■ Original article

Epileptic Disord 2021; 23 (6): 823-832

Epileptic Disorders

MiRNA-29a serves as a promising diagnostic biomarker in children with temporal lobe epilepsy and regulates seizure-induced cell death and inflammation in hippocampal neurons

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Received May 10, 2021; Accepted June 26, 2021

ABSTRACT

Objective. Temporal lobe epilepsy (TLE) in children is one of the most common refractory epilepsies. MicroRNAs (miRNAs) show abnormal expression in neurological disorders. The objective of this study was to determine changes in expression and the role of miR-29a in children with TLE.

Methods. Sixty-five TLE patients and 70 normal controls were recruited. The levels of miR-29a were quantified using qRT-PCR. An *in vitro* TLE cell model was established using primary hippocampal cells cultured in magnesium-free medium. Cell viability, cell apoptosis and inflammatory cytokine concentrations were evaluated. The luciferase reporter assay was applied to confirm the target gene, *HMGB1*.

Results. A low level of MiR-29a expression was observed in the serum of children with TLE, which demonstrated a negative association with the concentration of serum TNF-α, IL-6, and IFN-γ. The level of MiR-29a demonstrated high specificity and sensitivity in children with TLE. A low level of expression of miR-29a was also detected in the TLE cell model. MiR-29a over-expression reversed the decreased cell viability induced by TLE, and alleviated cell apoptosis. Release of TNF-α, IL-6, and IFN-γ induced by TLE was also inhibited by miR-29a over-expression. *HMGB1*, which was downregulated in the serum of TLE patients, was shown to be a target gene of miR-29a, and negatively correlated with miR-29a level.

Significance. The downregulation of serum miR-29a may serve as a non-invasive diagnostic biomarker for children with TLE. MiR-29a may be involved in the pathogenesis of TLE through regulation of neuronal apoptosis and neuroinflammation via targeting *HMGB1*.

Key words: miR-29a, hippocampus neuron, temporal lobe epilepsy, *HMGB1*, neuroinflammation

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doi:10.1684/epd.2021.1331

Epilepsy, a transient brain dysfunction syndrome, is characterized by unprovoked seizures caused by sudden abnormal neuronal discharges [1]. Temporal lobe epilepsy (TLE) is a common type of childhood epilepsy [2]. Due to resistance to a single antiseizure drug, children with TLE often need to take two or more drugs for clinical treatment, and currently, the treatment of children with TLE is increasingly being discussed [3-5]. TLE is a type of drug-refractory epilepsy, which is mainly treated by surgical resection [6]. In addition, compared with adults, the symptoms of TLE in children are greatly affected by age and brain development. The changing symptoms bring great challenges for doctors trying to identify and effectively treat children with TLE [3]. Recent studies have shown that immune system activation and an excessive inflammatory response play a persistent and important role in the spontaneous onset of TLE. Moreover, in the developing brain, the inflammatory response related to the nervous system can increase the frequency of seizures and the sensitivity of the brain to epilepsy, increasing the susceptibility to epilepsy [7]. Therefore, understanding the relationship between inflammation and the development of epilepsy is of great significance to the study of pathogenesis of TLE.

MicroRNAs (miRNAs), a group of single-stranded RNA molecules of approximately 21 to 23 nucleotides in length, regulate target gene expression by binding to their target messenger RNAs [8]. Most miRNAs can be detected by quantitative real-time polymerase chain reaction (qRT-PCR) and microarray methods [9]. Moreover, certain miRNAs have been reported to be abnormally expressed in various types of human diseases. A close association between miRNAs and the development and pathogenesis of diseases has been determined, reflecting their potential use in the diagnosis of diseases [9-15]. In recent years, dysregulation of miR-29a has been widely reported to be involved in neural development, brain damage and neurological disease repair [16-22]. Growing evidence indicates that a high-level of miR-29a promotes neurite outgrowth by decreasing PTEN [17]. MiR-29a is also involved in regulating neurite growth and neural stem cell development [23]. In cerebral ischemia-reperfusion injury, increasing miR-29a can delay neuronal death and thus prevent the occurrence of ischemia-reperfusion injury [22]. However, the role of miR-29a in epilepsy has not been examined. Therefore, in the present study, attention was focused on the role of miR-29a in TLE and its underlying potential mechanisms [24].

In our study, we first determined the expression level of miR-29a in the serum of children with TLE and evaluated its clinical diagnostic value as a biomarker for TLE. We subsequently examined the role of miR-29a in TLE based on a TLE *in vitro* model, focusing on its effect on proliferation of hippocampal neurons, apoptosis and inflammatory responses.

