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Original Article

The effect of miR-26a on temporal lobe epilepsy by regulating the HGMB1/1/NF-κB pathway

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Abstract: Seizures are associated with inflammatory pathways. HMGB-1/NF-κB is a common inflammatory pathway. miR-26a is involved in various diseases. However, the role of miR-26a in temporal lobe epilepsy (TLE) remains unclear. SD rats were divided into a control group and a TLE group. Lithium chloride and pilocarpine were used to induce a rat TLE model. Real time PCR was done to analyze the mmiR-26a expression in the temporal lobe tissue, western blot was performed to measure the NF-κB expression, and ELISA was conducted to detect the HGMB1 level. Human astrocytoma U251 cells were divided into a control group, a mir-26a mimics group, and a miR-26a siRNA group followed by an analysis of the miR-26a expression using real-time PCR, an analysis of the proliferation using an MTT assay, an analysis of the caspase-3 activity, an analysis of the of NF-κB and HGMB1 expressions using Western blot, and an analysis of the IL-1β and IL-6 secretions using ELISA. The miR-26a expression in the TLE rats was significantly increased with elevated secretions of the HMGB-1 and NF-κB expressions compared to the control group ($P<0.05$). The transfection of the miR-26a siRNA into the U251 cells significantly down-regulated the miR-26a expression, promoted cell proliferation, decreased caspase-3 activity, reduced the expression of NF-κB and HGMB1, and decreased the secretions of IL-1β and IL-6 ($P<0.05$). The transfection of the mir-26 mimics up-regulated miR-26a, inhibited cell proliferation, increased the caspase-3 activity, increased the expressions of NF-κB and HGMB1, and elevated the secretions of IL-1β and IL-6 ($P<0.05$). The miR-26a level is increased in TLE rats. The down-regulation of miR-26a expression can inhibit inflammation and apoptosis and promote neuronal proliferation by regulating the HGMB1/1/NF-κB inflammatory pathway.

Keywords: Temporal lobe epilepsy, Mir-26a, HGMB1, NF-κB, inflammation

Introduction

Epilepsy (EP) is a chronic nervous system disease. In epilepsy, an abnormal discharge of brain neurons leads to central nervous system dysfunction. Epilepsy is the second most common nervous system disease. Seizures can damage the central nervous system due to their repeated episodes and irregularities, leading to physical injury and great mental stress, and posing a serious threat to global health [1, 2]. The incidence of epilepsy in China has increased yearly, with nearly 10 million new patients per year, especially among children, so it is a major medical problem in China [3]. Epilepsy is a neurological disease related to neurobiology, psychology, and cognition, which can lead to epileptic seizures, causing cognitive dysfunction, anxiety, depression, and other symptoms [4]. The mechanism of epilepsy is complex and has not yet been fully elucidated, and the disease is associated with many causes such as heredity, neurostructural changes, infections, immunity, and metabolism [5, 6]. Recent studies have shown that seizures are closely related to inflammation, which can stimulate the nervous system, promoting neuronal apoptosis or a synapse remodeling of the nervous system, leading to increased excitability of the nervous system and lower thresholds for seizures [7, 8]. Due to the complicated pathogenesis of epilepsy, although the treatment methods are diverse, including drug treatment, surgical treatment, and other methods, the treatment effect is still poor [9]. Therefore, it is urgent to analyze the molecular targets that

can regulate epilepsy and then determine the pathogenesis of epilepsy and a basis for its treatment.

Epileptic seizures are associated with inflammatory pathways. HMGB-1 (high mobility group box-1)/NF-κB is a common inflammatory pathway [10]. HMGB-1 and NF-κB regulate cell proliferation and differentiation and are associated with tumors and autoimmune diseases, cardiovascular diseases, and inflammation [11, 12]. MicroRNAs, also known as miRNAs, small RNAs or microRNAs, are widely found in animals and plants and are small types of short-chain RNAs [13]. miRNAs can regulate mRNA degradation and protein translation by completely or incompletely pairing with target genes, or by inhibiting the expression of downstream transcriptional target proteins [14, 15]. Mir-26a is involved in inflammation and tumors [16]. However, miR-26a's role in temporal lobe epilepsy is unclear.

