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MiR-134 expression and changes in inflammatory cytokines of rats with epileptic seizures

W.-S. HUANG[1], L. ZHU[2]

[1]Department of Neurology, The Affiliated Hospital (Group) of Putian University, Putian, China
[2]Department of Neurology, Xuzhou Central Hospital, Xuzhou, China

Abstract. – OBJECTIVE: To investigate the expression of miR-134 and the change of inflammatory cytokines in seizure rats and to explore its relationship.

MATERIALS AND METHODS: A rat model of seizures was made by an intraperitoneal injection with kainic acid. ELISA kit for detection of seizures in rats was used. The changes of inflammatory cytokines (IL-1, IL-2, IL-6, TNF-α, IFN-γ) and the hippocampal neuronal cell growth were observed. The expression of miR-134 in brain tissue and serum samples of model group and control group was determined by reverse transcriptase-polymerase chain reaction (RT-PCR) quantitative determination.

RESULTS: After the intraperitoneal injection of kainic acid, the rat model of seizure was successfully established. Compared with the control group, the neuronal cells in the hippocampus of the model group showed evident pathological changes. Inflammatory cytokines in seizures rats showed that the increase of IL-2, IL-6, and TNF-α were larger, but the increase of IL-1 and IFN-γ was not obvious. The results of the RT-PCR quantitative analysis showed that the expression of miR-134 in brain tissue and serum of epilepsy rats was lower than that of the control group ($p<0.05$).

CONCLUSIONS: The expression of miR-134 would be down by the increasing of inflammatory cytokines in the epileptic seizure.

Key Words
Seizure fit rats, miR-134, Inflammatory cytokines, Hippocampal neurons.

Introduction

An epileptic seizure is a kind of brain dysfunction disease triggered by a series of etiologies, which is caused by over discharge due to a neuronal abnormality in a certain area of the brain. The main clinical symptoms are a temporary recurrent disturbance of consciousness and generalized or localized muscle spasms and convulsions. A large number of studies[1-3] have shown that cellular immunity and humoral immune function disorders occur during the pathogenesis of epilepsy. The body's immune mechanisms are involved in the pathogenesis of epilepsy.

Micro-ribonucleic acid (miRNA) is a non-coding conservative RNA with about 20-24 bp in length, which plays an important regulatory role in gene expression. As for gene regulation, one gene could be regulated by multiple miRNAs and multiple genes could be regulated by one miRNA[4,5]. MiRNA-134 (miR-134) is a highly conservative miRNA that widely exists in animal and human tissues, which is closely related to nervous system diseases and tumor diseases in the animal body.

Clinically, the autoimmune disorder is an important cause of epilepsy. After being injured, endothelial cells in brain tissues can release a variety of cytokines, activating T cells, and triggering immune responses in the brain. Then, the expression level of inflammatory regulators is up-regulated. This indicates that inflammatory cytokines are closely related to epilepsy.

This study aimed to explore the expression of miR-134 and changes in the serum level of inflammatory cytokines and to investigate the relationship between the changes in inflammatory cytokines and the expression of miR-134 in the rat model of epileptic seizures.

Materials and Methods

Experimental Animals

In this study, Sprague-Dawley (SD) rats (male, 20-25 weeks old, n=30) purchased from Hubei Provincial Laboratory Animal Research Center were taken as the experimental animals, and fed in an isolator with the temperature controlled at 25-28°C for preparation of the epileptic seizure rat model. This study was approved by the Animal Ethics Committee of Putian University Animal Center.

Corresponding Author: Liang Zhu, MM; e-mail: 254842054@qq.com

Main Reagents

Kainic acid (10 mg/bottle) was purchased from the Cayman Chemical Company (Ann Arbor, MI, USA). Enzyme-linked immunosorbent assay (ELISA) kit for inflammatory cytokines was purchased from the Sangon Biotech Co., Ltd. (Wuhan, China); primer synthesis kits, reverse transcription kits, and (SYBR Green) fluorescence quantitative kits were purchased from the TSINGKE Biological Technology Co., Ltd. (Kibbutz Beit Haemek, Israel); DY-1-type brain stereotaxic apparatus.

Preparation for the Model

Model group: 15 rats were randomly selected; kainic acid (10 mg/bottle) was diluted with 10 mL sterile saline; each rat was intraperitoneally injected with kainic acid (100 μL/100 g).

Control group: 15 rats were randomly selected; each rat was intraperitoneally injected with saline (100 μL/100 g); behavioral changes of the two groups were observed.