Materials and methods

Study population and sample collection

Sixty-five individuals with TLE were recruited (average age: 9.98 ± 2.58 years old), including 34 males and 31 females. TLE was diagnosed in accordance with the diagnostic standard of Practical Pediatrics, 7th edition [25], and the patients were finally diagnosed on the basis of their clinical seizure semiology, video-electroencephalography (EEG) monitoring, and high-resolution MRI results. According to the test results, 11 cases developed mesial sclerosis. Children with intracranial infectious diseases, craniocerebral trauma and intracranial space-occupying lesions were excluded. A 4-mL peripheral blood sample was collected from each subject within 12 hours after a seizure and then centrifuged. Subsequently, the serum samples were stored at -80°C for further analysis. In addition, 70 healthy children (mean age: 10.07 ± 2.62 years old) without TLE were included as the control group, including 37 males and 33 females. The age and gender were matched between the case and control groups. Peripheral venous blood specimens were collected from the antecubital vein after 12 hours of overnight fasting, and serum samples were separated and stored at -80°C for subsequent experiments. The protocol of this study was approved by the Ethics Committee of Binzhou Medical University Hospital, and written informed consent was collected from each subject.

Primary culture of hippocampal neurons from newborn rats

Newborn Wistar rats were used for the extraction of primary hippocampal neurons, and the cells were collected and cultured as previously described [1]. In brief, hippocampal tissue was isolated and shredded in an ice bath with PBS, and then digested with 0.5% trypsin in a 37°C water bath for 15 minutes. The digestion was stopped with FBS, and the tissue was gently blown and the filtrate was collected. Neurons were collected and placed on glass coverslips with medium, and then cultured in a humidified incubator (5% CO_2) at 37°C. After examining cell purity, cells were cultured with Neurobasal medium (Invitrogen, USA). Half of the medium was substituted with fresh medium every week.

Establishment of an *in vitro* TLE cell model

To mimic brain conditions in children with TLE, we cultured hippocampal neuron cells in magnesium-free

medium at 37°C [26]. After treatment for three hours, hippocampal epileptiform activity was induced, and the cells were returned to normal cell culture medium.

Transfection

The hippocampal neurons were randomly divided into four groups and transfected with different medium: No Mg^{2+} group, No Mg^{2+} + mimic NC group, No Mg^{2+} + miR-29a mimic group and control group (no treatment). All groups, except the control group, were cultured in magnesium-free medium. The MiR-29a mimic and the respective negative control (referred to as "mimic NC") were synthesized and supplied by Amspring (Changsha, China). Cell transfection was performed using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Twenty-four hours post transfection, cells were collected for further experiments.

RNA extraction and qRT-PCR

Trizol reagent (Invitrogen, USA) was applied to extract total RNA, which was reverse transcribed into cDNA using the TaqMan miRNA reverse transcription kit (Thermo Fisher Scientific) for miR-29a and the High Capacity cDNA Archive kit (Applied Biosystems) for *HMGB1*. With the cDNA as the template, quantitative real-time polymerase chain reaction (qRT-PCR) was then performed to quantify the expression level of miR-29a and *HMGB1* using the ABI 7300 real-time PCR system (ABI, USA). Relative gene expression of miR-29a and *HMGB1* was calculated based on the comparative delta CT ($2^{-\Delta\Delta Ct}$) method [9]; U6 and GAPDH were used as internal controls. The following sense and antisense primers were used. miR-29a: sense 5′-CTGATTTCTTTTGGTGTTCAG-3′/antisense 5′-AACCGATTTCAGATGGTGC-3′; sense 5′-CACCGTGGGACTATTAGGAT-3′/antisense 5′-GCT CACACTTTTGGGGATAC-3′; U6: sense 5′-GCTTCGGCAGCACATATACTAAAAT-3′/antisense CGCTTCACGAATTTGCGTGTCAT-3′; GAPDH: sense 5′-GGGAAGCTTGTCATCAATGG-3′/antisense 5′-CATCGCCCCACTTGATTTTG-3′.

MTT assay

MTT assay was used to assess cell viability according to a previous report [27]. Cells were seeded in 96-well plates at a density of 1×10^4 cells/well. The plates were incubated in 5% CO_2 at 37°C for three days, followed by exposure to 50 µL of MTT solution per well for a further three hours. Then, 150 µL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich; Merck) was added into each well to dissolve the formazan crystal. After gentle shaking for 10 minutes, the absorbance was measured at 490 nm using a microplate reader to determine cell proliferation. The cell proliferation rate was calculated based on the following formula: cell proliferation rate = ([average cell number in sample wells] / [average cell number in control wells]); and the cell proliferation rate of the untreated cells was normalized as 100%.

