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Original Research

Effect of miR-124 on PI3K/Akt signal pathway in refractory epilepsy rats

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Abstract: This research aimed to investigate the effect of miR-124 on the PI3K/Akt signaling pathway in refractory epilepsy rats. Ten healthy SD rats were selected as a control group, thirty-six successfully established model rats were randomly divided into the model group, mir-124-agomir, mir-124-antagomir, mir-124-agomir+ LY294002 group (combined group), with 9 rats in each group. PI3K or (and) Akt expressions were intervened respectively. The changes in attack latency and cognitive function were observed, and the correlation among miR-124, PI3K, Akt, and attack latency was analyzed. Up-regulation of the level of miR-124 could increase the seizure interval of rats (P< 0.05), improve the cognitive function of rats (P< 0.05), and promote the expression of PI3K, AKT. The level of miR-124, PI3K, Akt was positively correlated with the latent time of seizures in rats, and the level of miR-124 was positively correlated with the level of PI3K, Akt (P< 0.05). In conclusion, miR-124 can play a protective role in temporal lobe epilepsy by promoting PI3K/Akt signaling pathway, including the protection of cognitive function, which may be a potential target for clinical treatment of intractable epilepsy in the future.

Key words: miR-124; Refractory epilepsy; Rats; PI3K/Akt Signal Pathway.

Introduction

Epilepsy is a chronic disease with brain dysfunction. About 1% of epilepsy patients in the world have high morbidity and mortality. About 33% of epileptic patients have antiepileptic drug tolerance, and patients still cannot prevent epileptic seizures after being treated with two or more drugs, which is called intractable epilepsy and greatly limits the use and treatment of drugs (1-9). Although surgical treatment is an effective method for intractable epilepsy patients, epilepsy patients such as primary generalized epilepsy, non-localized or multifocal epilepsy or eloquent brain epilepsy are not suitable for resection (10, 11). Therefore, finding new therapeutic drugs or therapeutic targets is of great significance for the clinical treatment of intractable epilepsy.

miRNAs are a group of non-coding RNA with a length of about 19-24 nucleotides. They regulate regulation of post-transcription of gene expression by complementary base pairing with specific 3' untranslated region sequences (12). In recent years, some research reports have found that miRNAs play a very critical role in epilepsy. A study using the TaqMan low-density array showed that 51% of miRNAs detected in the normal hippocampus were down-regulated in the epileptic hippocampus (13). In the study by Peng et al. (14), it is reported that the expression patterns of miR-124 and miR-134 show almost the same dynamic changes in the three stages of development of temporal lobe epilepsy (acute, chronic and latent). In recent years, many studies have reported that silenced miR-134 has a better antiepileptic effect (15, 16). Few studies have reported whether miR-124 has a similar antiepileptic effect, and its specific mechanism of action is not completely clear (16-21).

This study explores the role of miR-124 in epileptic rats and its mechanism of brain protection, providing an experimental basis for finding new therapeutic targets for clinical treatment of epilepsy.

Materials and Methods

Research object

Altogether 50 SPF SD male rats were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd., with a strain code of 101 and production license of SCXK (Shanghai) 2017-0011. The rats were all fed with common nutritious feed (Beijing Zhecheng Technology Co., Ltd.). The drinking water was acidified water with a pH value of 2.5-3 after autoclaving. The average age of rats was (21± 2) days. The average body weight was (264.1± 21.5) g. The feeding temperature was 18-22 ℃, and the relative humidity was 40-70%. All the rats were separately fed in a feeding box, and the padding was changed regularly in the morning and evening every day. Environmental noise was less than 85 decibels, ammonia concentration was not more than 20 ppm, and ventilation was carried out 8- 12 times per hour. The nest was changed 1-2 times a week and cleaned and disinfected. The noise did not exceed 60dB, ammonia concentration did not exceed 14ppm, ventilation did not be less than 15 times per hour, and fluorescent lamps

Table 1. Racine grading in this experiment.

