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Regulatory effect of miRNA 320a on expression of aquaporin 4 in brain tissue of epileptic rats

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ABSTRACT

Objective: To study the expression of miRNA 320a in the brain tissue of epileptic rats and analyze its effect on the expression of aquaporin 4 (AQP4).

Methods: All rats were performed with the intraperitoneal injection of lithium chloride (3 mmol/kg) and then the intraperitoneal injection of pilocarpine (30 mg/kg) 24 h later (injected twice) to prepare the epileptic model of Wistar rats. Rats in the control group were injected with the equal volume of normal saline. According to the Racine scale, rats with over stage 3 of epilepsy were chosen and the brain tissue was separated quickly and then stored at −80 °C. The immunohistochemistry was used to detect the expression of aquaporin in the brain tissue of epileptic model and the Real-time PCR was employed to determine the difference in the expression of miRNA 320a and AQP4 in the brain tissue of rats between the epileptic model group and control group. Five 5-day neonatal Wistar rats were chosen to collect the cerebral cortex and their primary astrocytes were separated and cultured. They were transfected with miRNA mimic and imitated to the endogenous miRNA 320a to up-regulate the expression of miRNA 320a.

Results: In the model group, the expression of AQP4 was significantly higher than the control group ($P < 0.01$). However, the expression of miRNA 320a in the model group was lower than control group ($P < 0.05$), which was negatively correlated to AQP4. In the primary astrocytes, the transfection of miRNA 320a mimic could significantly reduce the expression of AQP4, while its inhibitor could up-regulate the expression of AQP4, which indicated that miRNA 320a could reduce the expression of AQP4.

Conclusions: In the primary astrocytes of rats, the miRNA 320a could inhibit the expression of AQP4 and after adding the inhibitor of miRNA 320a, the expression of AQP4 was up-regulated.

1. Introduction

MicroRNAs (miRNAs) are short, highly conserved small noncoding RNA molecule that is similar with small interfering RNA. miRNAs are 20–25 nucleotides long and regulate post-transcriptional messenger RNA expression, typically by binding to the 3′ untranslated region of the complementary messenger RNA sequence, resulting in translational repression and gene silencing. Studies have shown that thousands of human protein-coding genes are regulated by miRNAs, indicating that miRNAs played the key role in the regulation of cell growth and development [1,2]. miRNAs are widely expressed in tissues, including the brain tissue. According to previous researches, miRNA could regulate the expression of target gene and then regulate the proliferation and differentiation of brain cells, which would be of critical importance for the brain tissue to maintain the normal physiological process. The identification of target gene of miRNAs is the first step to study the biological functions of miRNAs. Though there have been plenty of researches on miRNAs, it's still a challenging work to identify the target gene of miRNAs, because each miRNA has thousands of target genes and the regulation of each target gene is different [3,4]. In recent years, some researchers found that miRNAs could be combined with 5′-untranslated region

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and promoter region [5–7], which made it even more difficult in studying miRNAs, but it might also provide the certain thoughts to understand the complicated biological functions of miRNAs.

Aquaporins (AQP) are the set of specific water channels and some small-molecule membrane proteins. The aquaporin 4 (AQP4) is mainly expressed in the brain tissue, which plays the key role in the regulation of water transportation in the brain [8]. The previous researches have shown that AQP4 could mediate the transportation of water molecule and may be involved in the physiological process of central nervous system. The change in the relative volume of gap between nerve cells might be related to the onset of epilepsy. As the AQP of central nervous system, AQP4 is of critical importance to maintain the normal ion concentration in the brain tissue. Therefore, it would be of clinical significance to discuss the effect of AQP4 expression on the occurrence and development of epilepsy [9]. miRNAs are the key factors to regulate the gene expression. Therefore, the study on the effect of miRNA on the expression of AQP4 would provide the certain thoughts for the medicine design of epilepsy. According to the bioinformatics database and research findings, AQP4 might be a potential target gene of miRNA 320a [10–15]. In this study, by constructing the epileptic model of rats, it was to study the expression of AQP4 and miRNA 320a in the brain tissue. Afterwards, the primary astrocytes were separated and cultured. Then they were transfected with miRNA mimic and miRNA inhibitor to regulate the expression of miRNA 320a, in order to study its effect and significance on the expression of AQP4.