Materials and methods

Experimental animals

Thirty, male, SPF-grade Sprague-Dawley rats, aged 8 weeks and weighing (200±20) g, were bought from our animal center and fed in the center. This study was approved by our unit ethics committee.

Reagents and instruments

Human astrocytoma U251 cells were preserved in our laboratory and stored in liquid nitrogen. Lithium chloride and pilocarpine were obtained from Sigma, USA. The PVDF membrane was from Pall Life Sciences, the EDTA was from Hyclone, USA, and the Caspase 3 active kit was purchased from the Nanjing Jiancheng Bioengineering Institute. The RNA extraction kit and the reverse transcription kit were from ABI, USA. The DMEM medium and the Qinglian double antibody were purchased from Hyclone, USA. The Dimethyl sulfoxide and the MTT powder were purchased from Gibco; trypsin-EDTA digest was purchased from Sigma, USA. The RNA extraction kit, the reverse transcription kit, and the lipo2000 transfection reagent were purchased from Invitrogen. The ECL reagent was from obtained from Amersham Biosciences Corp., the rabbit anti-mouse HGMB1 monoclonal antibody, the rabbit anti-mouse NF-κB monoclonal antibody, and the horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG were obtained from Cell signaling Corporation of the United States. The HMGB-1 ELISA test kit and the IL-1β and IL-6 ELISA test kits were purchased from the RD Corporation of the United States.

Animal model preparation and grouping

Twenty, healthy male Sprague-Dawley rats were reared for 2 weeks and randomly and equally divided into the control group and the TLE group, in which lithium chloride and pilocarpine were used to construct the rat TLE model. Following procedures established in earlier studies [17], 125 mg/kg of 6.35% lithium chloride was intraperitoneally injected, and after 18-20 hours, 1 mg/kg scopolamine was injected to reduce the side effects of the pilocarpine. After 30 minutes, an intraperitoneal injection of 150 mg/kg of 3.0% pilocarpine was performed. After 30 minutes, facial seizures began to occur, along with symptoms of the seizures, including rhythm nodding, forelimb clumps, hind limb standing, and even falling for more than 30 minutes, indicating a successful modeling.

Collecting samples and organization

After starting anesthesia using 10% chloral hydrate, blood was collected from the abdominal aorta and centrifuged at 2000 rpm to obtain serum. The rats were sacrificed, and their brain tissue was taken and stored at -80°C.

U251 cell culture and grouping

U251 human astrocytes were cultured in DMEM medium containing fetal bovine serum (containing 100 U/ml penicillin, 100 ug/ml streptomycin, 5.5 mmol/L glucose) at 37°C and 5% CO_2. The cultured U251 was divided into three groups: the control group, the miR-26a mimics group, and the miR-26a siRNA group, which was transfected with miR-26a mimics and miR-26a siRNA in the U251 cells, respectively.

Transfection of the miR-26a mimics and miR-26a siRNA in the U251 cells

The miR-26a mimics and the miR-26a siRNA were designed according to the miR-184

Table 1. Primer sequences

Gene	Forward 5'-3'	Reverse 5'-3'
GADPH	AGTGCCAGCCTCGTCTCATAG	CGTTGAACTTGCCGTGGGTAG
miR-26a	GATATCCATTTGGGGAGGGA	ATGGTGGTCCAGGTTCATTCTGT

