

## Original Article

# miRNA-10a-5p decreases pannexin-1 protein levels in rat hippocampi

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Received December 2, 2019; Accepted December 29, 2019; Epub April 15, 2020; Published April 30, 2020

**Abstract:** Objective: To explore the expression and significance of microRNA-10a-5p and pannexin-1 in the hippocampi of intractable epilepsy rats. Method: 48 Wistar adult male rats were randomly divided into a normal group (n=12), a sham operation group (n=12), a model group (n=12), and an experimental group (n=12). Among them, the normal group was not treated, the model and experimental groups used lithium chloride-pilocarpine to establish an intractable epilepsy (IE) model, and the sham operation group was treated with normal saline. The rat models were observed with electroencephalograms (EEG), and the lentiviral vector of microRNA-10a-5p was constructed. The lentiviral vector was injected into the hippocampi of the rats in the experimental group. The model and sham-operation groups were treated with normal saline, while the normal group was not treated. The expressions of microRNA-10a-5p and pannexin-1 in the rats' hippocampi were determined using qRT-PCR and WB. Results: The expressions of microRNA-10a-5p in the experimental and model groups were significantly lower than they were in the sham-operated and normal groups ( $P < 0.05$ ), and the expression of microRNA-10a-5p in the experimental group was significantly higher than it was in the model group ( $P < 0.05$ ). The expressions of the pannexin-1 protein in the hippocampi of the experimental and model groups was significantly higher than it was in the normal and sham-operated groups, while the expression of the pannexin-1 protein in the hippocampi of the experimental group was significantly lower than it was in the model group ( $P < 0.05$ ). There was a negative correlation between the expressions of microRNA-10a-5p and pannexin-1 in the hippocampi of the two groups, that is, the expression of pannexin-1 protein decreased with an increase of the expression of microRNA-10a-5p. Conclusion: The overexpression of microRNA-10a-5p can effectively reduce the expression of the pannexin-1 protein in the rat hippocampi, and it is expected to become a potential therapeutic target for the clinical treatment of IE.

**Keywords:** microRNA-10a-5p, pannexin-1, intractable epilepsy

## Introduction

Epilepsy is a neurological disorder caused by highly synchronous abnormal discharges of various brain neurons [1]. According to Theodore et al. [2], the incidence of epilepsy in North America is 50/100,000 per year, and it is more common in children under 5 years old and elderly people. A WHO report shows that there are 8 epilepsy patients among every 1000 people [3]. According to Mac's report, more than half of the world's 50 million epileptic patients live in Asia [4]. The treatment of epilepsy in the clinic is mainly done by prescribing drugs. Most patients have an obvious improvement after the drug treatment, but a few patients are still difficult to control after treat-

ment with various antiepileptic drugs, and their conditions deteriorate further, eventually developing into intractable epilepsy [5]. Intractable epilepsy (IE) refers to the regular treatment of patients for 2 years within a certain range of blood concentration by the appropriate use of antiepileptic drugs on the basis of excluding space occupying lesions or progressive central nervous system diseases, but the condition is still uncontrolled (at least 4 times/month), affecting the daily life of the patients [6].

With the gradual deepening of research, researchers have entered the molecular level of medical research. MicroRNAs are a hot research field nowadays. MicroRNAs are small (19-22 nucleotides) non-coding single-stranded RNA

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molecules. They are a kind of important non-protein-coding RNA. Regulating gene expression can cause the translation inhibition of the target mRNA or cutting the mRNA [7]. Recent studies have shown that microRNAs are closely related to tumors, immunity, and nervous system diseases [8-10]. As a member of the microRNA family, microRNA-10a-5p belongs to the microRNA-10 family and is located on 17p21.3. It has an abnormal expression in many malignant tumors and is closely related to tumor invasion [11]. Previous studies [12] have shown that microRNA-10a-5p can regulate the development of zebra fish neurons by inhibiting Mib1 through microRNA-10a/b, but whether microRNA-10a-5p plays a regulatory role in epileptic diseases has not been reported in the literature so far. The pannexin-1 protein is a gap junction protein which can form a gap junction half-channel on the cell membrane, thus realizing electrical coupling and information communication between cells [13]. In the study of Mylvaganam et al. [14], the pannexin-1 protein was highly expressed in the hippocampi of rats with epileptic seizures. Target Scan Release 7.2 software was used to predict the target gene of microRNA-10a-5p, and it was found that pannexin-1 had target binding sites to microRNA-10a-5p. But whether microRNA-10a-5p can regulate pannexin-1 and improve IE has not been reported.