2. Materials and methods

2.1. Materials

Wistar rats (purchased from Beijing HFK Bioscience Co., Ltd.); pilocarpine (from SIGMA-P6503); lithium chloride (from SIGMA-746460); miRNA Isolation Kit (mirVana-AM1560); miRNA Reverse Transcription Kit (TaqMan-4366596); miRNA mimic (mirVana-4464066); miRNA inhibitor (mirVana-4464084); Real-time PCR Fluorescent Quantitative Kit (SsoAdvanced SYBR Green Super mix, Bio-Rad); Lipofectamine 3000 transfection reagent (Invitrogen-L3000001); AQP4monoclonal antibody (Santa Cruz Biotechnology-sc-376445); horseradish peroxidase labeled secondary antibody (from Wuhan Boster Biological Technology Co., Ltd.); ECL kit (Millpore); PVDF film (Polyvinylidene fluoride-Millpore); MEM complete medium (GIBCO); fetal bovine serum (GIBCO). SsoAdvanced SYBR Green Super mix was purchased from Bio-Rad.

CO_2 constant temperature incubator (Thermo Scientific); MultiSkan FC enzyme-labeling instrument: Thermo Scientific; Fluorescent Quantitative PCR system: CFX96 Touch (Bio-Rad).

2.2. Methods

2.2.1. Construction of lithium chloride-pilocarpine epileptic model

Thirty healthy adult female Wistar rats with the weight of (200 ± 20) g were randomly divided into two groups, where 20 rats in the epileptic model group and 10 rats in the normal saline control group. Rats were fed in the standard animal cages, with 5 rats in each cage. Rats could eat and drink freely during the experiment. The feeding room had the good ventilation and natural lighting day and night. The room temperature was maintained at 18–25 °C.

The lithium chloride (1.5 mol/L) and pilocarpine (0.1%) were prepared with sterile normal saline into the solution. After 2 d of adaptive feeding, the laboratory rats were performed by the intraperitoneal injection of lithium chloride (3 mmol/kg) 24 h before the experiment. After 24 h, they were performed by the intraperitoneal injection of pilocarpine (30 mg/kg) at twice, with the time interval of 10 min [16,17]. Rats were observed for the onset of diseases and they were classified by Racie scale [18]. Rats with over level-3 epilepsy were chosen and their brain tissues were separated quickly.

2.2.2. Separation and culture of primary astrocytes of Wistar rats

Five 5-day neonatal healthy Wistar rats were anesthetized with the chloral hydrate. The cerebral cortex was separated and the tissue was digested in 0.25% trypsin at 4 °C and for 2 h. It was then filtered and ground under the sterile condition. After being diffused to the monolayer cell suspension, it was seeded in the 75 cm^2 culture flask. It was added with the complete MEM and 20% fetal bovine serum and then cultured at 37 °C and 5% CO_2. The medium was changed every 3 d.

2.2.3. Immunohistochemistry

The brain tissues were collected and cut into small blocks, with the thickness less than 5 mm. They were labeled and then fixed with 4% paraformaldehyde (to maintain the smooth and complete brain tissue, otherwise it might affect the cellular morphology). It was washed with the running water over night and dehydrated with the gradient alcohol. Tissues were embedded with the paraffin and cut into slices (with the thickness of 3–5 μm). The Canadian neutral balsam was used for the mounting.

The paraffin sections were dewaxed and washed by water finally. The hydrogen peroxide (3% H_2O_2) was used to mount the endogenous catalase. The AQP4 and monoclonal antibody kits were used for the immunohistochemical staining respectively, while the positive cells appeared as brown yellow. The immunohistochemical data was analyzed using Image-ProPlus. Choosing the area of interesting with the straining on the figure, the integrated option density of such region was then calculated. Afterwards, the effective areas were chosen and measured and then the ratio of integrated option density/area (density mean) of such region was calculated. The density mean was regarded as the semiquantitative parameter for the expression of AQP4.

