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Expressions of serum miR-146a and COX-2 in children with drug-resistant epilepsy and their correlation with prognosis

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Epilepsy is one of the most common chronic diseases of nervous system, and long-termanti-epileptic drug therapy leads to drug-resistant epilepsy in few cases. MicroRNA-146a is reported to influence development of drug-resistant epilepsy by regulating inflammatory response. Similarly, cyclooxygenase-2 (COX-2) is also known to play an important role in the early inflammatory response and neural excitation of brain tissue under ischemic and hypoxic conditions and alleviate epilepsy. Here, we investigated the expression of serum miR-146a and COX-2 in children with drug-resistant epilepsy and their correlation with prognosis. A total of 173 children with epilepsy were selected and divided into non-drug resistant group (110 patients) and drug-resistant group (63 patients) according to the diagnostic standard of International League Against Epilepsy (ILAE). All children with drug-resistant epilepsy received hemispheric insular transection, the prognosis was evaluated according to Engel classification, and the children were divided into non-recurrence group (51 patients) and recurrence group (12 patients) according to the 1-year follow-up results. qRT-PCR and ELISA was used to detect the expression level of miR-146a in serum and COX-2, respectively, and compared between the two groups. Receiver operating characteristic (ROC) curve was drawn to evaluate the predictive value of serum miR-146a and COX-2 expression levels in drug-resistant epilepsy, and logistic regression analysis was used to analyze the influencing factors of relapse in children with drug-resistant epilepsy. There were no significant differences in gender, age, family history of epilepsy, course of disease and seizure frequency between non-drug resistant group and drug-resistant group (P >0.05); compared with those in the non-drug resistant group, the expression levels of miR-146a and COX-2 in the drug resistant group were higher (P <0.05). The area under the curve (AUC) of serum miR-146a and COX-2 alone or combination in predicting drug-resistant epilepsy was 0.752, 0.757 and 0.836, respectively. The cut-off value of miR-146a in predicting drug-resistant epilepsy was 1.09, and the sensitivity and specificity were 74.60 and 65.50%, respectively. The cut-off value of COX-2 was 2.05 ng/L, the sensitivity and specificity were 65.10 and 80.90%, respectively. The sensitivity and specificity of the two methods were 68.30 and 86.40%, respectively, and the specificity was higher than that of single prediction. Compared with those in the non-recurrence group, the levels of serum miR-146a and COX-2 in the recurrence group were higher (P <0.05). Logistic regression analysis showed that high level of miR-146a and high level of COX-2 were risk factors for recurrence of drug-resistant epilepsy (P <0.05). Over all, our results suggest that the expression of serum miR-146a and COX-2 are related to the occurrence and prognosis of drug-resistant epilepsy, which may be used for early prediction and risk assessment of prognostic recurrence.

Keywords: Cyclooxygenase-2, MicroRNA-146a

Epilepsy is one of the most common chronic diseases of the nervous system clinically, and its onset is sudden and easily causes the panic of the population, resulting in serious injuries to the physiology and psychology of patients. There are about 50 million people with epilepsy worldwide, about 25% of the total global patients with childhood epilepsy, and about 40% of the new patients with epilepsy each year are children. Long-term antiepileptic drug therapy is the main clinical treatment for controlling the seizures of epileptic patients, and more than 20 drugs have been proved to be effective anti-epileptic drugs, but a proportion of epileptic patients, who are insensitive to antiepileptic drugs become drug-resistant epilepsy[1,2].

MicroRNAs (miRNAs), a class of endogenous noncoding RNAs with a length of approximately 22 nt, are widespread in eukaryotic cells, are highly conserved, and can negatively regulate their expression by binding to target genes[3]. MicroRNA-146a (miR-146a) can regulate body immune inflammatory response and is closely related to a variety of inflammatory diseases, which may participate in the occurrence and development of drug-resistant epilepsy by regulating inflammatory response[4,5]. Cyclooxygenase-2 (COX-2) is an important neuro-inflammatory factor that plays an important role in the early inflammatory response and neural excitation of brain tissue under ischemic and hypoxic conditions, downregulating its levels, which can inhibit the neurotoxicity of NO, reduce blood-brain barrier disruption, attenuate inflammatory damage of cerebrovascular tissue, and thus alleviate epilepsy[6,7].