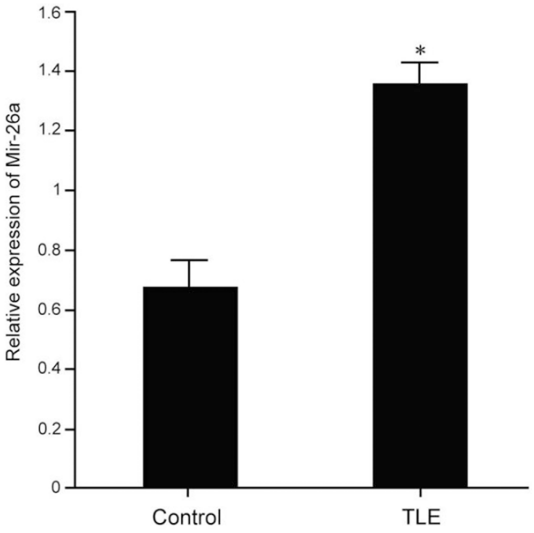

Figure 1. The expression of miR-26a in the brain tissue of rats with temporal lobe epilepsy compared with the control group, *P<0.05.

sequence and synthesized by the Shanghai Jima Company. The mir-26a mimics sequence was: 5'-UAAAUGUUGAUGAC-3', 5'-UAACUGCGUGUUGAUGAC-3'. The miR-26a siRNA sequence was: 5'-UAAUUGUGAUGAC-3'; 5' ACUGCCAAUUGGU-3'. The miR-26a mimics and the miR-26a siRNA were transfected into the U251 cells using lipo2000 transfection reagent. Logarithmic growth U251 cells were inoculated in a 6-well plate and cultured for 12 hours until the cell density was fused to 70-80% followed by addition of 5 μl lipo2000 to 200 ul serum-free medium for 15 a minute incubation. Then, 200 ul serum-free medium was added and mixed for a 15-minute incubation. The mixed lipo2000 was mixed with the miR-26a mimics and the miR-26a siRNA, respectively. The cells were cultured in a 5% CO_2 incubator, and the culture solution was changed at 37°C for 6 hours to continue the culture.

The effect of the MTT assay on the growth of the U251 cells

After 48 hours of cell culture, the cells were digested, counted, and seeded in 96-well plates. Five replicate wells were designed for each group, and 20 μl of 5 g/L MTT solution was added and incubated for 4 hours followed by the removal of the supernatant and the addition of 150 μl/well of DMSO until the purple crystals were fully dissolved, followed by measuring the absorbance (A) value at 570 nm.

Real time PCR measurement of the miR-26a expression

Under sterile conditions, lung tissue mRNA was extracted from the ice using Trizol reagent, and cDNA was synthesized using PCR according to the relevant primers (**Table 1**). The real-time PCR reaction conditions were: 52°C 1 minute, 90°C 30 seconds, 58°C 50 seconds, 72°C 35 seconds, for a total of 35 cycles. GAPDH was chosen as an internal reference. The gene expression was quantified using the 2-ΔCt method.

ELISA analysis of the IL-1β, IL-6, and HMGB-1 expressions

The serum of each group was used to measure the IL-1β, IL-6, and HMGB-1 levels using an ELISA kit according to the ELISA kit's instructions. Briefly, 50 μl standard was used to establish a standard curve. A 50 μl sample was added into a 96-well plate and then 50 μl of enzyme labeling reagent was added followed by the addition of 50 μl of developer A and subsequently 50 μl of developer B. After that, the reaction was stopped after adding 50 μl of the stop solution. The blank value was set to zero and the absorbance value (OD value) at 450 nm was determined.

Western blot analysis of the NF-κB and HGMB1 protein expressions

Lung tissue protein was isolated using a RIPA lysis buffer. After quantification, it was electrophoresed on a 10% SDS-PAGE, transferred to a PVDF membrane, blocked with 5% skim milk powder, and incubated with 1:1000 diluted HGMB1 and 1:500 diluted NF-κB monoclonal antibody at 4°C overnight. After PBST washing, 1:2000 goat anti-rabbit secondary antibody was added and incubated for 30 min in the dark followed by PBST washing, the addition of chemiluminescence color development for 1 min, and subsequent exposure. This assay was repeated four times.

