European Review for Medical and Pharmacological Sciences 2018; 22: 2372-2379

The effect of miR-146a gene silencing on drug-resistance and expression of protein of P-gp and MRP1 in epilepsy

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Abstract. – OBJECTIVE: To investigate the effect of miR-146a gene silencing on brain tissue and related drug-resistance proteins in rats and explore its resistance mechanism.

MATERIALS AND METHODS: A rat model of chronic refractory epilepsy was established. The rats were divided into four groups: Normal group, Model group, Negative control group and AntagomiR-146a group. Hematoxylin and eosin (HE) stain was used to detect brain histopathological changes. We examined the expression of mRNA of miR-146a, multidrug resistance (MDR1) and multidrug-resistant associated protein (MRP1) by RT-PCR. The expressions of protein of High motility group box 1 (HMGB1)/Toll-like receptor 4 (TLR4)/nuclear transcription factor-κB (NF-κB) pathway and P-glycoprotein (P-gp), MRP1 were detected by Western-blotting.

RESULTS: We demonstrated that the pathological lesion was lighter in antagomiR-146a group compared with the model group. The mRNA expression of miR-146a in AntagomiR-146a group was significantly decreased compared to the model group. Furthermore, the mRNA expression of MDR1 and MRP1 in AntagomiR-146a group was lower than that in the model group. In addition, the protein expression of HMGB1, TLR4, NF-κB and P-gp, MRP1 in AntagomiR-146a group was lower than that in model group.

CONCLUSIONS: These results demonstrated that miR-146a gene silencing can attenuate pathological changes and improve drug resistance in refractory epilepsy. Also, it is closely related to the HMGB1/TLR4/NF-κB signaling pathway regulation.

Key Words:
Gastric cancer, microRNA, miR-198, Toll-like receptor 4 (TLR4).

Abbreviations

AEDs = antiepileptic drugs; TLE = Temporal lobe epilepsy; miRNA = MicroRNA; P-gp = P-glycoprotein; MDR1 = multidrug resistance; MRP = multidrug = resistant associated protein; IP = intraperitoneal; PBS = phosphate-buffered saline; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; HE = Hematoxylin and eosin; HMGB1 = High motility group box 1; TLR4 = Toll-like receptor 4; NF-κB = nuclear transcription factor-κB; BCA = bicinchoninic acid; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF = polyvinylidene difluoride; BSA = bovine serum albumin; HRP = horseradish peroxidase; ECL = enhanced chemiluminescence; SD = standard deviation; THP = human monocytic leukemia cell line; TNF = tumor necrosis factor.

Introduction

Epilepsy is a chronic and serious neurologic disorder characterized by recurring unprovoked seizures that result from abnormal neurologic insult in the brain. In the world, the estimated proportion of the general population with active epilepsy is about 0.5-1%[1,2]. Although many new antiepileptic drugs (AEDs) came out over the past 10 years, epidemiological data indicate that 20-40% of the patients with newly diagnosed epilepsy will become refractory epilepsy[3]. Clinical trials demonstrated that cerebrovascular diseases, encephalitis and cortical dysplasia function as main causes of refractory epilepsy. Temporal lobe epilepsy (TLE) is the most common form of focal epilepsy[4]. Hippocampal sclerosis is a common pathologic finding in surgical specimens from patients with TLE[5]. Histologically, the segmental loss of pyramidal neurons in area CA1 is more severe than prominent neuronal loss in areas CA3 and CA4[6].

Previous studies[7,8] indicated that various populations were in poor health, suffering from

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epilepsy. It was found that dysregulation of epigenetic mechanisms is associated with human epilepsy syndromes. MicroRNA (miRNA) is a type of endogenous non-coding RNA that can regulate cell proliferation, differentiation, invasion, apoptosis and several other biological activities. Thereby, it is related to the development and disease in life course. Researchers[9] have found that miRNAs are closely related to refractory epilepsy. After ischemic stroke, intracerebral hemorrhage and kainic acid-induced acute seizures, the different miRNA expression was found in injured rat hippocampus[10]. Another work showed that antagomirs have identified contributions from miR-34a and miR-132 to seizure-induced neuronal death, whereas silencing miR-134 potently reduced status epilepticus, seizure-damage and the later occurrence of spontaneous seizures[11]. However, the pathogenesis underlying pharmaco-resistance in refractory epilepsy is still unclear. The limitation of AED access to the seizure focus by a range of efflux transporters is one of the possible mechanisms that have attracted growing interest. The prototype of this is P-glycoprotein (P-gp), which is encoded by the multidrug resistance (MDR1) gene. The association between multidrug transporter and epilepsy tolerance was concentrated on previous studies[12,13]. The multidrug-resistant associated protein (MRP) is an important multidrug transporter and MRP1 and MRP2 may be related to the drug-resistant mechanisms of refractory epilepsy[14]. There is little research about roles, targets, and mechanisms of single miRNA in the pathogenesis and pathophysiology of refractory epilepsy.