Racine Grading	Performance
0	No convulsions
I	Mouth and facial movement
II	Rhythmic nodding, facial clonus
III	Unilateral forelimb clonus
IV	Anterior bilateral limb clonus, standing
V	Standing and falling, tonic-clonic seizures all over the body

were periodically illuminated at intervals of 12 hours.

Establishment of rat model of intractable epilepsy

A random number table method was used to select 40 rats to construct a model. The temporal lobe epilepsy model was established by intraperitoneal injection of lithium chloride-pilocarpine (22). The dose of lithium chloride was 127 mg/kg, and methyl atropine bromide was intraperitoneally injected overnight at a dose of 1mg/kg, and pilocarpine was intraperitoneally injected 30min later at a dose of 50mg/kg. The neurobehavioral blood changes of rats were observed after 30min. Atropine and diazepam were injected intraperitoneally after epilepsy lasted for 1 hour. After controlling epilepsy into latency, Racine grading (Table 1) was performed (23). Rats with IV-V grading were considered to be a successful model. Thirty-six models were successfully modeled, and the success rate was 90%. 36 models were successfully established, with a success rate of 90%.

Intervention and grouping

miR-124 specific agonist (miR-124-agomir) and inhibitor (MiR-124-Antagonir) were purchased from Guangzhou RiboBio Co., LTD, brain stereotaxic was purchased from Stoelting Company of the United States, and 10 unmodeled rats were used as the control group. Thirty-six successfully modeled rats were randomly divided into a model group, miR-124-agomir, miR-124-antagomir, miR-124-agomir +LY294002 group (combined group) with 9 rats in each group. After intraperitoneal injection of 3.5% of chloral hydrate (50mg/kg) for anesthesia, the rats in miR-124-agomir and mir-124-antomir groups were exposed to the anterior fontanel by opening the skin. The anterior fontanel center was drilled 0.8mm backward from the center, 1.8mm beside the midline, and 3.7mm below the dura mater. Ten μl of mir-124-agomir, mir-124-antomir or LY294002 was injected here at an injection rate of 0.2μl/min.

Observation indicators

The changes in attack latency and cognitive function of rats after the intervention of miR-124 and PI3K/Akt were observed, and the relationship between miR-124, PI3K/Akt and attack latency of rats was analyzed.

Detection method

Morris water maze test

This test included two parts: positioning navigation and space exploration, lasting for 5 days. Positioning navigation experiment: every day at 9:00 AM, the platform was placed in the third quadrant, the rats were first allowed to stand on the platform for the 30s, and then put into the water from different quadrants facing the pool wall, and the time of finding and climbing the platform within 90s (escape latency period) was recorded. After finding the platform, the rats stood for the 30s and were taken off to rest. If the rat did not find a platform within 90 seconds, it would be artificially dragged to the platform and stayed for 15 seconds, and the escape latency would be recorded as 90 seconds. The rats were tested 4 times a day and the average value was taken. Space exploration experiment: the platform was removed on the 6th day, the rats were put into the water at the same water entry point, and the swimming track of the rats in the 90s was photographed with a camera, and the swimming time and distance in the quadrant (target quadrant) where the original platform was located were recorded. After the Morris water maze test was completed, 10% of chloral hydrate (100 mg/kg) was injected intraperitoneally to anesthetize the rats, and the rats were killed by neck amputation method, and the hippocampus tissue of the rats was taken immediately.

qRT-PCR

The miR-124 level was detected by qRT-PCR. After hippocampal tissue was ground into a slurry, the total RNA was extracted by TRIzol reagent (Thermofisher, China, item number: 1218355). The concentration of RNA was determined by ultraviolet spectrophotometer (Beijing Dingguo Changsheng Biotech Co., Ltd.) and the RNA purity A260/A280 value was required to be between 1.8 and 2.1. The integrity of RNA was analyzed by 3% of agarose gel electrophoresis (Gene line bioscience, Shanghai, China). First-strand cDNA synthesis: Transcript II Green Two-STEP QRT-PCR Supermix kit was purchased from TransGen Biotech, Beijing, China, with the article number of AQ301-01. cDNA amplification: Transcript II Two-STEP RT-PCR Supermix kit was purchased from TransGen Biotech, Beijing, China, with the article number of AH401-01. All operation steps referred to the kit instructions for details. The primer sequences were shown in Table 2.