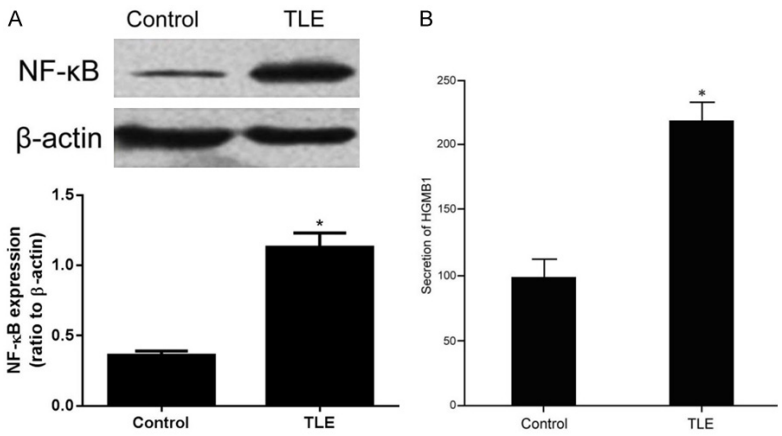

Figure 2. The expression of the HGMB1/NF-κB inflammatory pathway in temporal lobe epilepsy. A. The Western blot analysis of the NF-κB expression; B. The ELISA analysis of the HGMB1 expression, compared with the control group, *P <0.05.

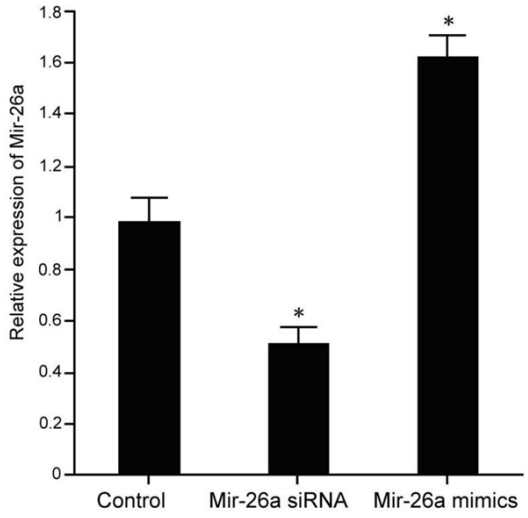

Figure 3. The effect of miR-26a on the expression of miR-26a in U251 cells. Compared with the control group, *P<0.05.

Caspase-3 activity analysis

The changes in the caspase-3 activity in each group of cells were examined according to the kit's instructions. Trypsin digested cells were centrifuged at 600 g at 4°C for 5 min and the cell lysate was lysed on ice for 15 min followed by centrifugation at 20000 g at 4°C for 5 min. After the addition of 2 mM Ac-DEVD-pNA, the OD value change at 405 nm was measured to calculate the caspase-3 activity.

Statistical analysis

The data are shown as the mean ± standard deviation (SD) and were analyzed using SPSS 16.0 software. Two-tailed unpaired Student's t-tests were performed to assess the differences between two groups, and a one-way ANOVA with a Bonferroni post hoc analysis was conducted to compare the differences among the three groups. P<0.05 indicated a statistically significant difference.

Results

miR-26a expression in the brain tissue of rats with temporal lobe epilepsy

The miR-26a levels in the brain tissue of rats with temporal lobe epilepsy were significantly higher than they were in the control group (P<0.05) (**Figure 1**).

The expression of the HGMB1/NF-κB inflammatory pathway in temporal lobe epilepsy

The temporal lobe epilepsy rats had significantly elevated HMGB1 levels compared to the control group (P<0.05). In addition, the NF-κB levels were also significantly increased (**Figure 2**).

The effect of miR-26a on the miR-26a expression in the U251 cells

The miR-26a siRNA transfection into the U251 cells significantly down-regulated the miR-26a (P<0.05). However, the miR-26 mimics transfection significantly up-regulated the miR-26a (P<0.05) (**Figure 3**).

The effect of miR-26a on cell proliferation

The transfection of the miR-26a siRNA into the U251 cells significantly promoted cell proliferation (P<0.05), which was significantly inhibited after the miR-26a mimics transfection (P<0.05) (**Figure 4**).

