in BV2 microglia cells, at 24 h after incubation, flow cytometry assay showed that siRNA-SIRT1 significantly increased the cell apoptosis rate of BV2 microglia cells compared with the siRNA-NC group. BCA protein assay showed that siRNA-SIRT1 significantly upregulated the activity of (C) caspase-3 and (D) caspase-9 compared with the siRNA-NC group in BV2 microglia cells. *P<0.01 vs. siRNA-NC. SIRT1, sirtuin 1; siRNA, small interfering RNA; NC, negative control.
increased by miR-135a-5p inhibitor. Collectively, the results indicated that SIRT1 was a target of miR-135a-5p in a KA-induced in vitro model of epilepsy. Subsequently, the role of SIRT1 and the interaction between SIRT1 and miR-135a-5p in epilepsy were investigated.

SIRT1 is an important type III histone deacetylase that regulates gene expression by catalyzing histone deacetylation and is necessary for SIRT1 to exert its deacetylase activity in the presence of NAD⁺ (34,35). SIRT1 is involved in numerous important physiological processes, including chromosome remodeling, transcriptional inhibition, energy metabolism, cell survival and apoptosis (28,29). SIRT1 has also been reported to be involved in epilepsy, Huntington's disease and other mental diseases (36). Moreover, SIRT1 protein expression levels and activities are altered at different time points following seizure (37-40). A study demonstrated that SIRT1 protein expression levels and activity levels are increased in patients and rats at 1 h post-seizure (37). However, another study indicated that SIRT1 protein expression was decreased at a longer time after seizure (38), which was consistent with the results of the present study. Collectively, the aforementioned results suggested that SIRT1 might serve different functions at different time points after a seizure and might also participate in epilepsy-induced cell apoptosis.

In addition, siRNA-SIRT1 effectively inhibited BV2 microglia proliferation and promoted microglia apoptosis. Moreover, SIRT1 inhibition attenuated, but did not completely block the effects mediated by miR-135a-5p inhibitor. There are a number of potential explanations for the aforementioned findings: i) There was a high level of SIRT1 protein expression following siRNA transfection, and the residual SIRT1 protein might be sufficient to regulate cell survival; ii) miR-135a-5p might serve roles via other target proteins, for example, it has been reported that potassium voltage-gated channel subfamily Q member 3, caspase activity and apoptosis inhibitor 1 (CAAP1) and other proteins are regulated by miR-135a-5p, and participate in cell proliferation and apoptosis (20,33); and iii) as aforementioned, SIRT1 protein expression levels and activity levels were dynamically altered post-seizure, thus inhibiting SIRT1 protein expression might trigger other cellular mechanisms, resulting in a compensatory effect.

In conclusion, the results of the present study suggested that miR-135a-5p participated in epilepsy-induced cell apoptosis via a SIRT1-related signaling pathway in a KA-induced BV2 microglia epilepsy model. Therefore, the present study suggested a potential therapeutic target and biomarker for post-epileptic intervention and provided an important experimental basis for the treatment of post-epileptic neural damage.

However, the present study had two key limitations: i) miR-135a-5p and SIRT1 overexpression experiments were not conducted and ii) experiments were not performed in multiple cell lines or in vivo.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.
Author's contributions

YW, ZhiY, KZ, YW and YZ performed the experiments and prepared the data analyses. ZhiY designed the experiments and prepared the final manuscript. YW and ZhiY confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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