Therefore, in this study, we explored the relationship between microRNA-10a-5p and pannexin-1 in an IE animal model, providing a reference for clinical scholars.

### Materials and methods

#### *Rat source*

48 Wistar adult male rats, 6-8 weeks old, (Beijing Witonglihua Laboratory Animal Technology Co., Ltd., China, SYXK (Beijing)) were purchased and fed in the animal experimental center for one week. The indoor temperature was 19-23°C and the relative humidity was 50-60%. They ate and drank water freely, avoided strong light and noise, and had a 24-hour daylight and night light system.

#### *Reagents and instruments*

TRIzol extraction kit, M-MLV Reverse Transcriptase, RIPA, BCA protein kit, T4 DNA Ligase,

imMedia™ Growth Medium, liquid, ampicillin (Thermo, A33251, 28025013, 89900, 23225, 15224041, Q60220, Shanghai, China) PrimeScript™ one-step RT-PCR kit, PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa, 6210A, RR055B, Dalian, China), Panx-1 mouse monoclonal antibody, beta-Actin, HRP-labeled goat anti-mouse IgG II antibody (R&D, MAB7097, MAB8929, HAF007, America), lithium chloride, pilocarpine (sigma, 746460, P6503, America) lentiviral vector and packaging plasmid (Jima Pharmaceutical Technology Co., Ltd., Shanghai, China), PCR instrument (ABI, 7500, America), High Speed Centrifuge (Xiangyi Centrifuge Instrument Co., Ltd., H1850, Hunan, China).

#### *Animal model construction*

The 48 rats were randomly divided into the normal group (n=12), the sham operation group (n=12), the model group (n=12), and the experimental group (n=12). The specific modeling scheme was as follows: 1 week after feeding, lithium chloride (127 mg/kg) was injected to increase the sensitivity of pilocarpine in the rats, and 16-20 hours after the injection, atropine (1 mg/kg) was injected in the rats. Thirty minutes after the injection of pilocarpine (30 mg/kg), the behavioral changes of the rats were observed. The rats were graded according to the grading criteria proposed by Racine [15] (Table 2). If no grade 4 or above occurred in the rats, pilocarpine (10 mg/kg) was added for 30 minutes each time until grade 4 or more reactions occur. The maximum dosage of pilocarpine is 60 mg/kg. When the rats had spasms of both forelimbs two or more times continuously, and no normal behavior was restored between the two seizures and they lasted for 60 minutes, they were considered to be a successful model and were included in experiment. The model rats were injected with 10% chloral hydrate (30 mg/kg) after a seizure of 60 minutes. After the injection, we observed them for 20 minutes. If the convulsion did not stop, we added 10% chloral hydrate (10 mg/kg) until the epilepsy stopped. The anesthetized rats were warmed to 25°C. After 30 minutes, a normal saline and 10% glucose injection (1 mL/100 g) were injected to supplement their energy. In the sham operation group, normal saline was used instead of pilocarpine, while the normal group was not treated. All the rats were given 10% chloral hydrate anesthesia at a specific time,

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**Table 1.** Primer sequence

Gene	Upstream primer	Downstream primer
microRNA-10a-5p	5'-CGCTACCCTGTAGATCCGAA-3'	5'-GTGCAGGGTCCGAGGT-3'
U6	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'

**Table 2.** Racine classification

Grade	Expression
Level 0	No sign of convulsion in rats
Level 1	Facial muscle spasms in rats, accompanied by mastication, movement and blinking
Level 2	Facial muscle spasms in rats, accompanied by rhythmic nodding, wet dog wobble
Level 3	Facial muscle spasms in rats with rhythmic nodding and forelimb spasm
Level 4	The facial muscles developed spasms, accompanied by rhythmic nodding, spasms of the forelimbs, and standing of the hind limbs
Level 5	Facial muscles developed spasms, accompanied by rhythmic nodding, forelimb spasms, hind legs standing and falling

and their brain wave activity was recorded by EEG.