2.2.4. Real-time PCR to detect the expression of miRNA 320a and AQP4

The miRNA Isolation Kit was used to extract the total RNA from the brain tissue, which was then stored at −80 °C. According to the instruction manual of TaqMan miRNA Reverse Transcription Kit, the reverse transcription was carried out using the specific primers of miRNA and its transcript cDNA was used as the template. The specific primers of miR-320a were:forward: 5′-ACTATGGAAAAGCTGGGTTGAGAG-3′; reverse: 5′-ATTCGTTGAGAGATCACAAGCGT-3′. The relative expression of miR-320a was normalized using the reference (U6). The

Table 1

Synthetic system of PCR.

Components	Volume per reaction
SsoAdvanced SYBR GreenSuper mix	5 μL
Forward primer (10 μm)	0.35 μL (350 nm)
Reverse primer (10 μm)	0.35 μL (350 nm)
cDNA template	100 ng
Nuclease-free water	Up to 10 μL

specific primers of AQP4 were:forward: 5′-ATCAGCGGTGGCCACATCAA-3′; reverse: 5′-GATGGGCCCAACCCAATATAT-3′, *GAPDH* as the reference. The double △Ct method was employed to calculated the relative expression of target gene: Relative expression of target gene = $2^{-\triangle\triangle Ct}$, △Ct = Ct (Target Gene)–Ct (Control); △(△Ct) = △Ct (sample)–△Ct (contro1). The synthetic system of PCR was shown in Table 1.

2.2.5. Transfection of miRNA mimic and miRNA inhibitor

After the passage of cultured primary astrocytes of rats one time 10 d later, they were seeded on the 96-well plate according to the cell density of 5×10^3/mL. At the time of cell adhesion, the cell density was maintained at 70%–80% during the transfection. FiftyμL serum-free medium was used to dilute 1 μL Lipofectamine 3000. The dilution of miRNA mimic and miRNA inhibitor was performed according to the instruction manual and the final density was 100 nm. The diluted miRNA mimic and miRNA inhibitor were mixed with Lipofectamine 3000 and then they were incubated at the room temperature for 20 min. The transfection reagent was added in the well and it was shook gently. After 4–6 h of culture, it was replaced by the new complete medium for the further culture. Twenty-four hours later, cells were collected for the Western blotting assay.

2.2.6. Western blotting assay

The collected cells were washed with PBS twice, 1 mL pipette was used to blow cells and then cell suspension was centrifuged to remove the supernatant. The precipitated cells were lysed with RIPA lysis buffer. Then the protease inhibitor cocktail was added. It was blew and mixed. After being put on the ice for 30 min, cells were lysed using the ultrasound. The probe-type ultrasound was used to produce the short impact with the appropriate frequency on the ice. The lysis mixture was centrifuged at 4 °C and 13 000 r/min for 20 min. The supernatant was transferred to the new centrifuge tube. Protein Assay Kit was employed to detect the protein concentration. The SDS-PAGE electrophoresis was performed on protein samples. The gel was soaked in the transfer buffer for 10 min of equilibrium. The electrodes were inserted, 100 V and 45–60 min. After the transfer, PVDF film was washed with TBS for 10–15 min. The film was placed in TBS/T blocking buffer containing 5% (w/v) skimmed milk powder and shaken at the room temperature for 1 h. Then the primary antibody with the appropriate degree of dilution was added [diluted with TBST containing 1% (w/v) skimmed milk powder]. It was incubated at the room temperature for 2 h and then the film was washed with TBST for 3 times and 5–10 min each time. The film was incubated with the secondary antibody (1:10000, horseradish peroxidase-labeled) that was diluted with TBST containing 0.05% (w/v) skimmed milk powder. It was incubated at the room temperature for 1 h and then the film was washed with TBST for 3 times and 5–10 min each time. It was exposed and then photographed to save the experimental results. The experiment was repeated three times. Quantity one v4.62 was used to measure the gray value of molecular band (trace tracking method of band). The optical density curve was drawn according to the optical density of different electrophoretic band. The area under the optical density curve was calculated as the quantitative basis for the electrophoretic bands and the statistical analysis was performed on collected data.