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In the present study, we investigated serum miR-146a and COX-2 expression and their prognostic relevance in children with drug-resistant epilepsy, in order to provide clinical reference for early prediction and recurrence assessment of drug-resistant epilepsy.

Materials and Methods

General data

A total of 173 children with epilepsy treated in Zhejiang Provincial Hospital of Chinese Medicine, Hangzhou from January 2017 to December 2019 were selected as the study objects, including 102 males and 71 females, aged 16 months ~ 6 years, with course of disease of 3 months ~ 4 years. The study objects were divided into two groups according to the diagnostic criteria of International League Against Epilepsy[8]: non-drug resistant group (110 patients) and drug-resistant group (63 patients).

Inclusion criteria:

(i) Children with non-drug resistant epilepsy: seizure frequency of 3 or more seizures in the previous 3 months while receiving epilepsy medication and persisting for more than 1 year after treatment with reasonable antiepileptic drugs; children with drug-resistant epilepsy: patients still not in a seizure free scenario (i.e., seizure free time shorter than 3 times the longest seizure free interval before treatment, or seizure free below 12 months of age) after adequate therapy and dose of two or more regimens; (ii) 12 months ≤ age ≤ 6 years; and (iii) Clinical data are complete.

Exclusion criteria:

(i) Secondary epilepsy caused by infection, tumor, trauma, and other causes; (ii) Those with comorbid progressive neurological disease or intracranial space occupying disease; (iii) Those with severe combined cardiac, hepatic, and renal dysfunction; (iv) Those who did not take the medicine regularly; (v) Those with comorbid immune disorders. All children with drug-resistant epilepsy underwent insular resection of the cerebral hemispheres and were divided into two groups according to the 1-year postoperative follow-up: non-recurrence group (n = 51), and recurrence group (n = 12).

In addition, the clinical data including gender, age, course of disease, family history of epilepsy, and seizure frequency of the study objects were collected. This study was approved by the ethics committee of the hospital and all guardians of the children gave written informed consent.

Methods

Serum miR-146 and COX-2 expression level detection

Fasting venous blood was collected from all study objects within 24 h of admission, and serum miR-146a expression levels were measured by qRT-PCR (quantitative real-time polymerase chain reaction) using the quantitative real-time polymerase chain reaction with Roche LightCycler 480 II fluorescent quantitative PCR instrument. Total RNA was extracted from serum by the Trizol method, reversely transcribed to cDNA, and used as a template in PCR amplification reactions. The miR-146a reaction system consisted of: 2 μL cDNA, TaqDNA polymerase 0.25 μL, 1 μL of upstream and reverse primers each, 2.5 μL of 10×PCR buffer, 0.5 μL of 10 mmol/L dNTPs. Then, double distilled water was added to 25 μL. Amplification reaction conditions: pre-denaturation at 95°C for 5 min, 40 cycles of 95°C for 30 s, 58°C for 15 s, and 72°C for 30 s, using U6 as internal reference gene, the specific operation was strictly performed according to the instructions, serum miR-146a expression levels were calculated by 2-2-ΔΔCt method, and primers and kits were purchased from R&D, USA. Serum COX-2 expression levels were measured by enzyme linked immunaosorbont assay (ELISA) using a Thermo Scientific Varioskan LUX multipurpose microplate reader, absorbance at 450 nm to was detected, drawing a concentration absorbance standard curve and calculating COX-2 expression levels in serum samples, the kits were purchased from R&D, USA, and the operation strictly followed the kit instructions. High and low levels were discriminated by the mean of serum miR-146a and COX-2 expression levels in children with drug-resistant epilepsy.