### *Lentiviral vector construction*

According to the mature gene sequence of rat microRNA-10a-5p (MIMAT0000648, 5'-UACCCUGUAGAUCCGAAUUG-3') in the microRNABase database, the microRNA-10a-5p shRNA sequence with BamH I and Xho I restriction sites and a corresponding negative sequence were designed and synthesized by the Jima Company (Shanghai, sequences are confidential). After annealing, T4 DNA ligase was used to connect to the pLenti-H1 vector overnight at 4°C. DH5 $\alpha$  competent cells were transformed and smeared on an LB plate (containing ampicillin). The colonies were identified using PCR. The reaction conditions of the PCR were as follows: It was pre-denatured at 95°C, denatured at 95°C for 20 s, annealed at 62°C for 20 s, and extended at 72°C for 30 s. The positive clones were cultured and verified by sequencing them. Subsequently, the constructed vector was co-transfected with 293T cells. After 72 hours of culture, the cell supernatants were filtered and collected using a high-speed centrifuge, and the virus titer was determined by hole-by-hole dilution titer.

### *Virus injection*

Four weeks after the successful rat modeling, the rats were fixed on the locator, and we opened their skulls by giving them 10% chloral hydrate (30 mg/kg) anesthesia. Lentivirus was injected into the rats' hippocampi in the experimental group with a syringe, and the rats in the model and sham-operation group were given

the same dosage of normal saline, and then sutured. The rats in the normal group were not treated. After the operation, the rats were put on a heating table at 25°C for warming. After they woke up, the rats were placed in a cage. After 2 weeks of feeding, 10% chloral hydrate (0.3 ml/100 g) was anesthetized and an air embolism was executed to collect the rats' hippocampi.

### *MicroRNA-10a-5p target prediction*

TargetScan release 7.2 software was used to predict the target gene of microRNA-10a-5p.

### *Western blot*

Total protein was extracted from the rat hippocampi using the RIPA splitting method. The protein concentration was determined using the BCA method. The protein concentration was adjusted to 4  $\mu$ g/mL. SDS-PAGE electrophoresis was used to separate the protein. After ionization, the protein was transferred to a PVDF membrane. The protein was dyed in a Lichunhong staining solution, washed with PBST for 5 minutes, sealed with 5% skimmed milk powder for 2 hours, and sealed overnight at 4°C with the first antibody (1:1000). The first antibody was removed by washing the membrane. The sheep anti-rat antibody, which was labeled with horseradish peroxidase (1:5000), was incubated at 37°C for 1 hour, and was rinsed with TBST 3 times, 5 min each time. Filter paper was used to dry the excess liquid on the membrane, ECL was used to make the protein light and develop in the darkroom. Scanning protein bands and analyzing the gray value in Quantity One software, the relative

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expression level of the protein is the gray value of the target protein band/GAPDH protein band.

### PCR detection

The total RNA was extracted from the collected rat tissues and cells using a TRIzol kit. The purity, concentration, and integrity of the total RNA were checked using ultraviolet spectrophotometer and agarose gel electrophoresis. The Total RNA reverse transcription was operated strictly according to the instructions of PrimeScript™ II 1st Strand cDNA Synthesis Kit. After the reverse transcription, some cDNA was collected, and another part was put aside to carry out the subsequent operations. The PCR reaction system: PrimeScript 1 step Enzyme Mix 1  $\mu$ L, upstream and downstream primers 0.5  $\mu$ L, 2X 1 step buffer 12.5  $\mu$ L, cDNA 1  $\mu$ L, and finally RNase Free dH<sub>2</sub>O were used to replenish it to 25  $\mu$ L. The PCR reaction conditions: it was pre-denatured at 94°C for 2 min, denatured at 98°C for 10 s, annealed and extended at 68°C for 2 min. A total of 30 cycles were conducted, U6 was used as an internal parameter, and 2<sup>- $\Delta\Delta$</sup>  was used to calculate the expression. The experiment was carried out three times (**Table 1**).

