

## Research Article

# Expression of miR-155 in Serum Exosomes in Children with Epilepsy and Its Diagnostic Value

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Received 30 December 2021; Accepted 22 February 2022; Published 26 July 2022

Academic Editor: Hongsheng Zhang

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**Objective.** This study was designed to analyze the expression of miR-155 in serum exosomes in children with epilepsy and to explore its diagnostic value. **Methods.** From March 2020 to March 2021, 43 hospitalized children with epilepsy admitted to the Department of Neurology of the hospital were included, and another 43 gender- and age-matched healthy children were randomly selected as the healthy control group during the same period. Then fasting serum samples of the two groups were collected to extract the exosomes. The morphology of the exosomes was evaluated under a transmission electron microscope, and the expression of specific protein markers on the surface was detected by Western Blot. In addition, the relative expression of miR-155 in serum exosomes in children with epilepsy with different courses of the disease and different degrees of abnormal electroencephalography (EEG) was compared, and the area under the receiver operating characteristic (ROC) curve (ROC-AUC) was used to evaluate the diagnostic value of miR-155. **Results.** A higher relative expression level of miR-155 in serum exosomes was obtained in the epilepsy group, as compared to the healthy control group ( $P < 0.05$ ), and the relative expression of miR-155 in serum exosomes in children with epilepsy was correlated with the course of the disease and the degree of abnormal EEG (both  $P < 0.05$ ). In addition, the expression of miR-155 in serum exosomes showed high diagnostic efficiency for epilepsy (AUC = 0.813,  $P < 0.05$ ). **Conclusion.** The expression of miR-155 in serum exosomes in children with epilepsy is up-regulated, and its level is related to the course of the disease and the degree of abnormal EEG, so miR-155 in serum exosomes may be used as a biomarker for the diagnosis and assessment of the severity of epilepsy.

## 1. Introduction

Micro ribonucleic acid (miRNA) is a non-coding RNA with a length of about 22 nt, which can bind to complementary sites on messenger RNA to reduce its stability, down-regulate translation, and regulate gene expression. In recent years, studies have found a variety of miRNAs differentially expressed in the serum of patients with epilepsy, which provides new research directions for the diagnosis and treatment of epilepsy [1]. Related animal experiments also revealed different expression levels of miRNA in the brain tissue and serum of rats in different stages of epilepsy [2]. Epilepsy is one of the main causes of children's disability that triggers dysfunction in children and compromises their quality of life [3]. Early diagnosis of epilepsy mainly relies on

medical history, electroencephalography (EEG), magnetic resonance imaging, biochemical examination, etc., which are of great significance for prognosis improvement and prevention of status epilepticus. Nonetheless, the effect of conventional EEG in capturing epileptic discharge waves is rather poor [4], and studies have shown that the brain magnetic resonance imaging results of most patients with epilepsy are negative [5]. Therefore, for non-invasive biochemical examination, it is of essential clinical significance to seek new high-sensitivity biomarkers for the early diagnosis of epilepsy. Exosomes are small vesicles containing complex RNA and proteins. In recent years, it has been reported that exosome miRNA is involved in the occurrence and development of a variety of central nervous system diseases including epilepsy [6]. Accordingly, it is speculated that

TABLE 1: Comparison of general data.

Group	No.	Age ( $\bar{x}\pm s$ , years old)	BMI ( $\bar{x}\pm s$ , kg/m <sup>2</sup> )	Gender (male, n/%)
Epilepsy group	43	8.56 $\pm$ 2.14	18.11 $\pm$ 2.13	26/60.47
Healthy control group	43	8.45 $\pm$ 2.18	18.15 $\pm$ 2.15	28/65.12
$t/\chi^2$ value		0.236	0.087	10.234
$P$ value		0.814	0.931	0.786

analysis of exosomes can help identify subtle changes in the physiological and pathological processes of patients with epilepsy. MiR-155 is a versatile RNA. One study has pointed out that the expression of miR-155 exerts a certain effect on seizures and progression [7]. Therefore, this study analyzed the expression of miR-155 in serum exosomes in children with epilepsy and explored its diagnostic value, aiming to provide more references for the diagnosis and treatment of epilepsy.

## 2. Materials and Methods

**2.1. Ethical Statement.** The study was reviewed and approved by the Linyi People's Hospital ethics committee, was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Children and their legal guardians signed informed consent forms.