Flow cytometric apoptosis assay

Cell apoptosis was detected using an Annexin V-FITC Apoptosis Detection kit (Keygen Biotechnology). All cells were harvested and washed with incubation buffer (containing 10 mmol/L HEPES/NaOH, pH 7.4, 140 mmol/L NaCl and 5 mmol/L $CaCl_2$). The cells were then washed with PBS and stained at room temperature for 15 mins with 5 µl Annexin V-FITC and propidium iodide (PI) staining solution. The apoptotic rates were measured by flow cytometry. Final apoptotic cell number was estimated as a total percentage of early apoptotic cells staining positive for Annexin V and negative for PI, and late apoptotic cells positive for both Annexin V and PI. The cell apoptosis rate was calculated as follows: cell apoptosis rate (%) = (apoptotic cell number / total cell number) × 100.

Enzyme-linked immunosorbent assay (ELISA)

The protein concentrations of TNF-α, IL-6, and IFN-γ in serum or cell culture supernatants were determined by ELISA using Biotrak Easy ELISA kits (Amersham Biosciences).

Luciferase reporter assay

Binding between miR-29a and the 3′UTR region of *HMGB1* was predicted based on sequence analysis using TargetScan software. *HMGB1* 3′-UTR double luciferase reporter plasmids of wild-type *HMGB1* (Wt) and mutated *HMGB1* (Mut) were designed and synthesized by Shanghai Sangon Biotechnology Co., Ltd., and the synthesized plasmid fragments were linked to pmiRglo reporter vectors, respectively. The constructed double luciferase recombinant plasmid (100 ng) combined with 50 nM miR-29a mimic or miR-29a inhibitor was co-transfected into hippocampal neurons using Lipofectamine 2000. After transfection, hippocampal neurons were cultured in a cell incubator at 37°C and 5% CO_2 for 24 hours. The luciferase activity of cells in each group was detected based on the dual luciferase reporter gene detection kit.

Statistical analysis

SPSS 24.0 software and GraphPad Prism 7.04 software were used for analysing all the data. The data were presented as mean ± standard deviation (SD) ($n \geq 3$). Chi-square test was used to compare categorical variables between groups. Student's t test

▼ **Table 1.** Clinical characteristics of the patients with TLE and healthy controls.

Parameters	Healthy controls (n = 70)	TLE patients (n = 65)	p value
Age (years)	10.07 ± 2.62	9.98 ± 2.58	0.847
Gender			
Male	37	34	0.949
Female	33	31	

or one-way analysis of variance was used to compare the differences between the two groups. Correlation of different clinical variables was examined using Pearson's correlation analysis. $P < 0.05$ was deemed statistically significant.

Results

Demographic and clinical characteristics of the study population

Table 1 summarizes the data on clinical characteristics of the two study groups. The age of the individuals in the healthy control group ranged from 4 to 17 years old, and ranged from 4 to 14 years in the TLE group. No significant difference was detected in terms of age and gender between the TLE patient and control group (p>0.05) (*table 1*).

Clinical value of miR-29a level in serum of children with TLE

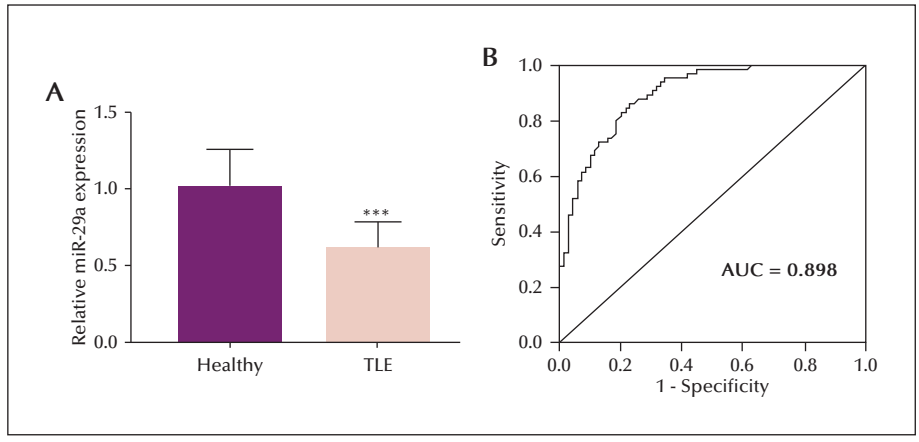
The qRT-PCR assay was used to evaluate the level of miR-29a in the serum of the study population. As shown in *figure 1A*, the level of miR-29a was significantly low in children with TLE compared with the control group (p<0.001), indicating the potential role of miR-29a in the pathogenesis of TLE.
According to the level of miR-29a in the two study groups, the ROC curve was established, with an area under the curve (AUC) of 0.898 (95% CI: 0.848-0.948) (*figure 1B*). The diagnostic sensitivity and specificity were 86.2% and 77.1% respectively, with a cut-off value of 0.810.