### Statistical analysis

In this study, the SPSS 20.0 software package was used to analyze the collected data. GraphPad Prism 7 was used to draw the data pictures. The measurement data were expressed as the means  $\pm$  standard deviations (mean  $\pm$  SD). ANOVA was used for the comparisons among groups. LSD-t tests were used for the comparisons between two groups. The Pearson test was used to examine the correlation between microRNA-10a-5p and pannexin-1.  $P < 0.05$  indicates that there is a significant statistical difference between the two groups.

## Results

### Successful modeling

There was no significant difference in the success rate of modeling between the model group and the experimental group ( $P > 0.05$ ).

### EEG detection shows an improved experimental group

There was no epilepsy and no behavioral change in the sham-operation group. The rats in the

model and experimental groups that received the pilocarpine injection had acute, static, and chronic phases respectively. An electroencephalogram (EEG) showed that the normal group had mainly 5-10 Hz alpha wave and no paroxysmal rhythm wave. The frequency of the brain wave in the sham-operated group was 7.25~10.25 Hz and the amplitude was less than 50  $\mu$ V. In the model and experimental groups, classical epileptic waves were observed in the acute phase, and the amplitude increased and the frequency of the background wave slowed down. There was no epileptic wave in the static phase, but the background wave slowed down and the amplitude increased. Periodic epileptic discharges were detected in the chronic phase, and the amplitude and frequency were lower than those in acute phase. This indicated that the intractable epilepsy rat model was successfully established. Lentiviral vectors were injected 4 weeks later. After 2 weeks' observation, it was found that there were no adverse reactions in the sham-operation and normal groups. The amplitude and frequency of the scene wave in the experimental group were significantly improved ( $P < 0.05$ ), the periodic epileptic discharges were reduced, and the situation in the model group remained unchanged.

### A higher expression of microRNA-10a-5p was observed in the sham operation and normal groups

The expressions of microRNA-10a-5p in the hippocampus tissues collected after the rats were sacrificed had differences among the groups ( $P < 0.05$ ). There was no significant difference between the sham operation group and the normal group ( $P > 0.05$ ), but the expressions of microRNA-10a-5p in the experimental and model groups were significantly lower than they were in the sham operation and normal groups ( $P < 0.05$ ). However, the expression of microRNA-10a-5p in the experimental group was significantly higher than it was in the model group ( $P < 0.05$ ) (**Table 3** and **Figure 1**).

### Target binding sites were found between microRNA-10a-5p and pannexin-1 3'-UTR

TargetScan release 7.2 online software was used to predict the target gene of microRNA-10a-5p. It was found that there were target binding sites between microRNA-10a-5p and pannexin-1 3'-UTR.

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**Table 3.** The expressions of microRNA-10a-5p in the rat hippocampi

Group	MicroRNA-10a-5p relative expression	F value	P value
Normal group (n=12)	1.015±0.021	180.159	< 0.001
Sham operation group (n=12)	0.987±0.035		
Model set (n=12)	0.422±0.084*#		
Experimental group (n=12)	0.732±0.105*#Δ		

Note: \* indicates that there were differences compared with the normal group (P < 0.05). # indicates that there were differences compared with the sham operation group (P < 0.05). Δ represents there were differences compared with the model group (P < 0.05).

*Higher expressions of pannexin-1 were observed in the experimental and model groups*

The relative expressions of pannexin-1 protein in the hippocampi of the rats in each group were detected by WB. The results showed that there were significant differences in pannexin-1 protein between the groups (P < 0.05). The relative expression of the pannexin-1 protein in the hippocampi of the rats in the normal and sham-operated groups had no significant difference (P > 0.05). The pannexin-1 protein levels in the hippocampi of the experimental and model groups were significantly higher than they were in the sham-operated group, but the pannexin-1 protein in the hippocampi of the experimental group was significantly lower than it was in the model group (P < 0.05) (**Table 4** and **Figure 1**).