Criteria for successful modeling: epileptic seizure symptoms (sluggishness, salivation, tremors, convulsions, etc.) were observed in model rats; rats in the control group behaved normally.

Detection of Inflammatory Cytokines

At 5 h after intraperitoneal injection of kainic acid or saline, 5 rats were randomly selected; 2 mL tail vein blood was taken and centrifuged at room temperature for 1 h, and the supernatant was removed. 200 μL serum was taken, and RNA was extracted from it using the TRIzol method. Meanwhile, the ELISA kit was used to detect the serum concentration of inflammatory cytokines [interleukin-1 (IL-1), IL-2, IL-6, tumor necrosis factor-alpha (TNF-α) and interferon-γ (IFN-γ)].

Pathological Examination

5% pentobarbital injection was prepared by sterile saline. 5 rats in the epileptic seizure model group and 5 rats in the control group were injected with the anesthetic (3 mg/100 g). The thoracic and abdominal cavities of rats were dissected, and their left ventricles were perfused with saline at room temperature until the rats died. The cranial cavity of rats was cut open to take brain tissues for preparing slices, while hematoxylin and eosin (HE) staining was performed. At the same time, some brain tissues were taken for RNA extraction.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Relatively Quantitative Analysis

According to the instructions of the RT-PCR Kit, RNAs extracted in 1.2.3 and 1.2.4 was reversely transcribed according to the reaction system in Table I. Relatively quantitative detection of miRNA: U6 RNA was taken as the internal control. The reverse transcription primers of miR-134 and U6: miR-134 (5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATAGGACCCCCTC-3' and U6 (5'-TGTTGGCGTGGAGTFG-3'). PCR primers were designed based on the conservative regions of miR-134 and U6, and the relatively quantitative detection of miR-134 was performed according to the instructions of (SYBR Green) fluorescence quantitative kits. Relatively quantitative detection system of miR-134: 25 μL GoTaq qPCR Master Mix (2×); 1 μL miR-134 forward primer; 1 μL miR-134 stem-loop RT Primer; 2 μL complementary deoxyribonucleic acid (cDNA); 21 μL RNase-Free H_2O; 50 μL in total.

Detection by Northern Blot

Brain tissues of the rats were homogenized and added with lysate for lysis, followed by the ultra-centrifugation at 30,000 rpm (4°C, 1.5 h). The precipitate was resuspended using 0.5 mL phosphate-buffered saline (PBS) to determine the total protein concentration. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted for samples, which were then transferred to polyvinylidene fluoride (PVDF) membrane. The primary antibodies were incubated overnight at 4°C, and then, incubated with horseradish peroxidase-labeled secondary antibodies for 1 h after washing. After the mixed solution of 3,3'-diaminobenzidine (DAB) and hydrogen peroxide were added, fluorography technique was used to get the images.

Table I. RT-PCR primer sequences of miR-134 and U6 RNA.

Primer name	Primer sequence
miR-134 forward chain	5'-GGTGTGACTGGTTGACCA-3'
miR-134 reverse chain	5'-TGCGTGTCGTGGAGTC-3'
U6 forward chain	5'-CTCGCTTCGGCAGCACA-3'
U6 reverse chain	5'-AACGCTTCACGAATTTGCGT-3'

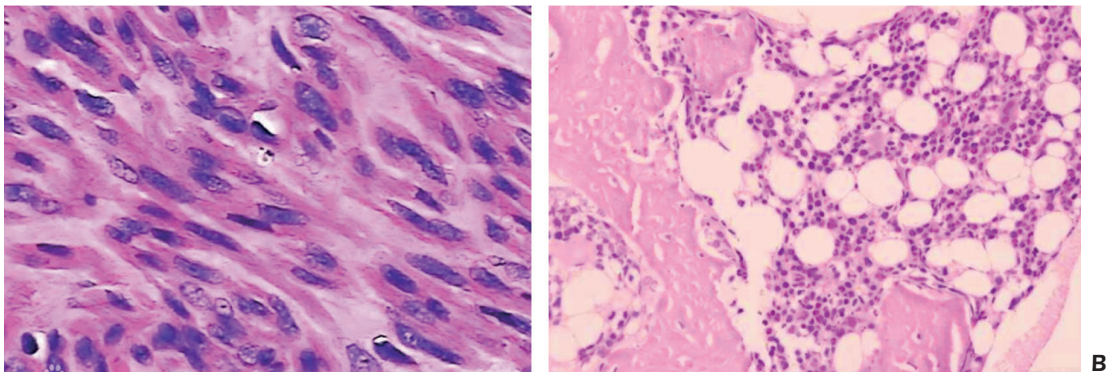

Figure 1. Pathological results of brain tissues of rats with epileptic seizures. **A**, Neuronal cells in various areas of hippocampus in normal saline. **B**, Neuronal cells in various areas of hippocampus in rats with epileptic seizures. HE staining on the CA3 area of rat hippocampus (Magnification: ×100).