Western blot

Sample buffer (4X) composed of bromophenol blue, glycerol, SDS, mercaptoethanol, etc (Shanghai Xin Yu Biotech Co., Ltd., item number: XY-31227-1), sample buffer to protein ratio was 3:1, and the sample buffer was heated in a water bath at 97 ℃ for 5min. BCA method was applied to detect protein concentration, the protein concentration was adjusted to 4μg/μL,

Table 2. Primer sequence for amplification of miR-124 and U6.

	forward primer	reverse primer
miR-124	5′-GATACTCATAAGGCACGCGG-3′	5′-GTGCAGGGTCCGAGGT-3′
U6	5'-GCGCGTCGTGAAGCGTTC-3'	5'-GTGCAGGGTCCGAGGT-3'

the protein was separated with 12% polyacrylamide gel electrophoresis with an initial voltage of 90 V, then the voltage was increased to 120V to move the sample to the appropriate position of the separation gel. The membrane was transferred with 100V of constant voltage for 100min after electrophoresis, and the sample was sealed at 37℃ for 60min. Next, the transfer membrane was placed in 5% skim milk for sealing, then the immune reaction was performed, the membrane was incubated with primary antibody (1: 1000) at 4 ℃ overnight, and rinsed three times with PBS with 5min each time on the next day, then ECL luminescent reagent was incubated with secondary antibody (1: 1000) for 1 hour at room temperature. After completion, ECL luminescent reagent was developed and fixed, and the strip scanned by the film was statistically analyzed by Quantity One software. The relative protein expression level = strip gray value/internal reference gray value. BCA protein kit and ECL light-emitting kit were all purchased from Thermo Scientific™, with the article numbers of 23250 and 35055. Rabbit anti-PI3K, AKT monoclonal antibody and goat anti-rabbit IgG secondary antibody were all purchased from Abcam in the United States, with the article numbers of ab32089, ab179463 and ab6721.

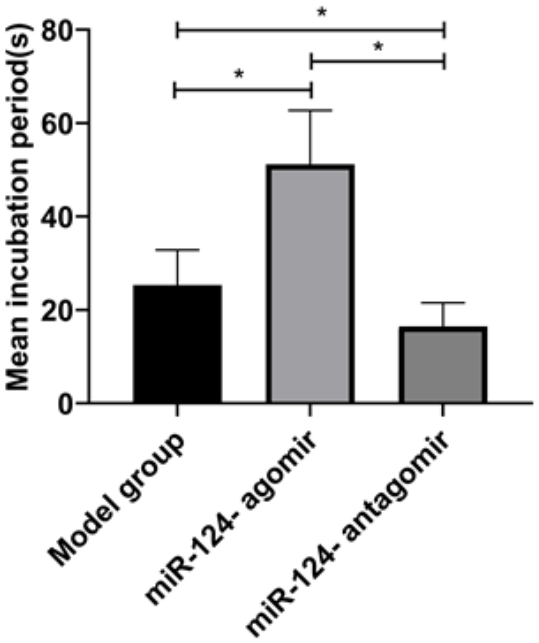

Figure 1. Average attack latency analysis of rats in the model group, miR-124-agomir group and miR-124-antagomir group. * indicates P< 0.05.

Statistical Analysis

SPSS 19.0 was used. The measurement data were expressed in percent and the $\chi 2$ test was used for the comparison of rates. The counting data were expressed by mean ± standard deviation (mean ± sd). The comparison between the two groups was conducted by t-test, the comparison among multiple groups was conducted by variance analysis, the back testing was conducted by LSD test, and Pearson correlation analysis was conducted to analyze the relationship between miR-124, PI3K, Akt levels and epileptic latency in rats. The *p*-value of less than 0.05 was regarded as statistical significance.