Correlation between serum miR-29a level and release of inflammatory cytokines

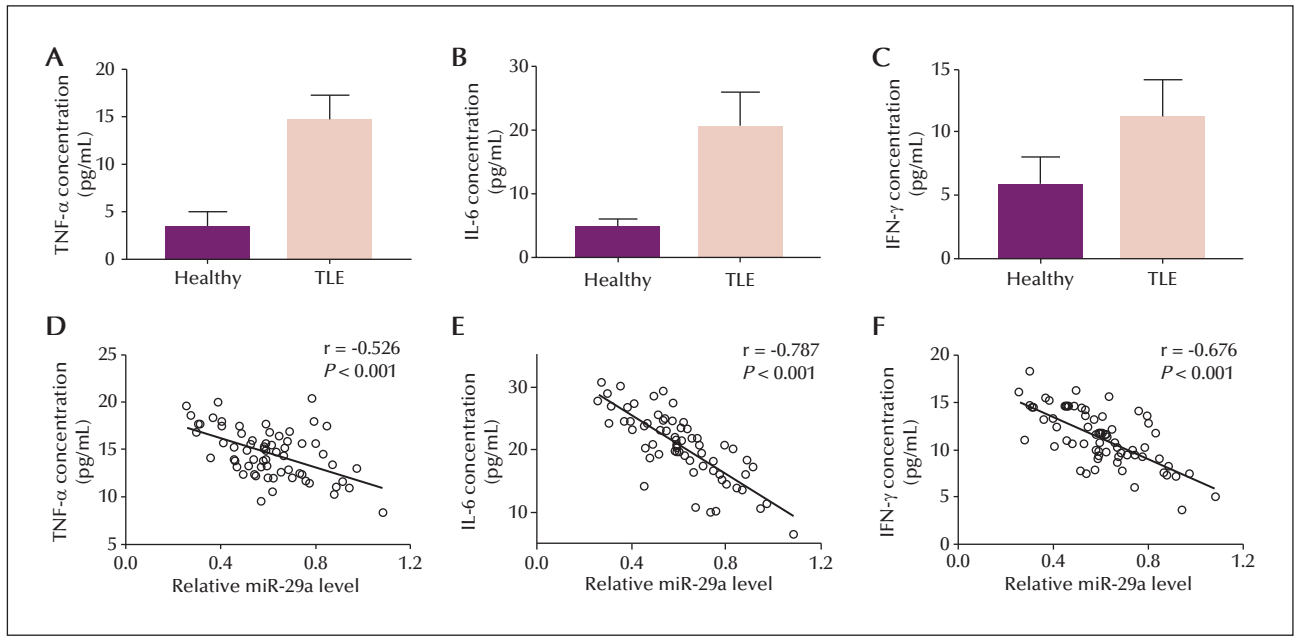
Based on the ELISA results, high concentrations of TNF-α, IL-6, and IFN-γ were detected in the serum of TLE patients in comparison to the control group (*figure 2A-C*). In addition, correlation analysis revealed that the level of serum miR-29a negatively correlated with the levels of TNF-α, IL-6 and IFN-γ, with correlation coefficients of -0.526, -0.787 and -0.676, respectively (*figure 2D-E*).

Level of miR-29a in the epileptiform discharge cell model

To mimic the TLE condition in children *in vitro*, primary hippocampal cells from newborn rats were cultured in magnesium-free medium to establish an epileptiform discharge model. The level of miR-29a was verified *in vitro*. As shown in *figure 3*, the level of miR-29a was low in the TLE cell model, and these data were consistent with the serum measurement results (p<0.001). Thus, miR-29a expression was clearly down-regulated under the TLE condition.