Statistical Analysis

SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used for data analysis. All quantitative data were expressed as mean ± standard deviation (SD). The comparison between groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ represented that the difference was statistically significant.

Results

Pathological Results of Brain Tissues of Rats With Epileptic Seizures

Pathological section results showed that there was a significant difference in the neuronal area of hippocampus between the epileptic seizure model group and the control group. As shown in Figure 1, hippocampal neurons were arranged in disorder in the epileptic seizure model group. Additionally, increased intercellular space, decreased shrinkage volume, necrotic lysis, unclear nucleoli and disappearance of cytoplasm were also observed in the epileptic seizure model group.

Inflammatory Cytokine Expressions in Serum of Rats With Epileptic Seizures

Detection results of the serum level of inflammatory cytokines by the ELISA kit showed that the levels of IL-1, IL-2, IL-6, TNF-α, and IFN-γ in the epileptic seizure group were significantly higher than those in the normal control group, among which IL-2, IL-6, and TNF-α in the former were about 3 times those in the latter, while IL-1 and IFN-γ were slightly increased (Table II).

Expression of miR-134 in Brain Tissues of Rats With Epileptic Seizures

Results of the relatively quantitative detection of miR-134 in brain tissues showed that, compared with that in brain tissues in the control group (mean ± SD: 16.9±1.67), the expression of miR-134 in brain tissues in the epileptic seizure model group (mean ± SD: 9.5±0.89) was significantly decreased ($p<0.001$) (Figure 2). This revealed that the relative expression level of miR-134 in brain tissues in the epileptic seizure model group was significantly reduced, indicating that miR-134 is lowly expressed in brain tissues of rats with epileptic seizures.

Table II. Comparisons of general data between the epileptic seizure group and the normal control group.

Group	IL-1 (pg/mL)	IL-2 (pg/mL)	IL-6 (pg/mL)	TNF-α (pg/mL)	IFN-γ (pg/mL)
Epileptic seizure group	25.7±7.4*	58.7±11.4*	197.8±12.2**	281.2±16.2**	27.6±10.2*
Normal control group	20.4±6.8	23.6±12.5	73.8±17.2	85.6±12.3	23.1±7.2

Compared with those in the normal control group, *$p<0.05$, **$p<0.01$ (n=5)

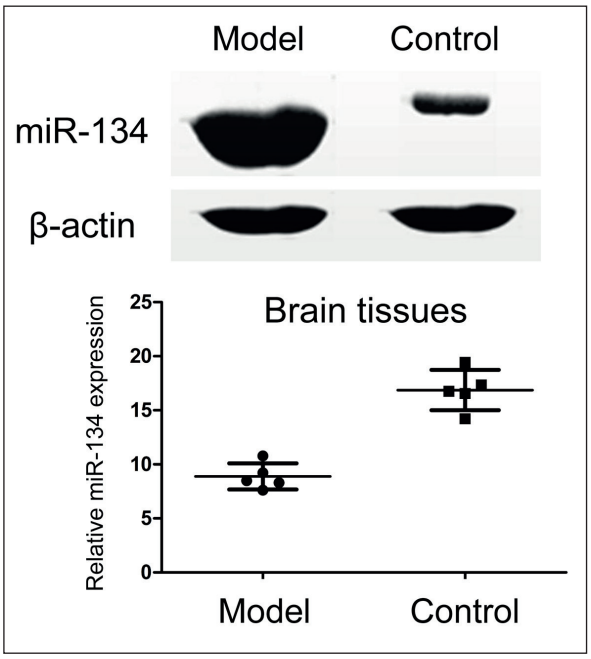

Figure 2. Expression of miR-134 in brain tissues of rats with epileptic seizures (*p<0.05; n=5).