2.3. Statistical analysis

The experimental data was treated using SPSS 11.5. Results were expressed by mean ± SD. The *t* test was performed for the comparison between groups, where $P < 0.05$ referred to the statistically significant difference.

3. Results

3.1. Construction of lithium chloride-pilocarpine epileptic model of rats

The epileptic model of Wistar rats that was constructed with the intraperitoneal injection of lithium chloride-pilocarpine possessed the actions of 5 stages as proposed by Racine (1. mouth and facial movement; 2. head nodding; 3. forelimb clonus; 4. rearing with forelimb clonus; 5. rearing and falling with forelimb clonus). The action of rats within 0–40 min after the injection of pilocarpine was summarized. During the model construction, the mortality of rats was a bit low, where 2 of 20 Wistar rats died and 4 failed (Table 2).

Table 2

Construction of lithium chloride-pilocarpine epileptic model.

Time (min)	Mouth and facial movement	Head nodding	Forelimb clonus	Rearing with forelimb clonus	Rearing and falling with forelimb clonus	Death	No epileptic symptoms
0–10	2	0	0	0	0	1	17
11–20	10	3	0	0	0	2	5
21–30	0	2	10	2	0	2	4
31–40	0	0	1	11	2	2	4

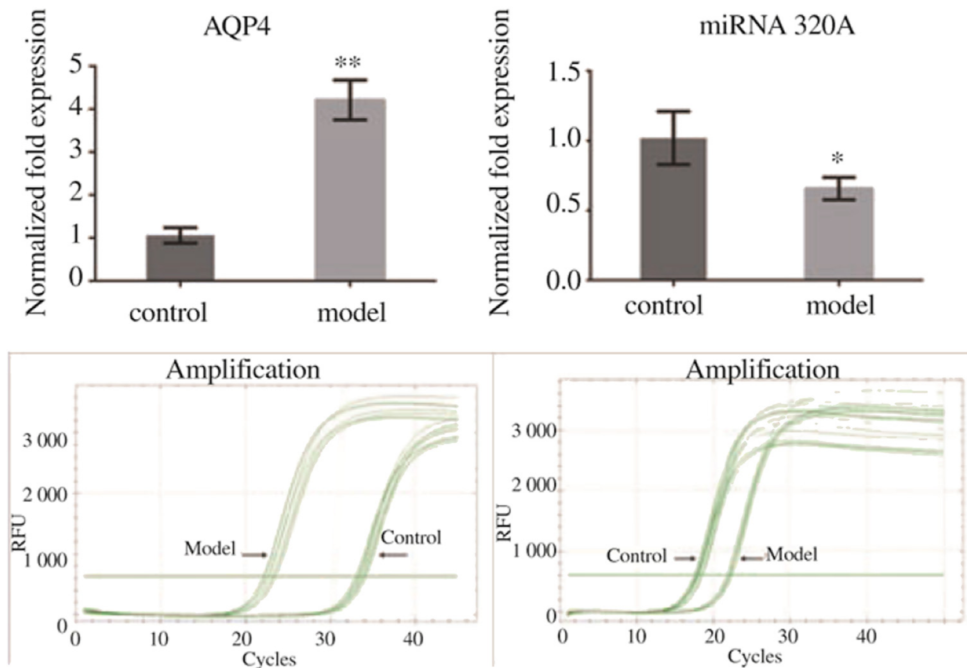

Figure 1. Difference in expression of miRNA 320a and AQP4. Compared with control group, $^{**}P < 0.01$, $^{*}P < 0.05$.

3.2. Difference in the expression of miRNA 320a and AQP4

Rats with the action over Racine's stage 3 were randomly divided into the epileptic model group ($n = 14$) and control group ($n = 10$). The brain tissues were separated quickly and then the total RNA was extracted. Real-time PCR was employed to detect the difference in the expression of miRNA 320a and AQP4 in the brain tissues between the epileptic model group and control one. Results were shown in Figure 1, where the expression of AQP1 in the model group was significantly higher than the one in the control group ($P < 0.01$). However, the expression of miRNA 320a in the model group was lower than the one in the control group ($P < 0.05$), which was negatively correlated to AQP4 (Figure 1).