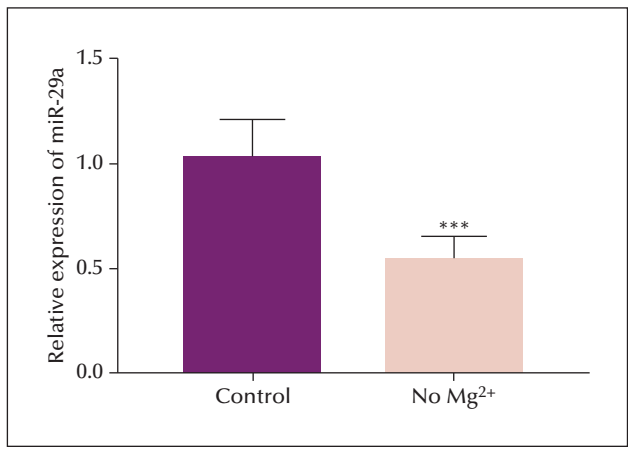
■ **Figure 1.** Level of serum miR-29a in children with TLE. (A) Low level of MiR-29a in the serum of TLE patients (***p <0.001). (B) The ROC curve for diagnostic evaluation is based on the serum levels of miR-29a in the TLE and healthy group.



■ **Figure 2.** Release of inflammatory cytokines in children with TLE. High concentrations of TNF-α (A), IL-6 (B), and IFN-γ (C) were detected in the serum of TLE patients in comparison to the control group. The level of serum miR-29a negatively correlated with the levels of TNF-α (D), IL-6 (E) and IFN-γ (F).

Effect of miR-29a on cell proliferation, apoptosis and inflammatory cytokine release

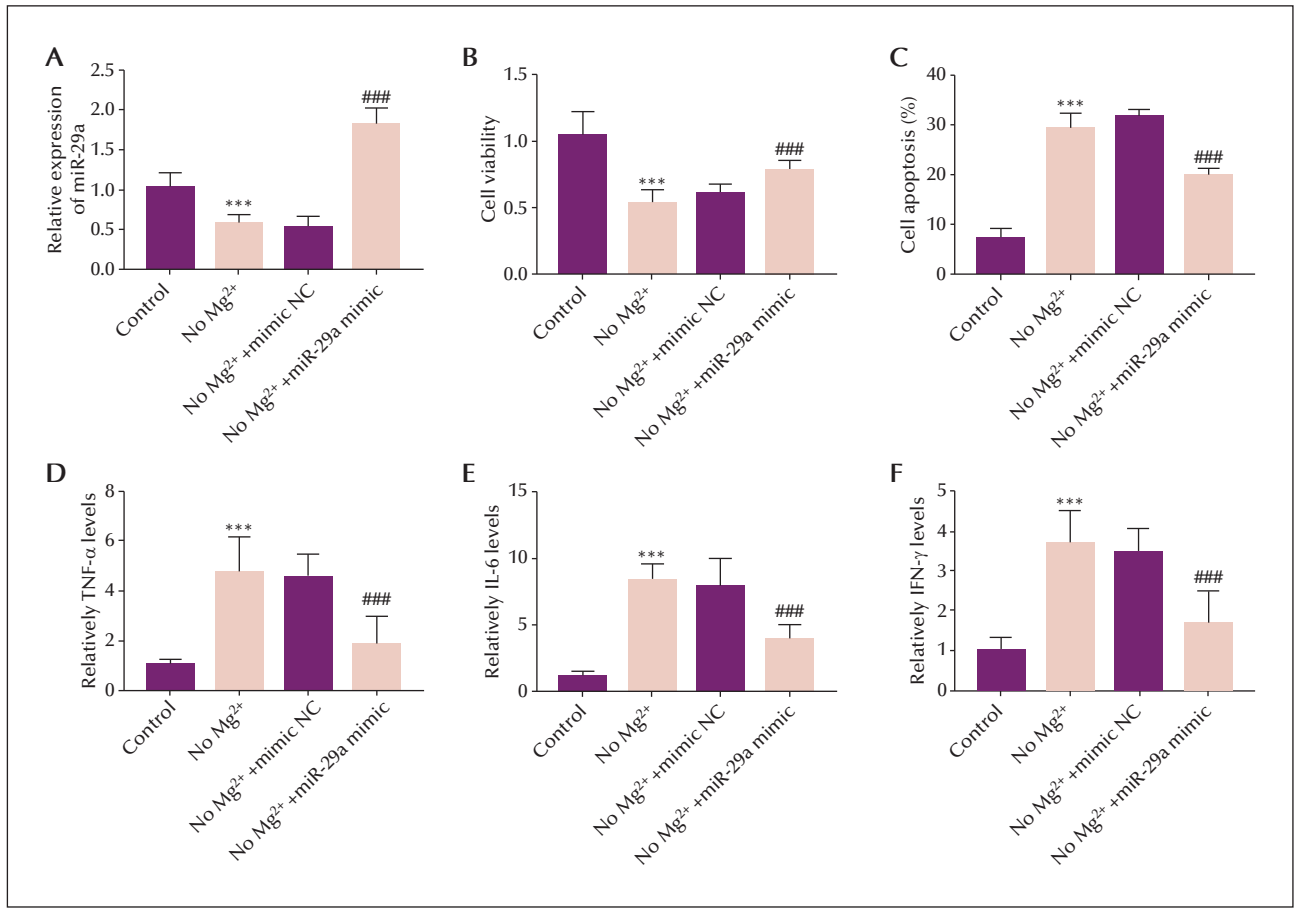
To further study the role of miR-29a in hippocampal cell proliferation and apoptosis, the level of miR-29a was regulated via cell transfection. The qRT-PCR results showed that transfection of a miR-29a mimic increased the level of miR-29a (p<0.001) (*figure 4A*). In addition, the viability of hippocampal cells in the magnesium-free group was significantly reduced, and cell apoptosis was significantly increased (p<0.001) (*figure 4B-C*). Based on the results of gain- and loss-of-function experiments, miR-29a over-expression was shown to significantly reverse decreased cell viability induced by TLE (p<0.001) (*figure 4B*). Moreover, up-regulating the expression of miR-29a also alleviated cell apoptosis (p<0.001) (*figure 4C*). In the magnesium-free group, the release of TNF-α, IL-6, and IFN-γ was also promoted, which was cancelled by miR-29a over-expression (*figure 4D-F*).