Follow-up

The children with drug-resistant epilepsy were followed up by telephone and outpatient, and the starting point of follow-up was the first postoperative day, and the follow-up period was 12 months in total and every 3 months thereafter. The children's prognostic status was assessed according to the Engel classification, with Engel grade I being recurrence free and grades II-IV being recurrence. All affected children were effectively followed up.

Statistical analysis

Data were processed with SPSS17.0. The measurement data were expressed as the mean ± standard deviation ($\bar{x} \pm s$), and comparisons between groups were performed by t-test; count data are

Table 1 — Comparison of baseline data between non-drug resistant group and drug-resistant group [($\bar{x} \pm s$) / N (%)]

Group	No. of cases (n)	Male [n (%)]	Age (years)	Fam. History of epilepsy [n (%)]	Course of disease (months)	Seizure frequency (frequency/ month)
Non-drug resistant group	110	67（60.91）	3.51±0.73	6（5.45）	19.76±4.92	20.27±5.18
Drug-resistant group	63	35（55.56）	3.64±0.85	5（7.94）	18.52±4.61	21.73±5.89
t/x^2	-	0.474	1.061	0.414	1.632	1.696
P	-	0.491	0.290	0.520	0.105	0.092

Table 2 — Comparison of serum miR-146a and COX-2 expression levels between (A) non-drug resistant group and drug-resistant group; and non-recurrence group and recurrence group ($\bar{x} \pm s$)

Group	No. of cases (n)	(A) non-drug resistant *vs*. drug-resistant		(B) non-recurrence *vs*. recurrence	
		miR-146a	COX-2 (ng/L)	miR-146a	COX-2 (ng/L)
Non-drug resistant group	110	1.02±0.25	1.81±0.32	1.02±0.25	1.81±0.32
Drug-resistant group	63	1.32±0.36	2.20±0.49	1.32±0.36	2.20±0.49
t	-	6.529	6.362	6.529	6.362
P	-	0.000	0.000	0.000	0.000

presented as number (n) and percentage (%) with chi-square test; receiver operating characteristic (ROC) curves were drawn to evaluate the predictive value of serum miR-146a and COX-2 expression levels in drug-resistant epilepsy; Logistic regression analysis was used to analyze the influencing factors of relapse in children with drug-resistant epilepsy. P <0.05 was considered statistically significant.

Results and Discussion

Comparison of baseline data between non-drug resistant group and drug-resistant group

There were no significant differences in gender, age, course of disease, family history of epilepsy or seizure frequency between the non-drug resistant group and drug-resistant group (P >0.05) (Table 1).

Comparison of serum miR-146a and COX-2 expression levels between non-drug resistant group and drug-resistant group

Serum levels of miR-146a and COX-2 expression were higher in the drug-resistant group compared to the non-drug resistant group (P <0.05) (Table 2).

Predictive value of serum miR-146a and COX-2 expression levels in drug-resistant epilepsy

The area under the curve (AUC) of serum miR-146a and COX-2 alone or combination in predicting drug-resistant epilepsy was 0.752 (95% CI: 0.673~0.830), 0.757 (95% CI: 0.676~0.837) and 0.836 (95% CI: 0.772~0.900), respectively. The cut-off value of miR-146a in predicting drug-resistant epilepsy was 1.09, and the sensitivity and specificity were 74.60 and 65.50%, respectively; the cut-off value of COX-2 was 2.05 ng/L, the sensitivity and specificity were 65.10% and 80.90% respectively; the sensitivity and specificity of the two methods were 68.30% and 86.40%, respectively, and the specificity was higher than that of single prediction (Fig. 1).