The effect of miR-26a on the caspase-3 activity in the U251 cells

The transfection of miR-26a siRNA into the U251 cells significantly decreased the caspase-3 activity compared to the control (P< 0.05). The transfection of the miR-26 mimics in

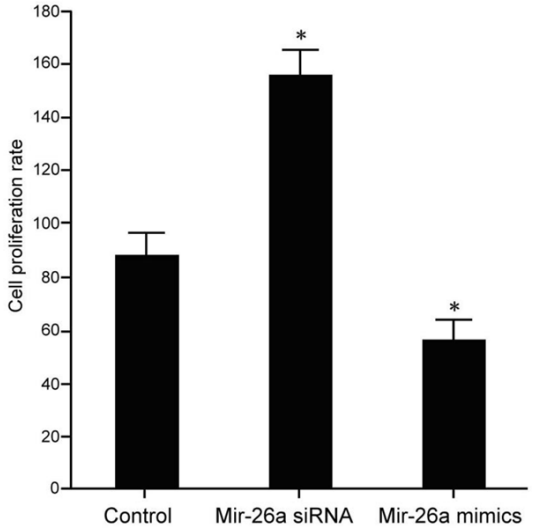

Figure 4. The effect of miR-26a on the proliferation of U251 cells. Compared with the control group, *P<0.05.

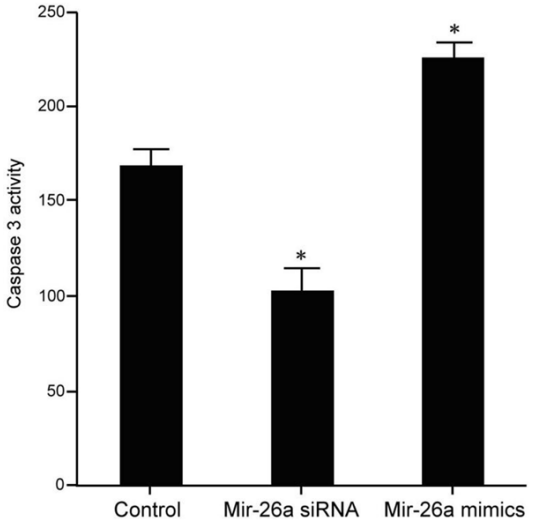

Figure 5. The effect of miR-26a on the caspase-3 activity in U251 cells. Compared with the control group, *P<0.05.

the U251 cells significantly increased the caspase-3 activity (P <0.05) (**Figure 5**).

The effect of miR-26a on the HGMB1/NF-κB inflammatory pathway

The transfection of the miR-26a siRNA into the U251 cells inhibited the HGMB1/NF-κB inflammatory pathway and decreased the NF-κB and HGMB1 expressions. The transfection of the miR-26 mimics in the U251 cells increased the NF-κB and HGMB1 expressions (**Figure 6**).

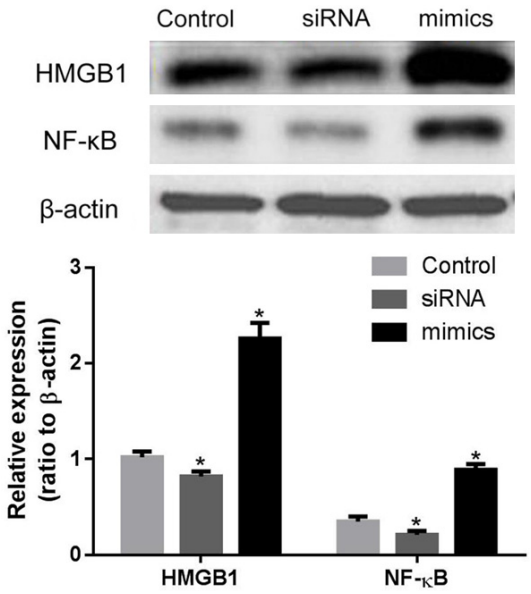

Figure 6. The effect of miR-26a on the expression of the HGMB1/NF-κB inflammatory pathway in U251 cells.