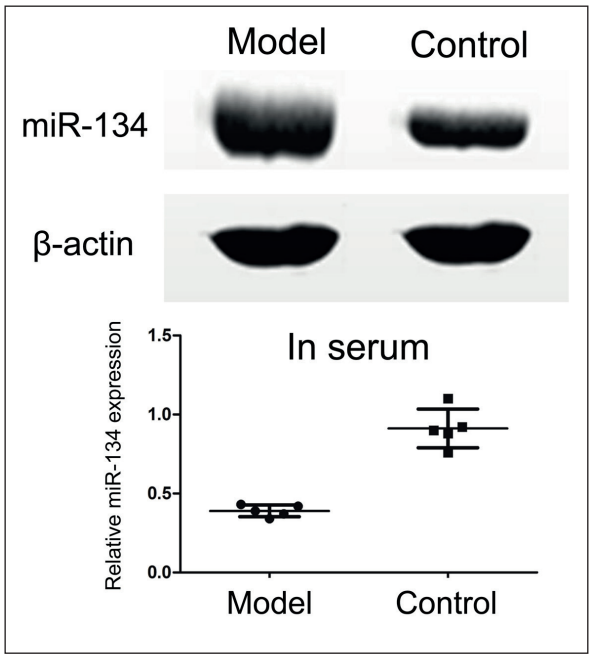

Figure 3. Expression of miR-134 in serum of rats with epileptic seizures (*p<0.05, **p<0.01; n=5).

Expression of miR-134 in Serum of Rats With Epileptic Seizures

Results of the relatively quantitative detection of miR-134 in rat serum showed that, compared with that in rat serum in the normal control group (mean ± SD: 0.39±0.09), the expression of miR-134 in rat serum in the epileptic seizure model group (mean ± SD: 0.91±0.11) was significantly decreased (p<0.001) (Figure 3). This indicated that miR-134 is also lowly expressed in the serum of rats with epileptic seizures compared with that in the serum of normal rats.

Dynamic Changes of miR-134 in Brain Tissues and Inflammatory Cytokines in Serum

In the epileptic seizure model group, sampling was conducted once at the interval of every 1 h during 1-6 h of injection with kainic acid, so as to determine the dynamic expression levels of miR-134 and inflammatory cytokines, respectively. As shown in Figure 4, during epileptic seizures of rats, the expression levels of inflammatory cytokines were constantly increased, while that of miR-134 was gradually decreased. Among the inflammatory cytokines, the increasing amplitudes of IL-2, IL-6, and TNF-α were relatively large, but those of IL-1 and IFN-γ were relatively small.

Discussion

An epileptic seizure is a common chronic disease in children's nervous system, seriously affecting children's lives and health. As a new type of regulatory molecule, miRNA affects the body's growth, development, metabolism and other important processes[6]. In particular, miR-134, a kind of specific miRNA in the brain, is closely related to the nervous system diseases of the animal body[7]. In addition, the occurrence process of epileptic seizures and body's immune system are inseparable. A large number of studies[8-10] have reported that inflammatory cytokines changed in epileptic brain tissue samples and serum, whether in the epileptic patients or epileptic animal models, indicating that the occurrence and development of epilepsy, are also closely correlated with inflammatory cytokines. Therefore, the study on the relationship of epileptic seizures with the expressions of inflammatory cytokines and miR-134 may provide new ideas for the treatment of epilepsy.

MiR-134 is expressed in neuronal cells and dendritic cells of the brain with various target proteins such as LIM domain kinase 1 (LIMK1), RNA binding proteins Pnm2, cyclic AMP response element-binding protein (CREB), double cortin (DCX), etc.[11]. MiR-134 regulates the brain development, synaptic plasticity, and dendritic morphology