**2.2. Clinical Data.** A total of 43 children with epilepsy admitted to the neurology department of the hospital from March 2020 to March 2021 were included into the epilepsy group. Inclusion criteria [8, 9]: (1) Patients at 0-14 years old; (2) Patients diagnosed as pediatric epilepsy [10]; (3) Patients who had not taken immunosuppressants, hormonal drugs, or other drugs that affect the results of this study in the past 3 months; (4) Children and their legal guardians signed informed consent forms. Exclusion criteria [8, 9]: (1) Patients with secondary epilepsy caused by head injury, etc.; (2) Patients with a history of brain injury or craniocerebral surgery; (3) Patients complicated with infectious diseases, endocrine system diseases, or malignant tumors. Among the 43 cases of children with epilepsy, there were 26 male and 17 female cases, aged 1-13 years old, with an average age of (8.56  $\pm$  2.14) years. In terms of disease course, there were 11 cases with the course shorter than 1 year, 15 cases with the course between 1 to 3 years, 10 cases with the course between 3 to 5 years, and 7 cases with the course longer than 5 years. In terms of the abnormal degree of EEG, there were 13 mild cases, 22 moderate cases, and 8 severe cases. Another 43 gender- and age-matched healthy children were randomly selected as the healthy control group during the same period. Among them, there were 28 males and 15 females, aged 1-14 years old, with an average age of (8.45  $\pm$  2.18) years.

### 2.3. Methods

**2.3.1. Main Reagents and Instruments.** Serum/plasma exosome extraction and RNA isolation kit (Cat. No.: YB-024-A, Shanghai Yubo Biotechnology Co., Ltd.), membrane seal-

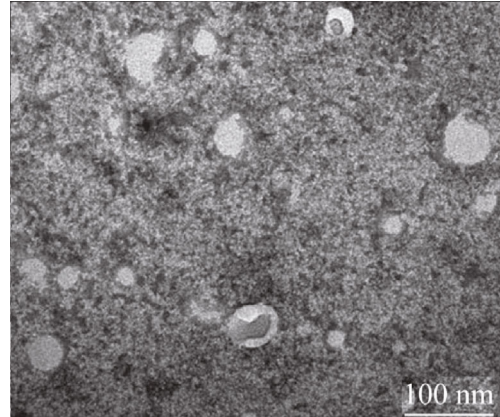


FIGURE 1: Morphology of serum exosomes in children with epilepsy under transmission electron microscope.

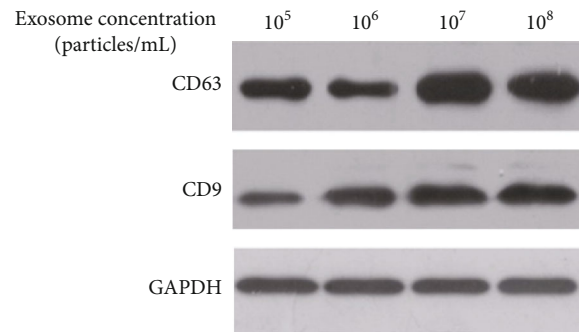


FIGURE 2: The expression of specific protein markers on the surface of serum exosomes in children with epilepsy.

ing fluid (article number: YT8900, Beijing Yita Biotechnology Co., Ltd.), rabbit monoclonal to anti-human CD63 antibody and rabbit monoclonal to anti-human CD9 antibody (article numbers: ab134045 and ab92726, Abcam, USA), ECL chemiluminescence detection kit (rabbit IgG) (article number: ASW2020, Shanghai Jizhi Biochemical Technology Co., Ltd.), serum/plasma miRNA extraction and isolation kit (article number: DP503, Beijing Tiangen Biochemical Technology Co., Ltd.), reverse transcription kit (article number: BTN60906, Beijing Biolab Technology Co., Ltd.), transmission electron microscope (model number: JEM-2100, JEOL Ltd.), and microspectrophotometer (model number: Nanodrop2000, Thermo Fisher Scientific). Real-time fluorescent quantitative PCR instrument (Thermo Fisher, USA; model: Applied Biosystems).