We investigated the effect of miR-146a gene silencing on brain tissue and related drug-resistance proteins in rats with refractory epilepsy by establishing chronic refractory epilepsy rat model, further exploring its resistance mechanism. An enhanced understanding of the underlying mechanisms of AED resistance will help in preventing or improving resistance and identify novel diagnostic and therapeutic strategies for refractory epilepsy.

Materials and Methods

Animals

SPF grade healthy Sprague-Dawley (SD) rats with weight 200-220 g were fed into the SPF animal room at 22-24°C with a relative humidity of 50-60%. The rats were adaptively fed with food and water freely at well-ventilated and quiet environment animal laboratory for one week. Ethical approval was given by the Medical Ethics Committee of The Affiliated Yantai Yuhuangding Hospital of Qingdao University.

Animal Model Establishment

The model was made by chemical point dyeing with lithium chloride-pilocarpine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA). The rats were injected intraperitoneally with lithium chloride (127 mg/kg) and atropine (1 mg/kg) 20 h later. 30 min after the injection of atropine, an intraperitoneal (IP) injection of pilocarpine (40 mg/kg) was given, followed by a 10% IP injection of pilocarpine (10 mg/kg) 30 min after that. The degree of epileptic seizures in rats was observed according to Racine Stage (Table I).

If there was no above grade 4 seizure episode in 30 min, IP injection of pilocarpine was given continuously every 30 min until the rats sustained epilepticus; next, the injection was stopped. 60 min after epilepsy, 10% chloral hydrate (300 mg/kg) was injected intraperitoneally to terminate the seizure. The rats entered into the incubation period that lasted 10-15 days after first 24 h of acute state of epilepsy. After 15 days, the rats entered into the chronic phase and were screened and monitored continuously with the image monitoring system. Detection time was from 8 am to 8 pm and observation time was 4 weeks (28 d). If the rats had spontaneous seizures episode after epilepticus in observation time, the rats were successful chronic refractory epilepsy models. If no seizures, the rats were abandoned.

Testing Group

10 rats were in the normal group and the successful preparation of refractory epilepsy model rats were randomly divided into three

Table I. The degree of epileptic seizures in rats.

0	1	2	3	4	5
Without any reaction	Facial clonus, blink, tonic, rhythmic chewing	Plus rhythmic nodding	Plus forelimb clonus	Plus hind legs standing	Plus fall

other groups. Test groups were as follows: normal group (Normal): normal rats, no treatment; model group (Model): successful model establishment of rats; Negative control group (NC): the AntagomiR Negative control (5'- USUSGUACUACACAAAAGUASCSUSGS -Chol-3', the whole chain 2'Ome modified) (Invitrogen, Carlsbad, CA, USA), by the same dose, dissolved into 200 ul of PBS, which was injected into the rats via the tail vein with continuous injection of three days; AntagomiR-146a group (AntagomiR-146a): the AntagomiR-146a (5'-ASASCCCAUGGAAUUCAGUUCSUSCSAS-Chol-3', the whole chain 2'Ome modified (Invitrogen, Carlsbad, CA, USA), by 20 mg/kg dose, dissolved into 200 ul of PBS, which was injected into the rats via the tail vein with continuous injection of three days. After 7 days, the rats were anesthetized by 10% chloral hydrate; the cerebrospinal fluid and brain tissue were taken, and the cortex and hippocampus tissue were separated. Then, they were partly stored in liquid nitrogen and in 4% paraformaldehyde.