■ **Figure 3.** Low level of miR-29a in primary hippocampal cells cultured with magnesium-free medium (*** p<0.001).

The relationship between miR-29a and *HMGB1*

Binding between miR-29a and the 3'UTR region of *HMGB1* was predicted based on sequence analysis using TargetScan software (*figure 5A*). The results of dual luciferase reporter gene assays further confirmed the regulatory effect of miR-29a on the *HMGB1* gene. In *HMGB1*-Wt transfected cells, luciferase activity in the miR-29a mimic group was significantly lower than that in the control group, which was higher in cells transfected with miR-29a inhibitor (p<0.01) (*figure 5B*). There was no significant difference in luciferase activity for *HMGB1*-Mut transfected cells among the different groups. In the magnesium-free group, high



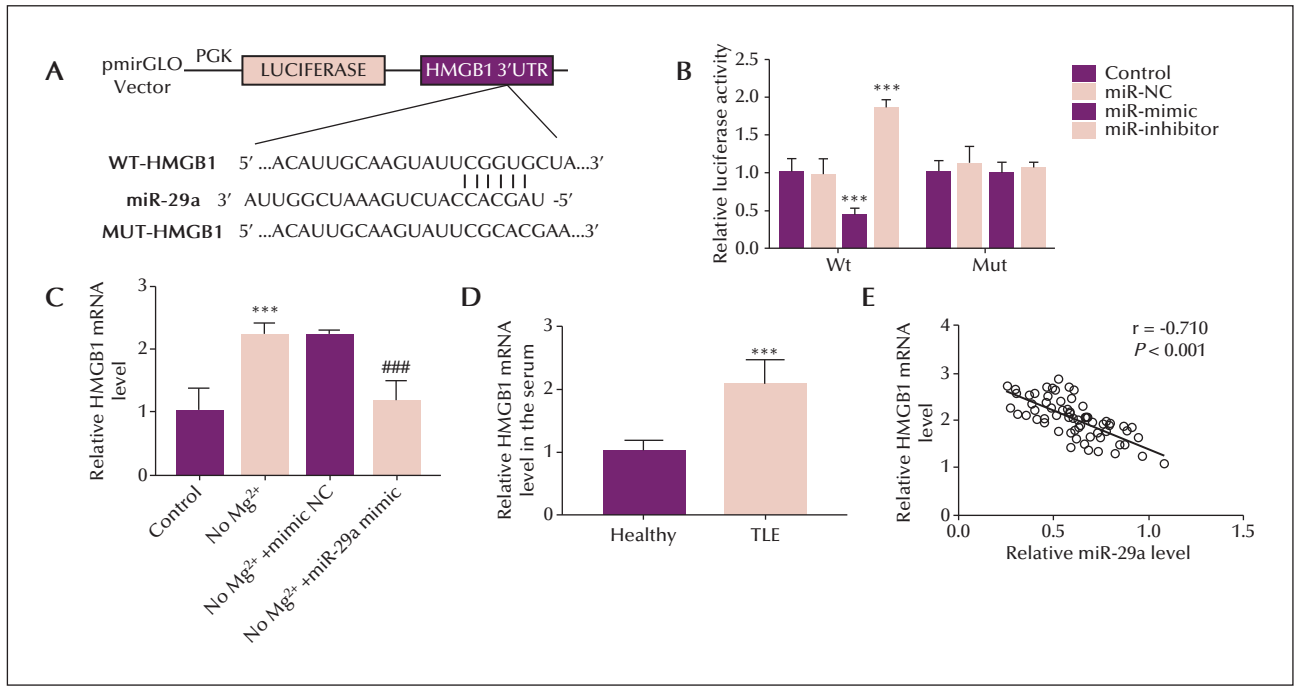
■ **Figure 4.** Effects of miR-29a on cell proliferation, apoptosis and inflammatory cytokine release. (A) Increase of miR-29a in response to transfection with miR-29a mimic. (B) MiR-29a over-expression significantly reversed decreased cell viability induced by TLE. (C) Up-regulation of miR-29a also alleviated cell apoptosis. (D-F) In the magnesium-free group, release of TNF-α, IL-6, and IFN-γ increased, but was cancelled by miR-29a mimic over-expression, compared with the control group (***p<0.001) and No Mg^{2+} group ($^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001).