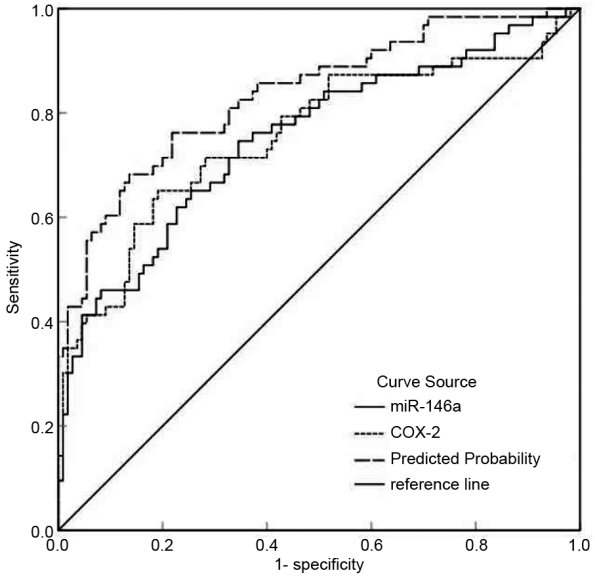

Fig. 1 — ROC curve of serum miR-146a and COX-2 in predicting drug-resistant epilepsy

Comparison of serum miR-146a and COX-2 expression levels between non-recurrence group and recurrence group

Compared with the non-recurrence group, the children with drug-resistant epilepsy in the recurrence group had higher serum miR-146a and COX-2 expression levels (P<0.05) (Table 2).

Impact factors of recurrence in children with drug-resistant epilepsy

The logistic regression analysis was performed with whether the children with drug-resistant epilepsy relapsed as dependent variables and the expression levels of miR-146a and COX-2 as independent variables. The miR-146a low and COX-2 low levels were assigned a value of 0, and the corresponding miR-146a high and COX-2 high levels were assigned a value of 1. The results of Logistic regression

Table 3 — Results of logistic regression analysis affecting recurrence in children with drug-resistant epilepsy

Independent variable	*B*	*S.E.*	*Wald*	*OR*	95% *CI*	*P*
miR-146a (low level *vs*. high level)	0.785	0.248	10.026	2.193	1.349~3.566	0.002
COX-2 (low level *vs*. high level)	0.801	0.217	13.629	2.228	1.456~3.409	0.000

analysis showed that high levels of miR-146a and high levels of COX-2 were risk factors for recurrence in children with drug-resistant epilepsy (P<0.05) (Table 3).

Epilepsy is caused by many factors; the occurrence mechanism is complex and has not been elucidated so far. With the continuous improvement of diagnosis and treatment technology, most children with epilepsy receive systematic formal antiepileptic drug treatment and the condition is alleviated to various degrees, but still about 30% of children with epilepsy experience recurrent seizures, evolve into drug-resistant epilepsy, and cause a huge economic burden to the child's family and society[1]. Drug-resistant epilepsy, also called refractory epilepsy, has an insidious onset and no specific clinical symptoms and signs, the evolution of the disease is more difficult to predict, and its treatment efficacy is often poor. Patients are extremely prone to seizures, clinical disability and lethality, which seriously threaten patients' daily life. Drug-resistant epilepsy has become the focus and difficult problem of current epilepsy treatment, and the clinical treatment methods of drug-resistant epilepsy mainly include surgical treatment, novel anti-epileptic drug treatment, Ketogenic diet treatment. Surgical approach of hemispheric disconnection is the most common treatment for drug-resistant epilepsy in children[9].