RT-PCR

The brain tissues were exacted after 7 days. The expression of miR-146a, MDR1 and MRP1 was detected by RT-PCR. Total RNA was extracted from the brain tissue by TRIzol. The optical density values of the RNA were measured by spectrophotometer at 260 nm and 280 nm respectively, the concentration was calculated and the purity was evaluated (MiR-146a with U6 as the internal reference, MDR1, MRP1 as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for the internal reference. The experiment was repeated 3 times; Primer design (Shanghai Shengong Bioengineering Co, Shanghai, China) (Table II).

Hematoxylin and Eosin (HE) Stain

The rat brain was fixed in 4% neutral formaldehyde solution for 24 h, which was dehydrated by ethanol, embedded in paraffin and sliced into 5 μm tissue sections for HE staining. The histopathological changes were observed under light microscope.

Western-Blot

The protein expression of High motility group box 1 (HMGB1), (Toll-like receptor 4) TLR4, nuclear transcription factor-κB (NF-κB), P-gp, MDR1 and MRP1 (Cell Signaling Technology, Danvers, MA, USA) were detected by Western-blot. The brain tissues of each group were collected and the total protein in the tissues was extracted according to the kit instruction. The protein samples concentrations were detected by bicinchoninic acid (BCA) protein quantification kit. Samples of each group were added up to 40 μg and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis separation, then transferred to the polyvinylidene difluoride (PVDF) membrane. The samples were closed with 5% fat-free milk for 1 h and added to 50 g/L bovine serum albumin (BSA). Each antibody was diluted (1:500). The membrane was washed by TBST (1 ml/L Tween-20) three times (5 min/times) after incubating at 4°C overnight, and then added to horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) labeled secondary antibody (1:5000). The membrane was incubated at room temperature for 2 h and was washed by TBST 3 times (1 min/times). Enhanced chemiluminescence (ECL) darkroom development was used. The protein expression level was normalized by β-actin, and the gray scale was scanned and quantified by software.

Statistical Analysis

All data were analyzed for variance by SPSS19.0 statistical software (Armonk, NY, USA). All data were represented as mean ± standard deviation (SD). Differences were considered significant if $p < 0.05$.

Table II. The sequence of primer design.

	F	R
miR-146a	5'-CAGTGCGTGTCGTGGAGT-3'	5'-GGGTGAGAACTGAATTCCA-3'
U6	5'-GCTTCGGCAGCACATATACTAAAAT-3'	5'-CGCTTCACGAATTTGCGTGTCAT-3'
MDR1	5'-GCCCATCCTGTTTGACTG -3'	5'-CGCTTCCTGGACGACCTT -3'
MRP1	5'-ATGGTGTCAGTGGTTTAGG-3'	5'-TGTGGGAAGAAGAGTTGC-3'
GAPDH	5'-CCATCACCATCTTCCAGGAG-3'	5'-CCTGCTTCACCACCTTCTTG-3'

Results

AntagomiR-146a Downregulated mRNA Expression Level of miR-146a

To validate the efficacy of AntagomiR-146a in downregulating mRNA expression level of miR-146a, RT-PCR was performed to detect the mRNA expression level of miR-146a in each group. The mRNA expression of miR-146a in the epileptic model group and the NC group was significantly higher than that in the normal group ($p < 0.01$) (Figure 1). In contrast, no significant difference of mRNA expression was observed between AntagomiR-146a group and normal group ($p > 0.05$). The expression of mRNA in AntagomiR-146a group was significantly lower than that in model group ($p < 0.01$). Conversely, no significant difference of mRNA expression was reported between NC group and model group ($p > 0.05$). The data suggested that the mRNA expression level of miR-146a in the epileptic rats and AntagomiR Negative control rats increased and AntagomiR-146a could downregulate mRNA expression level of miR-146a.

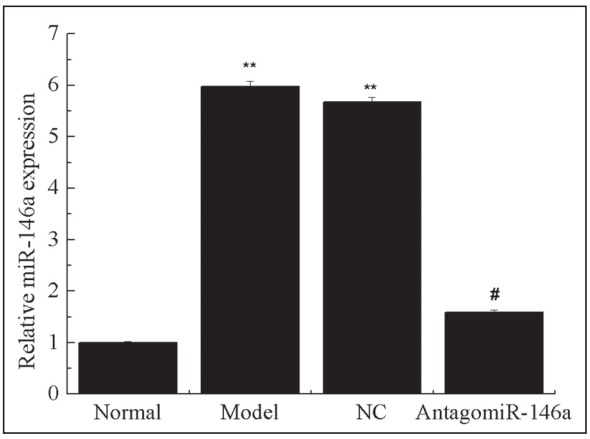

Figure 1. The detection of mRNA expression level of each group. *Note*: Compared with the normal group,*$p < 0.05$, **$p < 0.01$; compared with the model group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$.