3.3. Results of immunohistochemistry

The immunohistochemistry was employed to detect the expression of AQP4 at the tissue level in the separated brain tissue of rats. The positive cells appeared in brown yellow (Figure 2). Image-ProPlus was used in the analysis of immunohistochemical data. The density mean in the selected region was regarded as the semiquantitative parameter for the expression of AQP4, namely 0.49 ± 0.21 for the control group and 1.28 ± 0.25 for the epileptic model group.

3.4. Overexpression and inhibition of miRNA 320a

The primary astrocytes of rats were transfected with miRNA 320a mimic. Four hours after the transfection, the high expression of miRNA 320a could be detected, as stimulating the

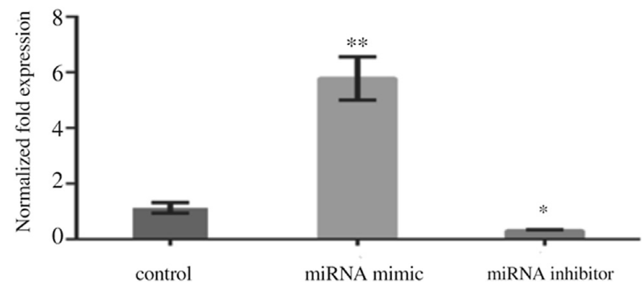

Figure 3. Overexpression and inhibition of miRNA 320a. Compared with control group, $^{**}P < 0.01$, $^{*}P < 0.05$.

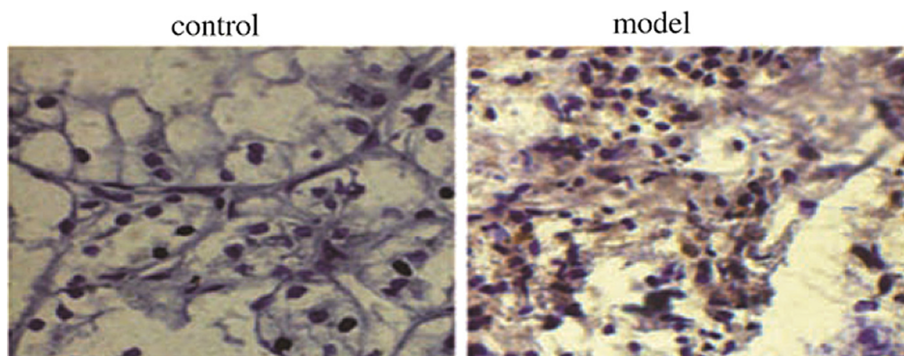

Figure 2. Expression of AQP4 in brain tissue using immunohistochemistry.

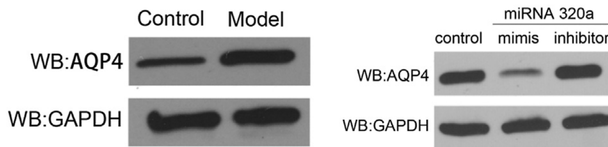

Figure 4. Expression of AQP4.

Table 3

Statistics of band signal analyzed by quantity one.

Gene	Brain tissue		Primary astrocytes		
	Control	Model	Control	Mimis	Inhibitor
AQP4	0.48	1.42*	0.83	0.32**	0.99
GAPDH	1.24	1.19	1.12	1.02	0.94

Compared with control group, $^{*}P < 0.05$, $^{**}P < 0.01$.

endogenous miRNAs. The miRNA inhibitor could specifically reduce the expression of miRNA. According to the experimental results, the miRNA inhibitor could reduce the expression of miRNA 320a by about 60% ($P < 0.05$) (Figure 3).

3.5. Effect of miRNA 320a on expression of AQP4

Similar with the results of Real-time PCR, the expression of AQP4 in the brain tissue of rats in the model group was significantly up-regulated. After the further separation and culture, the primary astrocytes were transfected with miRNA mimic and miRNA inhibitor to regulate the expression of miRNA 320a. Results showed that miRNA 320a mimic could significantly reduce the expression of AQP4, while its inhibitor could up-regulate the expression of AQP4, which indicated that miRNA 320a could reduce the expression of AQP4. Quantity one trace tracking was used to automatically calculate the relative expression (namely the signal of optical density of protein bands), which was normalized using the optical density of *GAPDH*) (Figure 4, Table 3).