**2.3.2. Serum Specimen Collection.** Before medication, 5 mL fasting venous blood was collected from each subject, placed

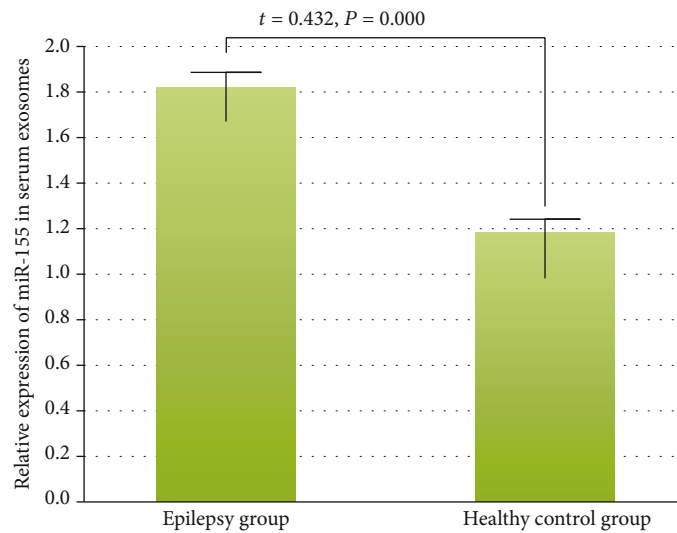


FIGURE 3: Comparison of the relative expression of miR-155 in serum exosomes.

at room temperature, and centrifuged at 3000 r/min for 10 min. The supernatant was then transferred into an EP tube and stored in a refrigerator at  $-80^{\circ}\text{C}$  for later use.

**2.3.3. Serum Exosome Extraction and Morphological Observation.** The serum sample was thawed in a water bath at  $25^{\circ}\text{C}$ , and centrifuged at 3000 r/min for 15 minutes at  $4^{\circ}\text{C}$  to remove cells or cell debris. Then the supernatant was collected, filtered with a filter membrane ( $0.22\ \mu\text{m}$ ), and placed in a new eppendorf (EP) tube, followed by addition of exosome extraction reagent, well mixing, and store at  $4^{\circ}\text{C}$  for 30 min. It was then centrifuged at 1500 r/min for 30 minutes at  $4^{\circ}\text{C}$  to aspirate the supernatant. Subsequently, it was centrifuged again at 1500 r/min for 5 min at  $4^{\circ}\text{C}$  to remove the residual liquid. Then, the diluent was added and mixed evenly with a pipette to completely dissolve the precipitate. The resuspended exosomes were transferred to the spin column and placed in the collection tube, followed by centrifugation at 2000 r/min for 5 minutes at  $4^{\circ}\text{C}$ . After discarding of the spin column, the serum exosomes were obtained. Afterwards,  $15\ \mu\text{L}$  of the exosomal solution was added on a 100-mesh sample-loading copper mesh, and placed at room temperature for 1 minute. Phosphotungstic acid was added dropwise and placed at room temperature for 5 minutes. The filter paper was used to absorb excess liquid from the edge, and the exosomes were evaluated and photographed under a transmission electron microscope.

**2.3.4. Western Blot Analysis for Detection of the Expression of Specific Protein Markers on the Surface of Exosome.** The Bicinchoninic Acid (BCA) method was used to quantify the protein in the exosome solution: The measurement wavelength of the microplate reader was set as 570 nm to determine the optical density (OD) value, and then a standard curve was drawn to calculate the protein concentration of each sample. The protein sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene fluorid (PVDF) membrane by the wet method, and immersed in the mem-

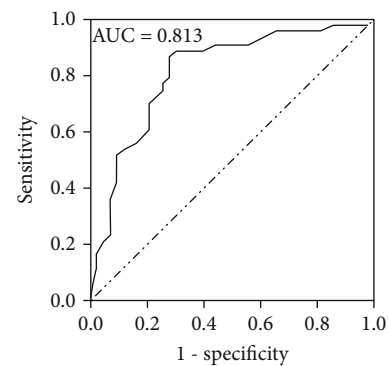


FIGURE 4: ROC curve analysis of miR-155 in serum exosomes in the diagnosis of epilepsy.

brane blocking solution. After incubation for 1 hour at room temperature, the primary antibody diluent was added and incubated overnight at  $4^{\circ}\text{C}$  (using Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control). Then the liquid was discarded and the membrane was rinsed 3 times with Tris-buffered-saline with Tween (TBST) solution, 10 minutes each time. After that, the secondary antibody diluent was added and incubated at room temperature for 2 hours in the dark, and then the membrane was rinsed 3 times with TBST solution, 10 minutes each time. Finally, the electrochemiluminescence (ECL) method was used for color development, the gel imaging system for observation and photographing of the target protein bands, and the Image J software for quantitative analysis.