levels of *HMGB1* were detected, and the level was decreased by transfection of miR-29a mimic (*figure 5C*). The mRNA levels of *HMGB1* were also detected in the serum of children with TLE, and were significantly high (*figure 5D*). A significant negative correlation was also detected between serum miR-29a and *HMGB1* levels in all TLE patients (r = -0.710, p<0.001) (*figure 5E*).

Discussion

TLE is a common form of epilepsy originating in childhood [1]. Because the clinical symptoms of TLE are complicated and not particularly specific, misdiagnosis of TLE in children may easily occur, leading to serious and long-lasting consequences [28]. Currently, EEG and neuroimaging are used to assist diagnosis of TLE. However, in order to obtain a definitive diagnosis, careful physical examination and detailed medical history taking should be performed. Therefore, the discovery of biomarkers is essential for the diagnosis and treatment of children with TLE.

In recent years, miRNAs have been shown to be involved in nervous system diseases, and several miRNAs have been reported to be abnormally expressed in epilepsy patients and may be involved in the progression of the disease [29-31]. According to the qRT-PCR results, miR-29a was detected at a low level in the serum of children with TLE. Similarly, more and more authors have indicated that miR-29a has a close relationship with neurological diseases.



■ **Figure 5.** Relationship between miR-29a and *HMGB1*. (A) Potential binding between miR-29a and the 3′UTR region of *HMGB1* based on sequence analysis. (B) Luciferase activity among the different groups (***p<0.001). (C) *HMGB1* mRNA level among the different groups, compared with the control group (***p<0.001) and No Mg^{2+} group ($^{###}p$<0.001). (D) *HMGB1* mRNA level in the serum of children with TLE (***p< 0.001). (E) Correlation between serum miR-29a and *HMGB1*.

Stary *et al*. found that over-expression of miR-29a resulted in the protection of CA1 neurons from delayed neuronal death [16]. Tan *et al*. have reported that YAP-regulated miR-29a promotes neurite outgrowth by decreasing PTEN in N2a cells [17]. Notably, a similar result was reported by Shi *et al*. showing that miR-29a promotes neuronal differentiation and decreases astrocyte differentiation of rat embryonic NSCs via targeting PTEN [18]. Moreover, Duan *et al*. reported that miR-29a modulates neuronal differentiation through targeting REST in mesenchymal stem cells [19]. Another study by Ma *et al*. showed that miR-29a is involved in the regulation of neurite outgrowths and the development of neural stem cells [20]. In the aforementioned studies, miR-29a was suggested to play an important role in neurogenesis and neurodevelopment. Thus, the up-regulation of miR-29a could promote neurogenesis and neurodevelopment. These data support our inference about the potential role of miR-29a in TLE. Due to the characteristics associated with serum testing, notably the stability, cost, rapidity, and non-invasiveness, more and more miRNAs have been considered as potential diagnostic tools for many diseases [32]. Thus, in the present study, the potential diagnostic value of serum miR-29a was also evaluated for TLE.