4. Discussion

The discovery of miRNAs is regarded as a landmark for a deeper understanding of life at the cellular and molecular level. With the thorough studies on miRNAs, it is found that the biological effects of miRNAs is more than the preliminary understanding. There have been many more researches on the regulation effect and method of miRNAs. Each miRNA has numerous target genes, while one gene will be regulated by several miRNAs during and after the translation. Therefore, studies on the target gene and mechanism of miRNAs will be of critical importance to reveal the effect of miRNAs in the occurrence and development of diseases. AQP are the set of specific water channels and some small-molecule membrane proteins. AQP4 is mainly expressed in the brain tissue, including two protein subtypes, namely M1 and M23. AQP4 can mediate the water transportation in the brain tissue, which plays a key role in maintaining the normal physiological functions of central nervous system. According to previous researches, the expression of AQP4 had been up-regulated during the epilepsy, which could promote the occurrence of cytotoxic cerebral edema. Because of its mediation of water transportation, it can remove the excessive hydrocephalus in the vasogenic cerebral edema. Meanwhile, AQP4 is also involved in the occurrence of neuromyelitisoptica. Thus AQP4 plays an important role in the steady state of nervous system.

The construction of epileptic model through the lithium chloride-pilocarpine could provide the pathological model to study the effect of miRNA 320a on the expression of AQP4 of epileptic rats. The epileptic model of Wistar rats that was constructed with the intraperitoneal injection of lithium chloride-pilocarpine possessed the actions of 5 stages as proposed by Racine (1. mouth and facial movement; 2. head nodding; 3. forelimb clonus; 4. rearing with forelimb clonus; 5. rearing and falling with forelimb clonus) [18]. During the model construction, the mortality of rats was a bit low, where 2 of 20 Wistar rats died and 4 failed. It could also stimulate the occurrence and formation of human epilepsy well, as the only animal model that is similar with the clinical characteristics of temporal lobe epilepsy [19]. Rats with over stage 3 of epilepsy were chosen to construct the animal model of epilepsy, in order to study the expression of AQP4 and miRNA 320a in the brain tissue. The total RNA was extracted from the separated brain tissue and then Real-time PCR was employed to detect the expression of AQP4 and miRNA 320a. Results showed that AQP4 had the high expression in the brain tissue of epileptic rats, which might be closely related to AQP4's mediation of water transportation. The status epilepticus was always accompanied with the cerebral edema [20–22]. According to previous researches, the deficiency of AQP4 could relieve the cerebral edema and improve the situation of local cerebral ischemia because of the accumulative toxicity of water molecule. In the animal models of vasogenic edema such as the brain tumor, the deficiency of AQP4 might make it impossible to transport the extracellular fluid outside the brain tissue in time and then apply more serious injuries of cerebral edema to the brain tissue [23].

According to the bioinformatics database and research findings, AQP4 might be a potential target gene of miRNA 320a [10–15], which indicated that miRNA 320a might regulate the expression of AQP4. The cell cycle gene (*POLR3D*) and transferrin receptor gene (*TFRC*) are two known target genes of miRNA 320a [24,25]. As the gene expression profile of fruit fly showed, part of AQP4 sequence is covered with *POLR3D* and *TFRC*. Thus it's reasonable to believe that AQP4 is a potential target gene of miRNA 320a. After observing the high expression of AQP4 in the brain tissue of rats in the epileptic model group, according to the further separation and culture, the primary astrocytes were transfected with miRNA mimic and miRNA inhibitor to regulate the expression of miRNA 320a, in order to study its effect and significance on the expression of AQP4. Results showed that miRNA 320a mimic could significantly reduce the expression of AQP4 ($P < 0.01$), while the transfection of miRNA 320a could up-regulate the expression of AQP4, which was in accordance with our previous findings, namely that miRNA 320a could reduce the expression of AQP4.

Conflict of interest statement

We declare that we have no conflict of interest.

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