**2.3.5. Real Time Fluorescent Quantitative Polymerase Chain Reaction (qRT-PCR) for Measurement of the Expression Level of miR-155 in Serum Exosomes.** miRNA was extracted from serum exosomes in strict accordance with the miRNA extraction kit instructions, and its concentration was determined by a micro-spectrophotometer. The cDNA was obtained according to the instructions of the reverse transcription kit, diluted by 10 times, and then added into a

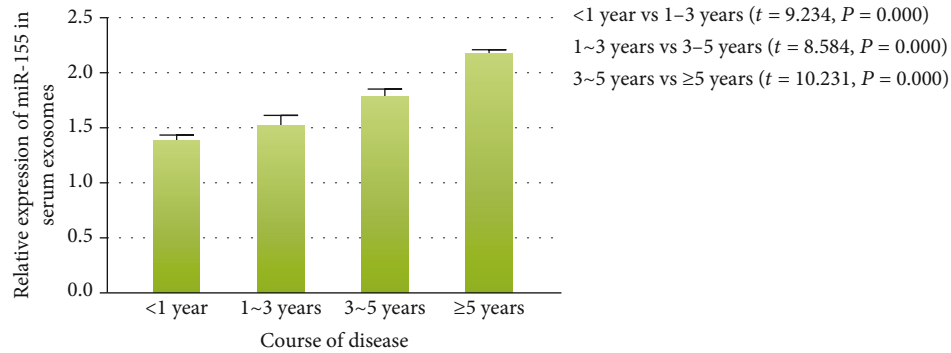


FIGURE 5: Comparison of miR-155 expression in serum exosomes in children with epilepsy in different courses of the disease.

dedicated PCR tube. The PCR reaction system consisted of  $10\ \mu\text{L}$   $2\times$  real-time PCR Master Mix,  $0.4\ \mu\text{L}$  Gene specific primer set,  $0.2\ \mu\text{L}$  Gene specific probe,  $0.2\ \mu\text{L}$  Taq DNA polymerase,  $2.0\ \mu\text{L}$  RT product, and  $7.2\ \mu\text{L}$  ddH<sub>2</sub>O, and the reaction conditions were as follows: pre-denaturation at  $95^\circ\text{C}$  for 3 min, denaturation for 12 s at  $95^\circ\text{C}$ , and annealing and extending for 40 s at  $62^\circ\text{C}$ , with U6 as the internal reference gene. Each sample was determined repeatedly three times. The fluorescence signal was then collected to measure the cycle threshold (Ct). The relative expression of the target gene was expressed by the  $2^{-\Delta\Delta\text{Ct}}$  method [11].

**2.4. Statistical Analysis.** SPSS20.0 software was used for data analysis. The measurement data in normal distribution were represented by  $(\bar{x}\pm s)$ , and compared between groups using the independent sample t-test. The count data were expressed by frequency or composition ratio; as the total number of cases in this study was  $\geq 40$  and the minimum theoretical frequency was  $> 5$ , the chi-square non-correction method was used for statistical analysis. The correlation was analyzed by Spearman rank correlation analysis. In addition, the area under the receiver operating characteristic curve (ROC) (ROC-AUC) was used to evaluate the diagnostic value.  $P < 0.05$  was considered statistically significant.

### 3. Results

**3.1. Comparison of General Data.** There were no significant differences in gender, age, and body mass index (BMI) between the two groups ( $P < 0.05$ ). See Table 1.

**3.2. Identification of Serum Exosomes.** It was found that the size of the serum exosomes obtained by centrifugation was mainly within a range of 50-100 nm under a transmission electron microscope, in line with the normal size range of exosomes of 30-200 nm, and they were round, quasi-circular or cup-shaped, uniform in size and with a complete lipid envelope, as shown in Figure 1. Western Blot analysis showed that the surface of the extracted 43 cases of serum exosomes all expressed CD63 and CD9, and as the concentration of exosomes increased, the relative expression of the above two protein markers was also up-regulated, as shown in Figure 2.