Inflammatory factors are one of the important mechanisms of epilepsy, which can promote neurons, glial cells and other cells to produce a large number of oxygen free radicals and inflammatory factors at the onset of epilepsy, causing brain tissue to be damaged again, aggravating the development of epilepsy, and forming a vicious cycle[6,10]. The miR-146 includes miR-146a, miR-146b, which differs in gene localization and play different roles. MiR-146a can affect multiple targets on the TLR-NF-κB signaling pathway, which in turn is involved in immune cell differentiation, apoptosis progression, and can exert negative feedback regulation on immune inflammatory responses[11]. Studies found that plasma levels of miR-146a were higher in children with sepsis and that their levels were closely correlated with the levels of inflammatory factors, such as TNF-α, IL-6 and IL-10 and the evolution of the disease, which can be used to evaluate prognosis in the near future[12]. miR-146a is closely related to epilepsy, and miR-146a-5p can regulate the release of inflammatory factors related to NF-κB signal transduction pathway in hippocampal neurons of epileptic rats, involved in the occurrence and evolution of epilepsy[4].

Studies found that plasma miR-146a was highly expressed in children with epilepsy and was related to the occurrence of epilepsy[13]. Another study found that miR-146a gene polymorphism was closely related to the occurrence of drug-resistant epilepsy, providing a reference for its mechanism[5]. Dysfunction of the immune system is one of the causes of epilepsy, COX-2 is mainly expressed on the cell membrane, it is not expressed or has low expression in the resting state, when the body appears hypoxia or inflammation, COX-2 expression is up-regulated[14,15]. COX-2 and its metabolite PEG2α can inhibit the expression of peroxisome proliferator-activated receptor γ by acting on mitogen activated protein kinase signaling pathway, which in turn promotes the disorder of Th cell subsets, the blood cerebrospinal fluid barrier is disrupted, and neural cells are induced to undergo inflammatory responses, eventually triggering epilepsy[16,17].

Studies found that high serum COX-2 levels in children with drug-resistant epilepsy may be involved in the development of drug-resistant epilepsy[16]. In the present investigation, it was found that compared with the non-drug resistant group, the serum levels of miR-146a and COX-2 expression in the drug-resistant group were all higher, suggesting that miR-146a and COX-2 are related to the development of drug-resistant epilepsy, and clinical attention should be paid to the children with epilepsy who have high levels of miR-146a and COX-2 to closely observe the development of the disease and prevent the occurrence of drug-resistant epilepsy.

Further investigation revealed that serum levels of miR-146a, COX-2 alone and the combination predicted drug-resistant epilepsy with AUCs of 0.752, 0.757, and 0.836, respectively, and the cut-off value of miR-146a alone in predicting drug-resistant epilepsy is 1.09, with a sensitivity and specificity of 74.60 and 65.50%, respectively; COX-2 alone predicted drug-resistant epilepsy with a cut-off value of 2.05 ng/L, with a sensitivity and specificity of

65.10 and 80.90%, respectively, and the combination of the two predicted drug-resistant epilepsy with a sensitivity of 68.30% and a specificity of 86.40%, respectively, compared with the prediction alone, the high specificity indicated that miR-146a and COX-2 might be used for the early diagnosis of drug-resistant epilepsy and play a clinical early warning role in order to change the treatment regimen early and reduce unnecessary adverse drug reactions.

In the present study, it was found that compared with the non-recurrence group, the children with drug-resistant epilepsy in the recurrence group had higher serum levels of both miR-146a and COX-2, and the high levels of both miR-146a and COX-2 were risk factors for recurrence in children with drug-resistant epilepsy, suggesting that miR-146a and COX-2 may be used for prognosis recurrence risk prediction in children with drug-resistant epilepsy, and the high levels of miR-146a and COX-2 may play an early warning role for prognosis recurrence.

Conclusion

The results of this study have demonstrated that children with drug-resistant epilepsy had higher serum levels of miR-146a and COX-2, both of which may be involved in the development of drug-resistant epilepsy. Logistic regression analysis suggests that higher level of miR-146a and high level of COX-2 were risk factors for recurrence of drug-resistant epilepsy. Findings of the present investigation provide a clinical reference for early prediction and prognosis of drug-resistant epilepsy recurrence, and also a new drug treatment target. However, the present study suffer from lack of dynamic monitoring of miR-146a and COX-2.

Conflicts of interest

Authors declare no competing interests.

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