The Pathological Lesion Decreased in AntagomiR-146a Group After miR-146a Gene Silencing

In the normal group, the neurons of CA3 and CA4 in the hippocampus of rats are arranged regularly, the cytoplasm is clear, the cell membrane and the nucleus are clear and the cells are intact (Figure 2). In the normal group, the neurons of CA3 and CA4 in the hippocampus of rats were arranged regularly, the cytoplasm was clear, the cell membrane and the nucleus were clear and the cells were intact. In the model group and the NC group, the neurons of CA3 and CA4 in the hippocampus of rats were scattered arrangement and the distance between the cells became larger. The incomplete, fragmented and swollen cells were seen and some cells shinked to triangles. The neurons began to degenerate and be necrotic. In contrast, the lesion was lighter, the cells were more neatly arranged, there was less fragmentation and swelling of cells, and the number of degenerated and necrotic neurons was less in antagomiR-146a group compared with the model group.

mRNA Expression of MRP1 and MRP2 in AntagomiR-146a Group Decreased After miR-146a Gene Silencing

RT-PCR was performed to detect the mRNA expression of MRP1 and MRP2 in each group. The mRNA expression of MDR1 and MRP1 in the epileptic model group and the NC group were significantly higher than those in the normal group ($p < 0.05$) (Figure 3). Conversely, the mRNA expression of MDR1 and MRP1 in the AntagomiR-146a compared to the normal group was not statistically significant ($p > 0.05$). The expression of MDR1 and MRP1 in AntagomiR-146a group was significantly lower than that in model group ($p < 0.05$). Furthermore, there was no significant difference of the mRNA expression of MDR1 and MRP1 between NC group and model group ($p > 0.05$). This data demonstrated that the mRNA expression of MRP1 and MRP2 reduced

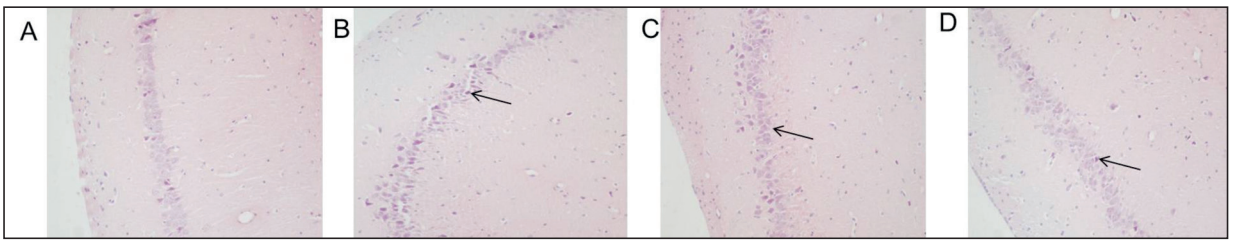

Figure 2. Histopathological changes in hippocampus of rats (× 400).