*microRNA-10a-5p was negatively correlated with pannexin-1 in the model and experimental groups*

A Pearson test was used to determine the expressions of microRNA-10a-5p and pannexin-1 in the hippocampi of the two groups. It was found that the expressions of microRNA-10a-5p in the hippocampi of the two groups was negatively correlated with the expression of pannexin-1, that is, the expression of pannexin-1 protein decreased with an increase in the expression of microRNA-10a-5p. The relationship showed no difference between the two groups (P > 0.05) (**Table 5** and **Figure 2**).

### Discussion

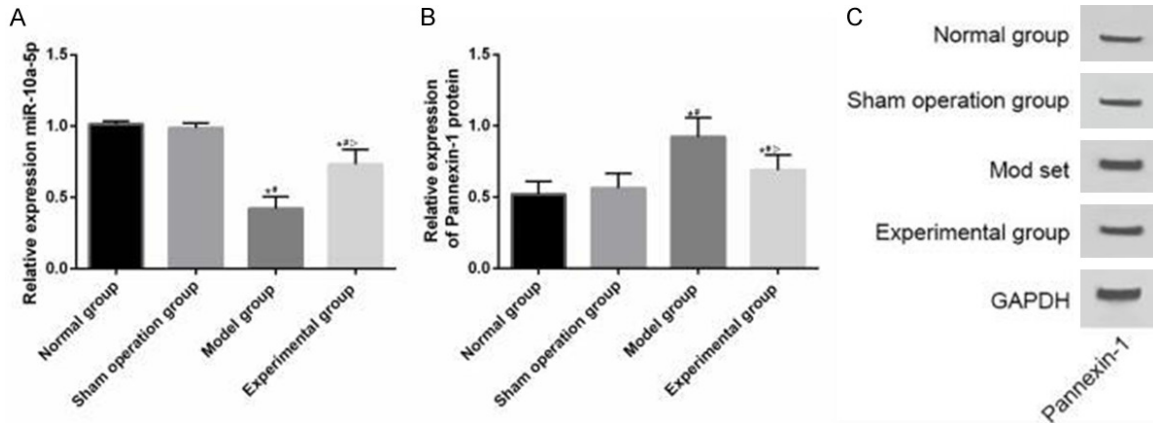
Epilepsy involves the abnormal or excessive over-synchronized discharge of brain neurons in patients. The clinical characteristics of epi-

lepsy are sudden and transient. Because the abnormal discharge neurons exist in different places in the brain, it causes many clinical manifestations of patients, such as motor, sensory and mental aspects [16]. Some documents show that more than 2.4 million people are diagnosed with epilepsy every year, while other data show that more than 70 million people are affected by epilepsy worldwide, and more than 90% of these

patients are in developing countries [17]. As a country with a high incidence of epilepsy, China's data show that about 9 million patients are affected by epilepsy, and the number of epilepsy patients increases at the rate of 400,000 to 600,000 per year. The diseased population of epilepsy is U-shaped, with the majority of patients being elderly and children [18]. Most epilepsy patients can achieve good control through long-term drug treatment, but some people have no noticeable response to drug treatment, clinically known as IE [19]. At present, the mechanism of intractable diseases is not clear, so it is particularly important to explore its specific mechanisms for clinical treatment reference.

Pannexin-1 protein is a gap junction protein that has the same topological structure as other gap proteins. Many scholars believe that pannexin-1 can construct gap junction channels on cell membranes [20]. Studies have shown that [21, 22] pannexin-1 widely exists in pyramidal cells and Purkinje cells of the central nervous system and can form electrical coupling, which is the basic structure of electrical synapses and plays a key role in the synchronous activities of conducting neurons. In this study, we established an IE rat model using lithium chloride-pilocarpine. As an agonist of the M-cholinergic receptor, pilocarpine can directly stimulate cholinergic receptors and plays a cholinergic role after injection. It is a commonly used reagent in epilepsy modeling. Repeated low-dose lithium chloride injections can cause spontaneous recurrent epilepsy in the model, which is very similar to human temporal lobe epilepsy, and is an ideal IE model [23]. It was found that there was no epilepsy or behavioral changes in the sham-operated