Neuroinflammation is clearly linked to the pathophysiology of seizures and epilepsy with a bidirectional interaction. Inflammation is the most common pathophysiological reaction and is associated with the occurrence and progression of epilepsy [7]. On the other hand, seizures themselves can induce inflammatory responses in the brain. Clinical evidence shows that there is obvious inflammatory cell infiltration in the hippocampus of TLE patients, and inflammation is considered to be a key factor in the pathogenesis of TLE [7, 33]. In the present study, high concentrations of TNF-α, IL-6, and IFN-γ were detected in the serum of TLE patients. In addition, correlation analysis revealed that the level of serum miR-29a negatively correlated with the release of inflammatory cytokines. Thus, miR-29a may be involved in the regulation of the inflammatory response.

In order to further explore the effect of miR-29a on the progression of TLE, we constructed a TLE model based on a hippocampal cell model of newborn rats. A low level of miR-29a was found in the TLE cell model, consistent with the results from the clinical serum

samples. Additionally, gain- and loss-of-function experiments indicate that the upregulation of miR-29a enhances cell viability in hippocampal cells cultured in magnesium-free medium, and reduces cell apoptosis. Moreover, miR-29a over-expression also inhibited the release of inflammatory cytokines induced by TLE. Similarly, based on a study in mice by Roshan *et al*., brain-specific knockdown of miR-29a resulted in dysregulation of neuronal cell death and ataxia in mice [34]. The occurrence of TLE is associated with abnormal behaviour of hippocampal cell proliferation and apoptosis [35]. Combined with the cell experiments, we conclude that miR-29a may be involved in the pathogenesis of TLE through regulation of apoptosis of hippocampal neurons and neuroinflammation.

High-mobility group box 1 (HMGB1), a member of the chemokine family, can induce enrichment of chemokines and complement components. HMGB1 can affect the stability of nerve electrical impulse transmission of neurons [36]. In epileptic seizures, the release of HMGB1 is found to accompany the phenomenon of reactive gliosis and neurodegeneration [37]. Over-expression of HMGB1 can activate macrophages and monocytes and promote damage related to inflammatory cells in brain tissue, contributing to the progression of epilepsy [38]. Whereas disruption of HMGB1 inhibits neuroinflammation and neurodegeneration after status epilepticus [37]. In the current study, *HMGB1* was shown to be the target gene of miR-29a. High levels of *HMGB1* were also detected in the serum of TLE patients, demonstrating a negative correlation with serum miR-29a level. Furthermore, in coriaria lactone-induced epilepsy, HMGB1 is suggested to mediate microglia activation via the TLR4/NF-κB pathway [39]. In addition, miR-129-5p is reported to be involved in the development of autoimmune encephalomyelitis (AE)-related epilepsy by targeting HMGB1 through the TLR4/NF-kB signalling pathway in a rat model [40]. Therefore, it is speculated that miR-29a may play a role by targeting and down-regulating the expression of *HMGB1*, thus participating in the regulation of neuronal apoptosis and neuroinflammation during the progression of TLE, and TLR4/NF-kB signalling may play an important role in the underlying mechanism.

In summary, we suggest that a low level of miR-29a may be a biomarker for the diagnosis of TLE in children. MiR-29a participates in the regulation of neuronal apoptosis and neuroinflammation during the progression of TLE via targeting *HMGB1*. A limitation of the present study is that it is *in vitro*-based and *in vivo* experiments were not performed. Future studies on the regulatory role of miR-29a in the mouse model of TLE may provide stronger evidence for the involvement of miR-29a in the pathogenesis of TLE. ■

Supplementary material.
Summary slides accompanying the manuscript are available at www.epilepticdisorders.com.

Disclosures.
The authors declare no conflicts of interest.

Funding.
This study was supported by the University-level Project (BY2017KJ21).

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TEST YOURSELF

(1) Which method was used to evaluate the diagnostic value of serum miR-29a for temporal lobe epilepsy (TLE)?
A. ROC curve analysis
B. qRT-PCR
C. Luciferase reporter assay

(2) What are the common vectors for luciferase reporting assays?
A. pmirGLO
B. T7
C. PGK

(3) What type of data can be analysed by Pearson's correlation analysis?
A. Normally distributed variable
B. Categorical variable
C. Continuous variable

Note: Reading the manuscript provides an answer to all questions. Correct answers may be accessed on the website, www.epilepticdisorders.com.