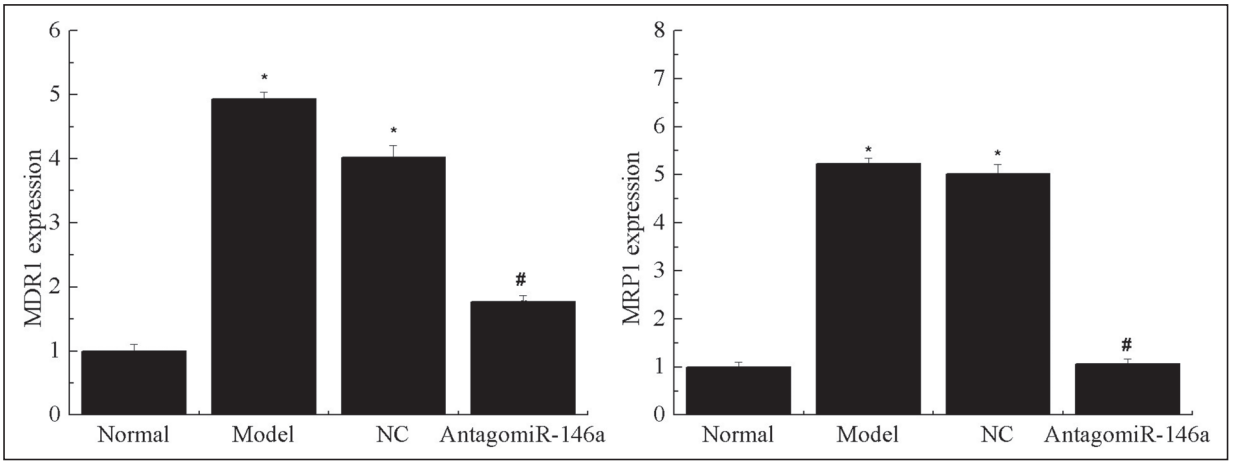

Figure 3. The detection of the expression of mRNA MDR1 and MRP1 of each group. *Note:* Compared with the normal group, $*p < 0.05$, $**p < 0.01$; compared with the model group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$.

in AntagomiR-146a group was significantly decreased after miR-146a gene silencing.

The Protein Expression of P-gp and MRP1 in AntagomiR-146a Group Decreased After miR-146a Gene Silencing

Western blot was used by detecting protein expression and the results were represented as P-gp/β-actin and MRP1/β-actin. The protein expression of P-gp and MRP1 in model group and NC group were significantly higher than those in normal group (Figure 4). The difference was statistically significant ($p < 0.01$). In contrast, there was no significant difference of protein expression between AntagomiR-146a group and normal group ($p > 0.05$). The protein expression of P-gp and MRP1 in AntagomiR-146a group was significantly lower than that in model group. The

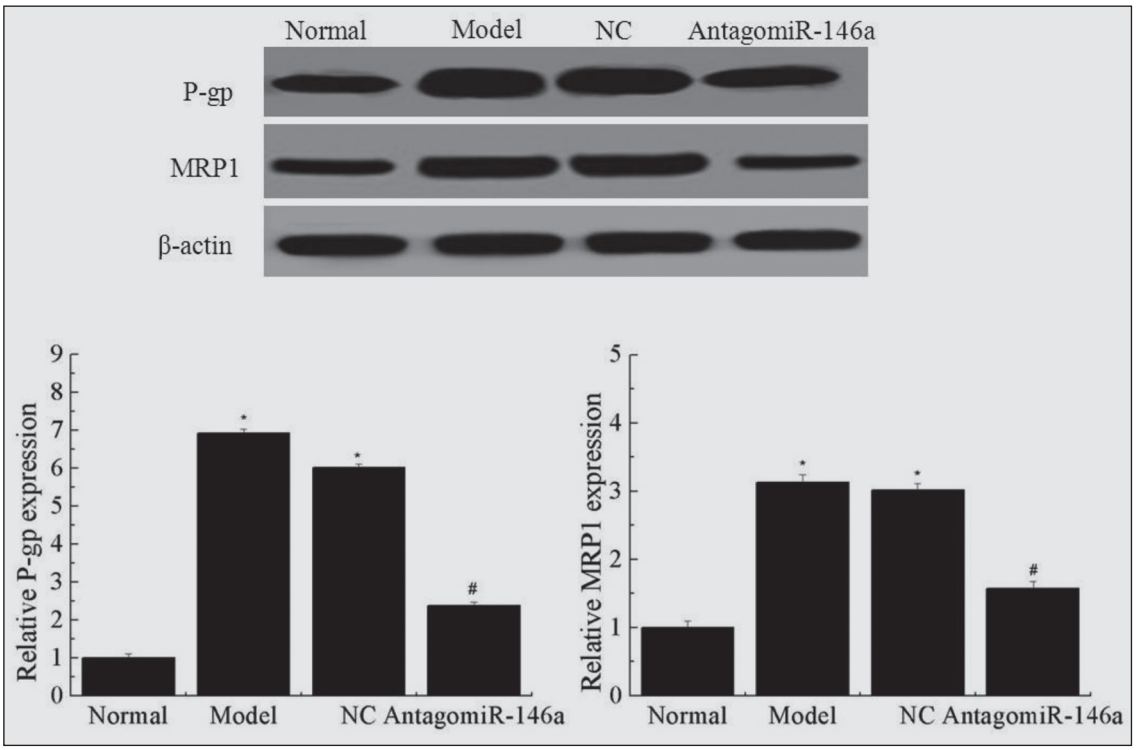

Figure 4. The detection of the protein expression of P-gp and MRP1 of each group. *Note:* Compared with the normal group, $*p < 0.05$, $**p < 0.01$; compared with the model group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$.