Results

Average seizure latency of epilepsy

The average attack latency of the model group, miR-124-agomir and mir-124-antomir groups was statistically different ($p< 0.05$). The average attack latency of the model group, miR-124-agomir group, and mir-124-antomir group were (25.32 ± 7.52) s, (51.16 ± 11.57) s, and (16.48 ± 5.03) s, respectively. The average attack latencies of the model group and miR-124-agomir group were longer than that of mir-124-antomir ($p < 0.05$), and that of miR-124-agomir was longer than model group ($p < 0.05$) (Figure 1).

Cognitive Function

There were statistical differences in the escape latency, target quadrant cumulative time and cumulative distance of the four groups of rats on the 5th day (P< 0.05). The latency of the control group, miR-124-agomir, model group and miR-124-antagomir rats increased successively (P< 0.05), while the cumulative time and cumulative distance decreased successively (P< 0.05) (Figure 2).

miR-124 Expression Level

The expression levels of miR-124 in the hippocam-

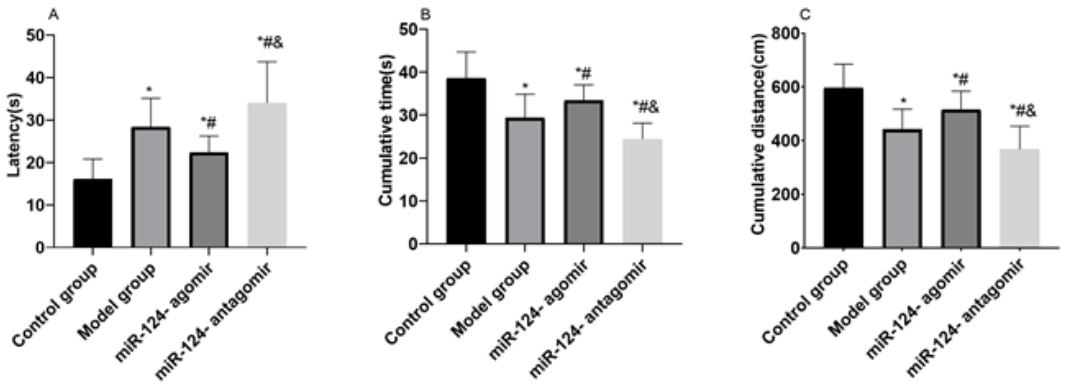

Figure 2. Cognitive function analysis of rats in the control group, model group, miR-124-agomir group, and miR-124-antagomir group. **A:** Analysis of escape latency on the 5th day in the four groups. **B:** Analysis of Target Quadrant Cumulative Time on the 6th day in the Four Groups. **C:** Analysis of Target Quadrant Cumulative Distance on the 6th day in the Four Groups. * indicates that compared with the control group, p< 0.05; # indicates that compared with the model group, P< 0.05; & indicates that compared with the miR-124-agomir group, P< 0.05.

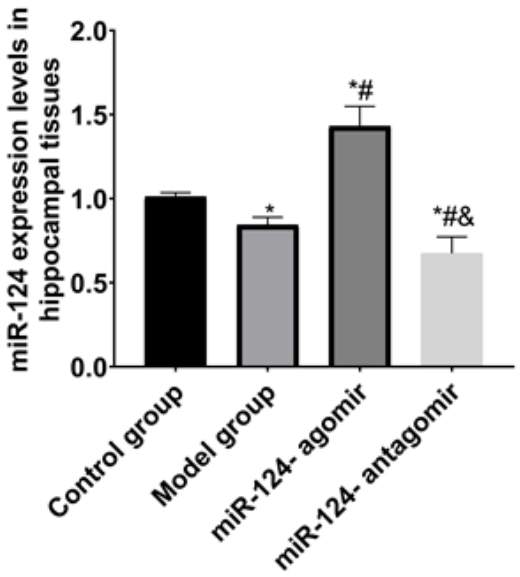

Figure 3. Analysis of the expression level of miR-124 in the control group, model group, miR-124-agomir group and miR-124-antagomir group. * indicates that compared with the control group, P< 0.05; # indicates that compared with the model group, P< 0.05; & indicates that compared with the miR-124-agomir group, P< 0.05.