TABLE 2: Correlation between miR-155 in serum exosomes and course of disease and abnormality of EEG.

	r value	p value
Courses of disease	0.876	0.002
Abnormality of EEG	0.911	0.001

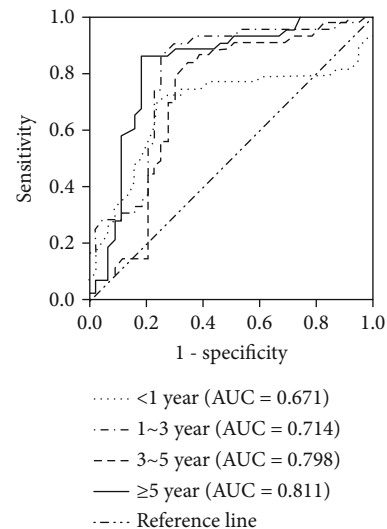


FIGURE 6: ROC curve analysis of miR-155 in serum exosomes in the diagnosis of epilepsy in different courses of the disease.

**3.3. Comparison of the Relative Expression of miR-155 in Serum Exosomes.** The epilepsy group showed a higher expression level of miR-155 in serum exosomes than the healthy control group ( $P < 0.05$ ). See Figure 3.

**3.4. ROC Curve Analysis of miR-155 in Serum Exosomes in the Diagnosis of Epilepsy.** The specificity and sensitivity of miR-155 in serum exosomes in diagnosing epilepsy were 78.24% and 84.11%, respectively, and the AUC was 0.813. See Figure 4.

**3.5. Comparison of miR-155 Expression in Serum Exosomes in Children with Epilepsy in Different Courses of the Disease.** As the course of epilepsy prolonged, the expression level of miR-155 in serum exosomes in children with



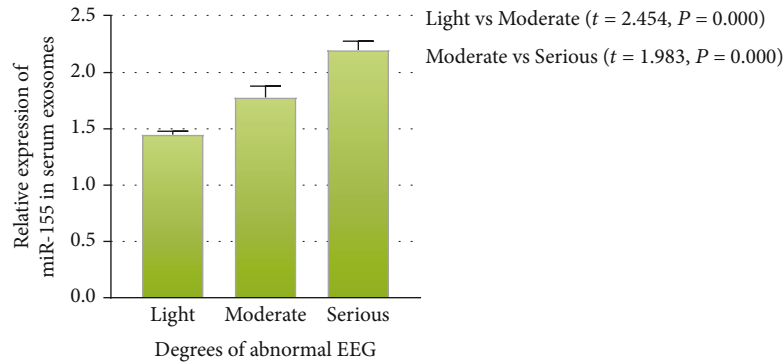


FIGURE 7: Comparison of miR-155 expression in serum exosomes in children with epilepsy with different degrees of abnormal EEG.

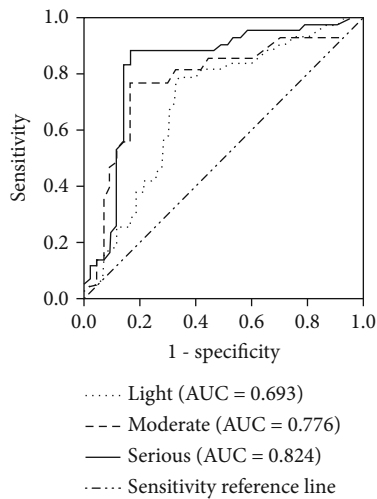


FIGURE 8: ROC curve analysis of miR-155 in serum exosomes in the diagnosis of epilepsy with different degrees of abnormal EEG.

epilepsy gradually increased ( $P < 0.05$ ), so it was positively correlated with the course of the disease ( $r = 0.876$ ,  $P = 0.002$ ). See Figure 5 and Table 2.

**3.6. ROC Curve Analysis of miR-155 in Serum Exosomes in the Diagnosis of Epilepsy in Different Courses of Disease.** The AUC of miR-155 in serum exosomes diagnosed as epilepsy with a course of less than 1 year, 1 to 3 years, 3 to 5 years, and  $\geq 5$  years were 0.671, 0.714, 0.798, and 0.811, respectively. See Figure 6.