difference was statistically significant ($p < 0.01$). There was no significant difference of protein expression between model group and NC group ($p > 0.05$). The data suggested that the protein expression of P-gp and MRP1 in AntagomiR-146a group was significantly decreased after miR-146a gene silencing.

The Protein Expression of HMGB1, TLR4 and NF-κB in AntagomiR-146a Group Decreased After miR-146a Gene Silencing

To explore whether the expression of HMGB1/TLR4/NF-κB pathway-related protein in AntagomiR-146a group decreased after miR-146a gene silencing, Western-blot was performed to detect the protein expression. The results were expressed as HMGB1/β-actin, TLR4/β-actin and NF-κB/β-actin (Figure 5). The results showed that HMGB1, TLR4 and NF-κB protein levels in model group and NC group were significantly higher than those in normal group ($p < 0.01$ or $p < 0.05$). In contrast, the protein expression of HMGB1, TLR4 and NF-κB in the AntagomiR-146a compared to the normal group was not statistically significant ($p > 0.05$). The protein expression of HMGB1, TLR4 and NF-κB in AntagomiR-146a group was significantly lower than that in model group ($p < 0.01$). Furthermore, there was no significant difference of expression of protein HMGB1, TLR4 and NF-κB between of NC group and model group ($p > 0.05$). The data demonstrated that the protein expression of HMGB1, TLR4 and NF-κB in AntagomiR-146a group was significantly decreased after miR-146a gene silencing.

Discussion

Nowadays, there are many people suffering from epileptic[15,16]. However, the mechanism of antiepileptic drugs resistance for refractory epilepsy remains to be elucidated. Recently, miRNAs are recognized to be key regulators in the pathogenesis and potential treatment of epilepsies. Nudelman et al[17] first found connection between miRNA-132 expression and seizures. Then, another investigation[18] found that miR-146a was increased and persistent in reactive astrocytes, suggesting that miR-146a plays a role in the controlling of the astroglial inflammatory response occurring in TLE. However, targets and mechanisms of miRNAs in the pathogenesis and signaling inflammatory pathways of refractory epilepsy were rarely studied. We investigated the effect of miR-146a gene silencing on brain tissue and related drug-resistance proteins in rats with refractory epilepsy by establishing chronic refractory epilepsy rat model and explored its resistance mechanism. We demonstrated that the pathological changes decreased in antago-