pus of the four groups of rats were statistically different (P< 0.05). The expression levels of miR-124 in the hippocampus of the control group, miR-124-agomir, model group and miR-124-antagomir rats decreased successively (P< 0.05) (Figure 3).

PI3K/Akt Expression Level

The expression levels of PI3K and Akt in the hippocampus of the four groups of rats were statistically different (P< 0.05). The expression levels of PI3K and Akt in the hippocampus of the control group, miR-124-agomir, model group and miR-124-antagomir rats decreased successively (P< 0.05) (Figure 4).

Correlation Analysis

Pearson correlation analysis showed that miR-124,

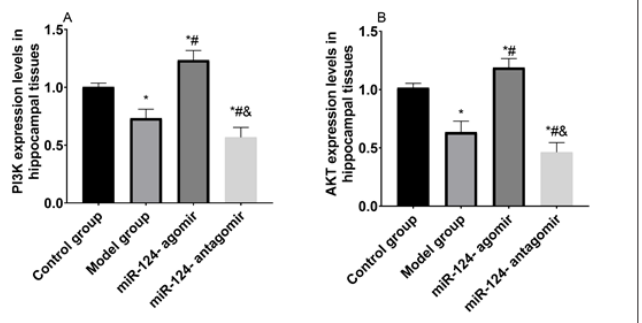

Figure 4. Analysis of PI3K/Akt expression level in the control group, model group, miR-124-agomir group and miR-124-antagomir group. **A:** Analysis of PI3K Expression Level in Four Groups of Rats. **B:** Analysis of Akt Expression Level in Four Groups of Rats. * indicates that compared with the control group, p< 0.05; # indicates that compared with the model group, P< 0.05; & indicates that compared with the miR-124-agomir group, P< 0.05.

PI3K and Akt levels were positively correlated with epileptic latency in rats (P< 0.05), and miR-124 was positively correlated with PI3K and Akt levels (P< 0.05) (Figure 5).

Analysis Results of Rescue Experiment

On the 5th day, the escape latency of the rats in the combined group was higher than that in the miR-124-agomir group (P< 0.05), and the average attack latency, target quadrant cumulative time and cumulative distance were lower than those in the miR-124-agomir group (P< 0.05). PI3K and Akt levels were also lower than those in the miR-124-agomir group (P< 0.05), and miR-124 expression had no difference (P> 0.05) (Figure 6).

Discussion

miR-124 has been known as a miRNA that is specifically expressed in brain tissue and is initially considered as a key regulator of neuronal differentiation and nervous system development (14, 24, 25). Recent studies