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**Figure 1.** The Relative expressions of microRNA-10a-5p and pannexin-1 protein in the rat hippocampi in each group. A. There was no significant difference in the expressions of microRNA-10a-5p between the sham-operation and normal groups ( $P > 0.05$ ), but the expression of microRNA-10a-5p in the experimental and model groups was significantly lower than it was in the sham-operated and normal groups ( $P < 0.05$ ), and the expression of microRNA-10a-5p in experimental group was significantly higher than it was in the model group ( $P < 0.05$ ). B. There was no significant difference in the relative expression of the pannexin-1 protein between the normal and sham-operation groups ( $P > 0.05$ ), but the expression of the pannexin-1 protein in the hippocampus of the experimental and model groups was significantly higher than it was in the sham operation and normal groups, and the expression of the pannexin-1 protein in the hippocampi of the experimental group was significantly lower than it was in the model group ( $P < 0.05$ ). C. Pannexin-1 Western Blot. \* indicates that there were differences compared with group A ( $P < 0.05$ ). # indicates that there were differences compared with group B ( $P < 0.05$ ). Δ indicates that there were differences compared with group C ( $P < 0.05$ ).

**Table 4.** The expressions of the pannexin-1 protein in the rat hippocampi

	Normal group (n=12)	Sham operation group (n=12)	Model group (n=10)	Experimental group (n=10)	F	P
The expression of the pannexin-1 protein	0.521±0.089	0.563±0.102	0.922±0.135* <sup>#</sup>	0.689±0.108* <sup>#</sup> Δ	29.452	< 0.001

Note: \* indicates that there were differences compared with the normal group ( $P < 0.05$ ). # indicates that there were differences compared with the sham operation group ( $P < 0.05$ ). Δ indicates that there were differences compared with the model group ( $P < 0.05$ ).

**Table 5.** The expressions of microRNA-10a-5p in the rat hippocampi in the two groups

Group	r	P
Model group (microRNA-10a-5p VS pannexin-1 protein)	-0.762	0.010
Experimental group (microRNA-10a-5p VS pannexin-1 protein)	-0.698	0.025

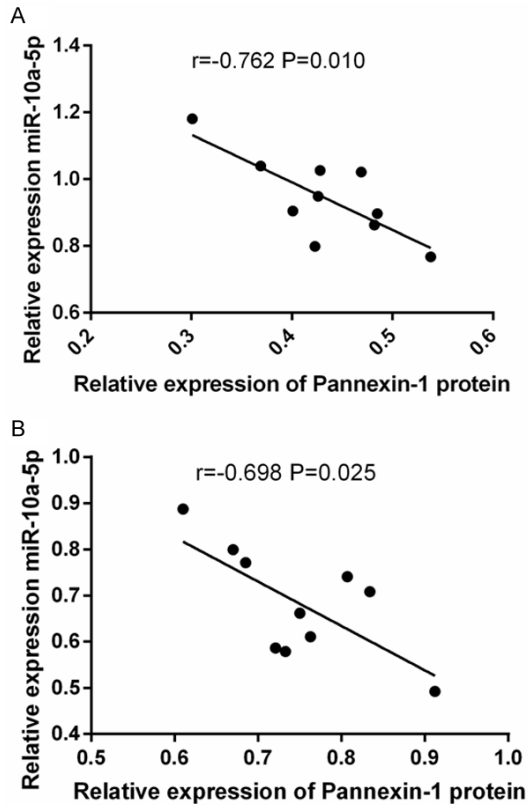
group compared with the model and experimental groups. There were obvious acute, static, and chronic phases in the model and experimental groups, and classical epileptic waves were observed in the acute phase. There was no epileptic wave in the static phase, but the amplitude showed an increasing trend, while periodic epileptic discharges were detected in the chronic phase. This indicated that the intractable epilepsy rat model was successfully established. The expression of pannexin-1 protein in the hippocampi of rats in the model group was detected. The expression of the pannexin-1 protein in hippocampi of the rats in the normal and sham operation groups was signifi-

cantly lower than it was in the model group, which indicated that pannexin-1 protein was differently expressed in IE. Studies by Aquilino et al. [24] showed that the expression of

pannexin-1 protein in several animal epileptic models and resected human epileptic brain tissues was higher than it was in normal brain tissues. Our study confirmed that pannexin-1 protein was increased in the hippocampi of epileptic animal models, but the regulation of its expression is unclear.