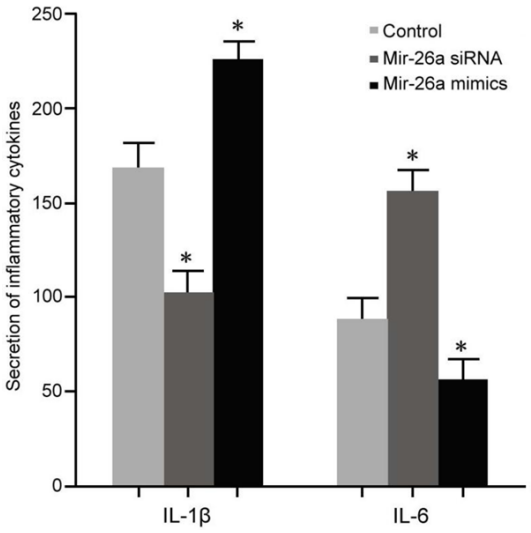

Figure 7. The effect of miR-26a on the secretions of the inflammatory factors in U251 cell supernatants. Compared with the control group, *P<0.05.

miR-26a's effect on the secretions of the inflammatory factors

The transfection of miR-26a siRNA in the U251 cells significantly inhibited the IL-1β and IL-6 secretions in comparison with the control (P<0.05). The transfection of the miR-26 mimics in the U251 cells significantly increased the secretions of IL-1β and IL-6 (P<0.05) (**Figure 7**).

Discussion

Epilepsy is mainly manifested as brain tissue dysfunction, limb convulsions, behavioral disorders, and sensory disturbances, which can cause irreversible damage to the brain tissue [18]. miRNA has a wide range of mechanisms and can participate in the regulation of the growth and development of the body, so that the body's ability to adapt to the environment is enhanced [19]. miRNAs play an important role in the pathological process, especially in tumors, and they can play the role of oncogenes or tumor suppressor genes, but the regulation of miRNAs in epilepsy is less clear [20]. Epilepsy is closely related to inflammation [7, 8], and miR-26a is involved in the inflammatory pathway, but whether miR-26a plays a role in epilepsy has not been determined. In this study, a rat temporal lobe epilepsy model was established to evaluate the role of the HGMB1/NF-κB inflammatory pathway in a rat temporal lobe epilepsy model, and the miR-26a level was increased in the temporal lobe epilepsy model, suggesting the involvement of miR-26a in seizures.

Further, this study analyzed the possible regulatory mechanisms of miR-26a in epilepsy. In vitro cell culture experiments confirmed the transfection of miR-26a siRNA in U251 cells, down-regulated the miR-26a expression, promoted cell proliferation, and decreased apoptotic activity, while it transfected miR-26 mimics in U251 cells, up-regulated miR-26a expression, inhibited cell proliferation, and increased the apoptotic activity, indicating that miR-26a may regulate neuronal apoptosis and survival, which may affect the occurrence of epilepsy. As an important inflammatory pathway, HMGB-1/NF-κB binds to its specific receptor RAGE and plays a role in the stability of chromosome structure and the regulation of transcription and translation [21, 22]. Under pathological conditions, a massive release of HMGB-1 occurs, which activates the downstream signaling pathway NF-κB, which leads to the activation of the body's immune system, and an immune response against the pathogen, resulting in an increase in the release of inflammatory factors, causing inflammatory damage and aggravating the occurrence of epilepsy [14]. This study demonstrates that the up-regulation of miR-26a expression in U251 cells inhibits NF-κB and HGMB1 expressions, increases inflammatory factor secretions, down-regulates miR-26a in U251 cells and promotes NF-κB and HGMB1 expressions, indicating a negative correlation between the miR-26a level and the activation of the HMGB-1/NF-κB inflammatory pathway.

Conclusion

miR-26a expression is increased in temporal lobe epilepsy rats. The down-regulation of miR-26a expression can inhibit inflammation and apoptosis and promote nerve cell proliferation by regulating the HGMB1/1/NF-κB inflammatory pathway.

Disclosure of conflict of interest

None.

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