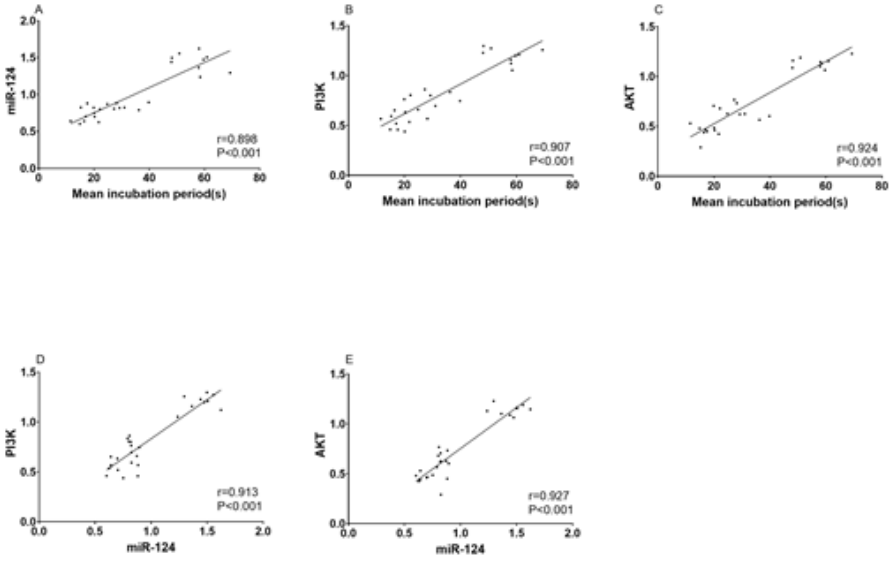

Figure 5. Correlation analysis. **A:** Correlation between miR-124 and mean latency in rats. **B:** Correlation between PI3K and Mean Latency in Rats. **C:** Correlation between Akt and Mean Latency in Rats. **D:** Correlation between miR-124 and PI3K expression. **E:** Correlation between miR-124 and Akt expression.

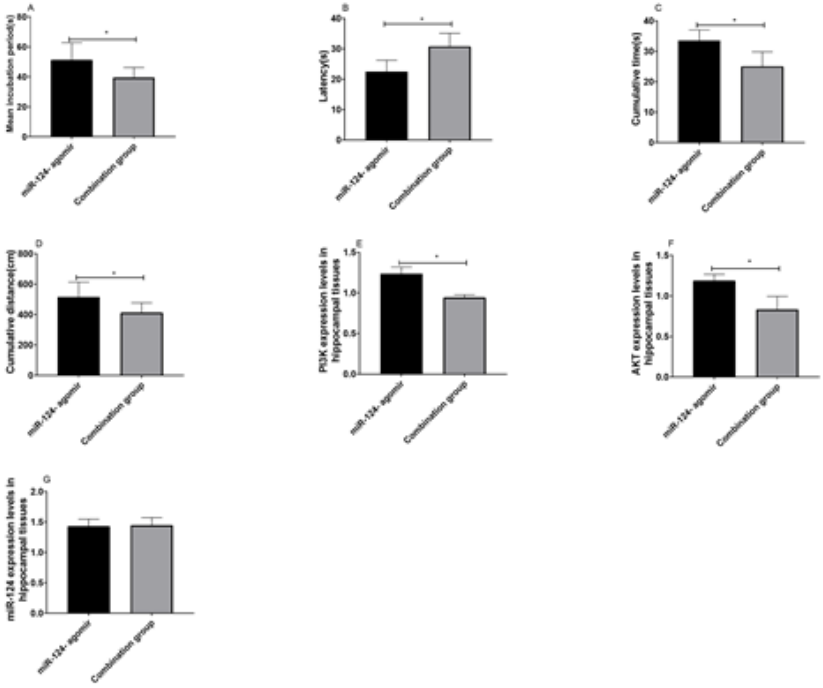

Figure 6. Rescue experiment. **A:** Comparison of average attack latency between the two groups of rats. **B:** Comparison of escape latency on the 5th day between the two groups of rats. **C:** Comparison of accumulative time in the target quadrant between two **groups of rats. D:** Comparison of accumulative distance in the target quadrant between two groups of rats. **E:** Comparison of PI3K level between two groups of rats. **F:** Comparison of AKT levels between the two groups of rats. **G:** Comparison of miR-124 levels between the two groups of rats. * indicates that P< 0.05.

have found that the up-regulation of miR-124 has an anti-epileptic effect. Based on some works, it is found that miR-124 mimetic pretreatment reduces the severity of epileptic seizures in rats during pilocarpine-induced status epilepticus and pentylenetetrazole induced seizures. Although it has been found that miRNAs are deregulated in many animal models of epilepsy, the molecular mechanism of miRNAs regulating epilepsy is still unclear. Some studies have reported that miR-124 inhibits RHO-associated expression of coiled-coil binding protein kinase 1 by activating PI3K/Akt signaling pathway, and promotes neuronal synaptic elongation (26). Some studies have also reported that miR-124 activates PI3K/Akt signaling pathway to play a neuroprotective role in ischemic stroke (27). Therefore, this may also be one of the mechanisms by which miR-124 plays a protective role in epilepsy. This study explored this issue.