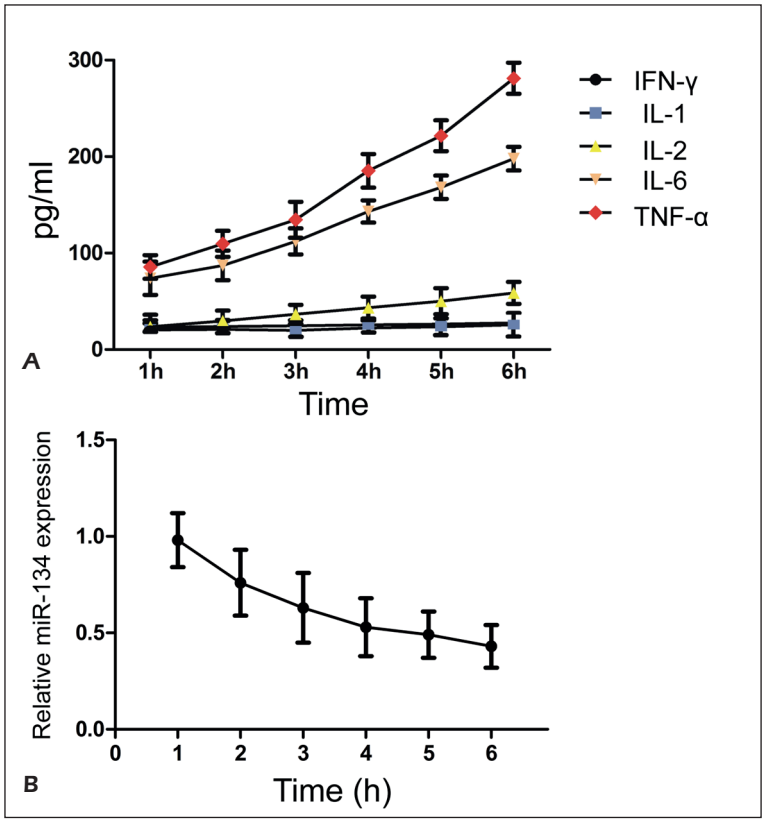

Figure 4. Diagram of changes in the expression levels of various inflammatory cytokines (**A**) and miR-134 (**B**) in rats with epileptic seizures at different time points (*p<0.05, **p<0.01; n=5).

through these target proteins. MiR-134 also regulates dendritic spines that are postsynaptic targets for excitatory synapses. The size and number of dendritic spines are indicators for measuring synaptic efficacy. Dendritic spine remodeling is closely associated with learning, memory, epilepsy, and other neuropsychiatric disorders. For example, miR-134 can promote the development of the vertebrate central nervous system (including neurons, axons, and dendrites)[12]. At present, there are relatively few studies on the expression of miR-134 in the process of epileptic seizures, and the relevant studies mainly focus on tumor-related fields. Therefore, relatively quantitative detection was conducted for the expression level of miR-134 in the process of epileptic seizures, which was of great significance.

We found that the increasing amplitudes of IL-2, IL-6, TNF-α and other inflammatory cytokines in the process of epileptic seizures were relatively large, proving that the process of epileptic seizures is closely related to the body's immune function.

IL-6 has a significant pro-inflammatory effect. It can be rapidly diffused and released when the body is subjected to external stimuli, so as to exert its pro-inflammatory effect and protect the body[13]. A study[14] has shown that IL-6 protects the brain injury caused by acute epileptic seizures, but its long-term overexpression can aggravate epilepsy and even cause changes in tissue structures such as gliosis and neuronal reduction. Lehtimaki et al[15] found that IL-6 is mainly released by cerebral blood vessels after epileptic seizures, and IL-6 level in peripheral blood is subsequently increased through blood circulation. Besides, IL-6 level in rats of the recurrent tonic-clonic seizure group is significantly higher than those in rats of the single tonic-clonic seizure group, the focal seizure group, and the normal control group, so he believed that the increasing amplitude of IL-6 level is related to epilepsy itself, which is consistent with the conclusion of this study.

IL-2 is a member of the γc family. Its main function is to promote lymphocyte proliferation, fully regulate the adaptive immune response, and maintain relatively stable lymphocytes, which are mainly secreted by activated T cells through autocrine or paracrine pathways[16]. Natural killer (NK) cells, T cells, and other immune cells in the immune system are induced by IL-2 and cause activation-induced cell death. T cell production and the exertion of its functions are regulated by IL-2, T cells in differentiation are susceptible to IL-2, and IL-2 exerts a certain therapeutic effect on the body affected by

the disease. Yang et al[17] reported that a large number of lymphoid hyperplasia, severe lymphadenopathy, splenomegaly, and other T cell-related autoimmune diseases can be found in the model of mice with defective IL-2. De Sarro et al[18] showed that IL-2 has a certain therapeutic effect on the sound-induced epilepsy, and has a certain inhibitory effect on the chemical reagent-induced epilepsy model, proving that IL-2 plays an important role in the process of epileptic seizures. The experimental results showed that IL-2 level in rats of the epileptic seizure group was 2 times of that in rats of the normal control group, indicating that serum IL-2 and IL-6 will be greatly increased in the process of epileptic seizures with the activation of the immune mechanism, and they play roles in mitigation in the epileptic seizure process.

TNF-α can induce the activation of T cells, B cells, and monocyte-macrophage cells, which can increase the production of IL-2, IL-6, and other relevant cytokines and promote the expression of antigen presenting cells into leukocyte class II antigens. At last, those inflammatory reactions participate in epileptic seizures[19,20].

Conclusions

We observed that the expression of miR-134 and inflammatory cytokines showed a certain rule with each other. During the process of epileptic seizures, T cells were stimulated to increase the level of inflammatory cytokines *in vivo*, while the expression of miR-134 was inhibited, and its expression level was significantly decreased.

Conflict of Interest:

The authors declared no conflict of interest.

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