**3.7. Comparison of miR-155 Expression in Serum Exosomes in Children with Epilepsy with Different Degrees of Abnormal EEG.** Results showed that the more severe the abnormality of EEG in children with epilepsy, the higher the expression of miR-155 in their serum exosomes ( $P < 0.05$ ), so the expression level of miR-155 in serum exosomes was positively correlated with the abnormality of EEG ( $r = 0.911$ ,  $P = 0.001$ ). See Figure 7 and Table 2.

**3.8. ROC Curve Analysis of miR-155 in Serum Exosomes in the Diagnosis of Epilepsy with Different Degrees of Abnormal EEG.** The AUC of miR-155 in serum exosomes to diagnose mild, moderate, and severe degrees of abnormal EEG were 0.693, 0.776, and 0.824, respectively. See Figure 8.

## 4. Discussion

Research data has shown that the prevalence rate of epilepsy in children is about 15 times higher than that of adults [12]. The pathological changes of epileptic seizures are highly complicated, and most of the changes are considered to be related to the abnormal activity and discharge of brain cells, glial cell proliferation, and neuronal cell death [3]. Electroencephalogram is currently the most widely used and relatively sensitive auxiliary diagnostic method [13], but its application effects are undermined by the potential failure of short-range EEG in recording epileptiform waves [14] and the huge time and economic costs of continuous video EEG monitoring [15]. On the contrary, the blood biochemical test is characterized by high specificity and simple operation compared with EEG. MiRNAs are small RNA molecules that are widely found in cells and can regulate gene expression. One recent study has found that miRNAs can extensively regulate cortical development, inflammatory response, neuron and glial cell function, apoptosis, etc., and thus play an important role in the occurrence and development of epilepsy as a key regulator [16]. MiR-155 widely exists in eukaryotes, which can inhibit the expression or translation of target genes at the post-transcriptional level and participate in a variety of physiological and pathological processes. One study has concluded that miR-155 can regulate the expression of AP-1 in astrocytes and promote its transcription, thus playing a key role in pentylentetrazole-induced epilepsy [17]. Exosomes naturally exist in body fluids such as blood and saliva. It has been confirmed that exosomes containing miRNAs related to cell sources can pass through biological barriers to transfer functional nucleic acid molecules between cells, thereby exerting various biological functions. Nevertheless, the miRNA in the blood has poor stability and can be decomposed by ribonuclease, while the miRNA in exosomes is highly stable. It has been pointed out that exosome biomarkers have high sensitivity and specificity in the diagnosis of many diseases [18]. Thus, it is speculated that miRNA in serum exosome is expected to be a non-invasive molecular marker of epilepsy in children.

After identification, it was found that the size of exosomes in the serum of children with epilepsy extracted in this study was mainly within the range of 50-100 nm, which

was in line with the normal size range of exosomes [19], and the specific protein markers were expressed on the surface, which indicates that this study has successfully isolated exosomes from serum samples. In this study, the expression of miR-155 in children with epilepsy was found to be up-regulated, indicating that the high expression of miR-155 in serum exosomes is related to the occurrence of epilepsy and that it may be considered as an early diagnostic marker and therapeutic target, whereas the molecular mechanism of its action still requires further analysis. According to comparison results of the expression of miR-155 in serum exosomes in children with epilepsy in different courses of the disease, with the prolongation of the disease course, the expression level of miR-155 gradually elevated, indicating that the changes of miR-155 expression are related to the progression of epilepsy in children, which is of great significance for the prognosis evaluation of children. Furthermore, the more severe the abnormality of EEG in children, the higher the expression level of miR-155 in their serum exosomes, which suggests that the detection of miR-155 in serum exosomes could be a supplement to the diagnosis of epilepsy and improve the early diagnostic effect.

In summary, the expression of miR-155 in serum exosomes in children with epilepsy significantly elevated, and its level was related to the course of the disease and the degree of abnormal EEG, so it could be used as a new marker for the diagnosis and assessment of the severity of epilepsy. Yet, this study presents a limitation that mechanism of miR-155 in epilepsy has not been explored, and further analysis is scheduled in the future.

## Data Availability

The datasets used during the present study are available from the corresponding author upon reasonable request.

## Conflicts of Interest

The authors declare that they have no conflict of interest.

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