Temporal lobe epilepsy is common refractory epilepsy (28). In this study, a rat model of temporal lobe epilepsy was established by lithium chloride-pilocarpine. The research results showed that after the intervention of miR-124 agonist, the latency of epilepsy in rats was significantly prolonged, while miR-124 inhibitor could significantly reduce the latency of epilepsy in rats, and also had a certain promotion effect on cognitive impairment in rats. miR-124 agonist played a protective role. The detection results of miR-124 expression level in rat hippocampus also showed that miR-124 agonist significantly up-regulated miR-124 expression, the miR-124 inhibitor significantly inhibited miR-124 expression, and the miR-124 level was significantly linearly and positively correlated with epileptic latency in rats. These research results once again verified that miR-124 has a better antiepileptic effect.

PI3K/Akt signaling pathway has been verified to be closely related to the occurrence and development of epilepsy (29, 30). Our results also showed that compared with the control group, PI3K and Akt protein levels in the hippocampus of the epileptic model group were significantly inhibited, while the up-regulation of miR-124 could relieve this inhibition. We found through rescue experiments that, inhibition of PI3K/Akt signaling pathway by LY294002 inhibitor could effectively block the anti-epileptic effect of miR-124, including the protection of cognitive function in rats. These results showed that PI3K/Akt signaling pathway was another mechanism of miR-124 anti-epileptic effect. This may be related to miR-124 protecting brain cells by promoting the PI3K/Akt signaling pathway. Wang et al (31) reported in the study that miR-124 reduces cerebrovascular endothelial cell apoptosis and ROS generation by regulating PI3K/AKT signaling pathway. Kang et al (32) also reported in the study that miR-124 inhibits Tau protein hyperphosphorylation by regulating PI3K/AKT signaling pathway, thus hindering the formation of neurofibrillary tangles to play a neuroprotective role. miR-124 promotes neuronal synaptic elongation by regulating the PI3K/AKT signaling pathway (26), which may be the further mechanism of action. This will be further verified in our future research.

There were also some deficiencies in this study. We did not detect the survival of hippocampal tissue cells. In addition, we did not further verify whether there is a targeted regulatory relationship between miR-124 and PI3K/AKT signaling pathway. However, we found a targeted binding site between AKT and miR-124 by predicting miR-124 downstream target genes through Target scan 7.2, and we will further verify it in future studies. The pathogenesis of epilepsy is complex, and the role of miR-124 in epilepsy is also complex. Manna et al. (33) reported in the study that the allele or genotype distribution of the polymorphism of miR-124

rs531564 was not associated with the genetic susceptibility to temporal lobe epilepsy. Brennan et al. (34) also reported in the study that miR-124 has a dual role in epilepsy. mir-124 saves NRSF-mediated gene inhibition of key neurons by blocking the up-regulation of NRSF and weakens epilepsy, but at the same time promotes epilepsy through inflammation. Therefore, more research is needed to fully understand the role of miR-124 in epilepsy.

To sum up, miR-124 can play a protective role in temporal lobe epilepsy by promoting the PI3K/Akt signaling pathway, including the protection of cognitive function, which may be a potential target for clinical treatment of refractory epilepsy in the future.

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Availability of data and materials
The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions
JH, XT, HC, QH and XL led the conception and design of this study. JH, XT, GC, LM and CJ were responsible for the data collection and analysis. HC, GC and LM were in charge of interpreting the data and drafting the manuscript. JH and XL revised critical perspectives for important intellectual content. The final version was read and adopted by all the authors.

Ethics approval and consent to participate
The study was approved by the Ethics Committee of Youjiang Medical College for Nationalities.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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