As a research hotspot in recent years, microRNA is a kind of non-coding single-stranded RNA with length of 19-22 nt. It is the main regulator of the activity in organisms nowadays. MicroRNA does not have the function of translating proteins. It regulates the expression of target genes through the transcription of 3'UTR

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**Figure 2.** The relationship between the expressions of microRNA-10a-5p and the pannexin-1 protein in the hippocampi of the model and experimental groups. A. There was a negative correlation between the expressions of microRNA-10a-5p and the pannexin-1 protein in the hippocampi of the model group ( $r=-0.762$ ,  $P=0.010$ ). B. There was a negative correlation between microRNA-10a-5p and the pannexin-1 protein in the hippocampi of the experimental group ( $r=-0.698$ ,  $P=0.025$ ).

base sequences complementary pairing [25]. More and more studies show that microRNA is involved in the occurrence and development of epilepsy. The study of Hu et al. [26] showed that microRNA-34a has a targeted neuroprotection on hippocampal neuronal apoptosis after the epileptic state by establishing the expression profile to detect the hippocampus of the temporal lobe epilepsy rat model. Other studies showed that the expression of microRNA-210 is significantly overexpressed in the epileptic rat model [27]. MicroRNA-10a-5p is considered an anti-oncogene and is closely related to the occurrence and development of digestive tract tumors [28]. There has been no previous report on the relationship between microRNA-10a-5p and epilepsy, but it was found that pannexin-1 had target binding sites to microRNA-10a-5p by

predicting the target gene of microRNA-10a-5p. Therefore, it was found that the expressions of microRNA-10a-5p in the hippocampi of the rats in the control and sham-operated groups were significantly higher than the expressions in the model group. This indicates that the expression of microRNA-10a-5p may be related to the occurrence and development of IE. Moreover, we found that the expression of microRNA-10a-5p was negatively correlated with the expression of the pannexin-1 protein in model rats, and we speculated that there might be a regulatory relationship between microRNA-10a-5p and the pannexin-1 protein. Therefore, the successful IE rats were injected with lentiviral vectors in vivo. It was found that the expression of microRNA-10a-5p in the hippocampal tissue of the rats increased significantly after the injection of lentiviral vectors, but the expression of the pannexin-1 protein decreased significantly. This suggests that the up-regulation of microRNA-10a-5p can effectively inhibit the expression of the pannexin-1 protein in rat hippocampi. In the study of Miercelo et al. [29], an epilepsy model was established by injecting kainic acid into mice. The epileptic symptoms of mice were relieved by knocking out the pannexin-1 gene. Our experiment inhibited the expression of the pannexin-1 protein by up-regulating the expression of microRNA-10a-5p, which is expected to become a potential therapeutic target for the clinical treatment of IE.

Although a relationship between microRNA-10a-5p and pannexin-1 was found in this study, there are still some shortcomings. First, the relationship between microRNA-10a-5p and pannexin-1 has not been further confirmed by a double luciferase report. Secondly, no long-term observation of rats has been carried out, and the specific changes are not clear. Finally, as a basic experiment, this study has not carried out any related projects in the clinic. Therefore, we hope to increase our experimental time in future research, so the experimental project can confirm the relationship between Mi-10a-5p and pannexin-1, and confirm the results of this study through clinical experiments.

In conclusion, the overexpression of microRNA-10a-5p can effectively reduce the expression of the pannexin-1 protein in rat hippocampi, and it is expected to become a potential therapeutic target for the clinical treatment of IE.

## Acknowledgements

This work was supported by the Shenzhen Dapeng New Area Industrial Development Special Fund Project: functional MRI based study of the mechanism of interactive head for brain remodeling (Project No. YL20170202).

## Disclosure of conflict of interest

None.

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