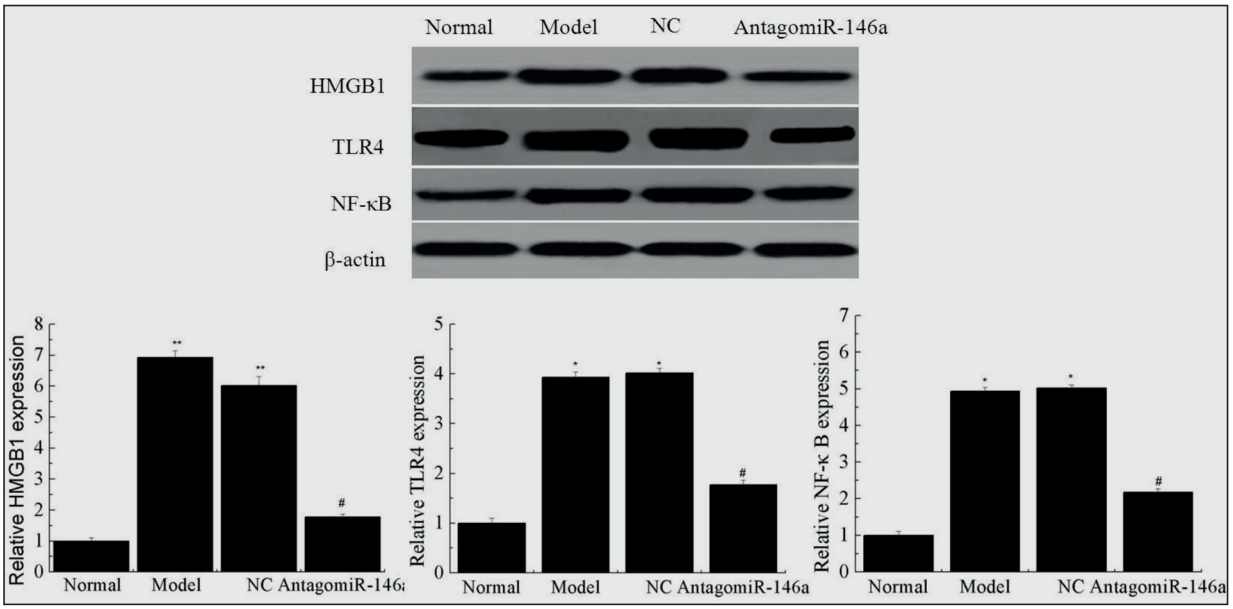

Figure 5. The detection of the protein expression of HMGB1, TLR4 and NF-κB. *Note:* Compared with the normal group, $*p < 0.05$, $**p < 0.01$; compared with the model group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$.

miR-146a group after miR-146a gene silencing. The lesion was lighter, the cells arranged in neat relatively, the less fragmentation and swelling of cells were observed and the number of degenerated and necrotic neurons were less in antagomiR-146a group compared with the model group.

Our data also suggested the mRNA expression of MDR1 and MRP1 and the protein expression of P-gp, MRP1 in AntagomiR-146a group were significantly lower than that in model group after miR-146a gene silencing. MRP1 and MRP2 were related to drug transport. Our findings are in essential agreement with previous reports[19] that suggested overexpression of multiple drug resistance genes in endothelial cells from patients with refractory epilepsy. It is generally agreed that a higher expression of MRP1 and MRP2 in children with intractable epilepsy may be associated with the drug-resistant mechanism of intractable epilepsy[20]. Another recent work[21] seemed to confirm the notion that the efflux transporter P-gp is indeed increased in the drug-resistant epilepsy. Our data not only agreed with previous findings, but also suggested that MDR1 and MRP1 were targets of miR-146a and AntagomiR-146a miR-146a can downregulated the expression of MDR1 and MRP1 after miR-146a gene silencing.

MiR-146a has been implicated to function in inflammation, innate immunity, and cancer, as well as to regulate mitochondrial functions involving inflammation-aging[22,23]. HMGB1 responds to pathogens, both exogenous and endogenous, to activate TLR4, leading to phosphorylation of NF-κB and increased production of proinflammatory molecules in endothelial cells[24]. The influence of miRNAs on the mammalian response to microbial infection was found by miR-146 and was identified as an immune system regulator. Exposure of human monocytic leukemia cell line (THP)-1 cells to lipopolysaccharides (LPS) results in rapid induction of the expression of both miR-146a and miR-146b[24]. Further characterization of miR-146a/b revealed that it is induced through TLR and the induction is NF-κB dependent[25]. Our findings demonstrated that the protein expression of HMGB1, TLR4 and NF-κB in AntagomiR-146a group was lower than that in model group after miR-146a gene silencing. These results suggest that miR-146a gene silencing plays a role in reducing proinflammatory signaling, attenuating inflammatory pathways mediated by TLR4/NF-κB and tumor necrosis factor (TNF)-α. Recently, there is growing interesting that circulatory miRNA expression profiling can be used as valuable molecular marker for detection of epilepsy. MiR-106b-5p may provide a new noninvasive biomarker to improve the current diagnosis of epilepsy[26]. Our research may provide a novel potential option of miR-146a as a diagnostic biomarker for refractory epilepsy.

Conclusions

We showed that miR-146a gene silencing can attenuate pathological changes and improve drug resistance in refractory epilepsy, related to the HMGB1/TLR4/NF-κB signaling pathway regulation. The outcome suggests that miR-146a gene silencing can be a promising potential diagnostic and therapeutic target for refractory epilepsy.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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